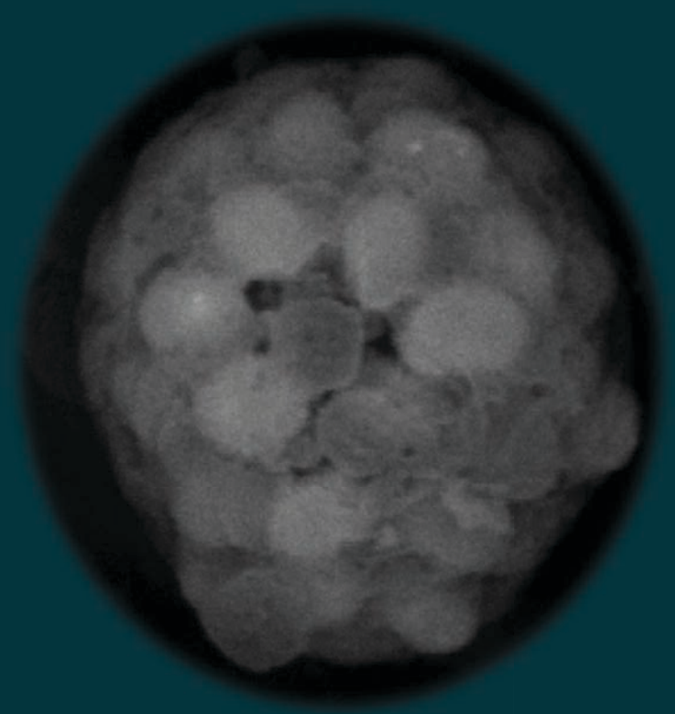
Growth and dynamics of *Hemimycale columella* (Demospongiae, Poecilosclerida) and the relationship with its microbial symbionts

Crecimiento y dinámica de *Hemimycale columella* (Demospongiae, Poecilosclerida) y la relación con sus simbiontes microbianos

Elena Fernández Gómez



Leyre Garate Amenabarro
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Memoria presentada por Leyre Garate Amenabarro para optar al grado de Doctor por la Universidad de Barcelona

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A mis padres

A Naiara y Asier, mis hermanos

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"Your world is nothing more than all the tiny things you've left behind". JC.

Directors' report

Mª Jesús Uriz Lespe and Andrea Blanquer Pérez, supervisors of the Doctoral Thesis by Leyre Garate Amenabarro "Growth and dynamics of *Hemimycale columella* (Demospongiae, Poecilosclerida) and the relationship with its microbial symbionts", declare that Leyre Garate Amenabarro has participated actively in the five chapters that constitute her Doctoral Thesis. The five chapters included in the Doctoral Thesis correspond to five scientific articles. Specifically, the tasks in which she has participated are:

- Planning of the objectives
- Design and execution of experiments in field and in laboratory
- Collecting samples for experiments
- Analyses of samples and statistical analyses of data
- Redaction of manuscripts and figures preparation

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Papers that have been already published, submitted or in preparation:

Chapter 1

Uriz MJ, Garate L, Agell G (2017) Molecular phylogenies reveal a new cryptic Hemimycale species in the Mediterranean and show the polyphyly of the genera Crella and Hemimycale (Demospongiae: Poecilosclerida). *PeerJ* 5:e2958 DOI: 10.7717/peerj.2958. Multidisciplinary Sciences **Q1. Impact factor**: **2.18**

Uriz MJ, Garate L, Agell G (2017) Redescription and establishment of a holotype and three paratypes for the species *Hemimycale mediterranea* sp. nov. *PeerJ* (in press). Multidisciplinary Sciences **Q1. Impact factor**: **2.18**

Chapter 2 (in press)

Garate L, Blanquer A, Uriz MJ Contrasting biological features in morphologically cryptic Mediterranean sponges. *PeerJ*. Multidisciplinary Sciences **Q1. Impact factor**: **2.18**

Chapter 3

Garate L, Sureda J, Agell G, Uriz MJ (2017) Endosymbiotic calcifying bacteria across sponge species and oceans. *Scientific Reports* 7:43674 DOI: 10.1038/srep43674. Multidisciplinary Sciences **Q1. Impact factor: 5.525**

Chapter 4 (in preparation)

Contrasting microbiomes in sponges harbouring calcifying bacteria.

Chapter 5

Garate L, Blanquer A, Uriz MJ (2015) Calcareous spherules produced by intracellular symbiotic bacteria protect the sponge Hemimycale columella from predation better than secondary metabolites. *Marine Ecology Progress Series* 523: 81-92 DOI: 10.3354/meps11196. Marine & Freshwater Biology **Q1. Impact factor: 2.4**

Structure

The thesis has been divided in five chapters dealing with the five main objectives addressed. Chapters 1 and 2 refer to the ecological and biological aspects of the sponge *Hemimycale columella* and *H. mediterranea*. In chapter 1, the identification and description of a new *Hemimycale* cryptic species was carried out. Chapter 2 compares the growth dynamics and survival of these two cryptic species in two different habitats, evaluating the relationship of the sponges' biological features with the respective environmental factors. Chapters 3 and 4 investigate the symbiosis between calcifying bacteria and *H. columella,* as well as the microbiomes of the sponge species observed with calcium carbonate spherules. Chapter 3 describes the accumulation of calcibacteria at the *H. columella* periphery, identify these calcibacteria in other sponge species, and explores their phylogeny. Chapter 4 compares the microbiomes of several sponge species harbouring bacteria-mediated calcareous spherules. Finally, chapter 5 encompasses the potential deterrent role of the calcibacteria of *H. columella* against several potential predators in the sponge habitats.

Chapter 1: Molecular phylogenies reveal a new cryptic *Hemimycale* species in the Mediterranean and show the polyphyly of the genera *Crella* and *Hemimycale* (Demospongiae: Poecilosclerida).

Chapter 2: Contrasting biological features in morphologically cryptic Mediterranean sponges.

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INTRODUCCIÓN

POR QUÉ ESPONJAS MARINAS

Las esponjas son los animales vivos más antiguos que existen en la Tierra (Love et al. 2009), de los que existen aproximadamente 8.500 especies, mayoritariamente marinas, aunque se estima que puedan ser descubiertas unas 29.000 en los próximos años (Appeltans et al. 2012; Van Soest et al. 2012). Son un componente importante en los ecosistemas bentónicos, dominante en hábitats de zonas templadas y frías (Uriz et al. 1992; McClintock et al. 2005; Wulff 2012), donde intervienen en numerosos procesos: proveen micro-hábitats para otros animales, estabilizan el sustrato, transfieren nutrientes del sistema pelágico al bentónico, son fuente de alimento para otros animales, albergan simbiontes que intervienen en ciclos biogeoquímicos y producen multitud de compuestos químicos con interés farmacológico (e.g. Dunlap & Pawlik 1996; McClintock et al. 2005; Sipkema et al. 2005; Bell 2008; de Goeij et al. 2013; Zhang et al. 2013). Las esponjas se han adaptado a un amplio rango de condiciones ambientales para sobrevivir a través del tiempo y de los océanos. Son capaces de obtener nutrientes de la fracción particulada y disuelta, modifican su forma para hacer frente a la hidrodinámica del agua, y se reproducen sexual y asexualmente para asegurar la persistencia de las especies (Ayling 1980; Bell & Barnes 2000a; de Goeij et al. 2008).

El hecho de que las esponjas sean organismos sésiles ha contribuido a que produzcan cantidad de compuestos químicos bioactivos, que usan para competir con otros organismos y evitar que crezcan sobre ellas (e.g. Uriz et al. 1995), y para defenderse de los depredadores. Entre estos últimos, se encuentran crustáceos, equinodermos, peces y tortugas (Meylan 1988; Cerrano et al. 2000; Santos et al. 2002; Loh & Pawlik 2009). Además de los compuestos químicos, las esponjas también pueden defenderse con sus espículas, formadas por siliceo o carbonato cálcico (e.g. Uriz et al. 2003; Hill et al. 2005).

Dada la importancia de las esponjas en los sistemas bentónicos, es crucial identificarlas correctamente para conocer todas las características biológicas del ecosistema (patrones de reproducción, la dinámica de crecimiento, la interacción con otras especies) (Knowlton et al. 1993; Prada et al. 2014; De Meester et al. 2016) que pueden condicionar el funcionamiento del ecosistema (Loreau 2004), esencial a la hora de realizar estudios de conservación y establecer políticas de gestión (p. ej. Forsman et al. 2010). En los casos en que los caracteres morfológicos (fenotípicos) no son suficientes para identificar nuevas especies, las herramientas moleculares (genéticas) han resultado básicas para diferenciar especies crípticas (p. ej. Blanquer & Uriz 2007; de Paula et al. 2012). Además, cuando las especies crípticas son simpátricas, es decir, cuando no pueden diferenciarse por su distribución, conocer las características biológicas y ecológicas es decisivo para confirmar la existencia de nuevas especies y entender los mecanismos que les permiten coexistir (Blanquer et al. 2008b; Payo et al. 2013).

Las esponjas están continuamente interaccionando con el ambiente a través de los continuos cambios en el crecimiento (incremento y recesión), y experimentando episodios de fusiones y fisiones (Garrabou & Zabala 2001; Tanaka et al. 2002; Blanquer et al. 2008; De Caralt et al. 2008). Su estrategia de "*es necesario que todo cambie para que todo se mantenga como está*" les ayuda a lidiar con la competición, depredación, y otros modos de interacción (Cebrian & Uriz 2006), y determina la forma y tamaño de las esponjas en un hábitat determinado, permitiéndoles una larga persistencia.

La temperatura y/o la disponibilidad de nutrientes se han identificado como dos factores ambientales relevantes en su interacción con el crecimiento de las esponjas. La temperatura puede afectar positivamente o negativamente según la especie de esponja estudiada (Tanaka 2002; Duckworth et al. 2004; Page et al. 2005; Blanquer et al. 2008b; De Caralt et al. 2008; Koopmans & Wijffels 2008). La disponibilidad de nutrientes es decisiva en el crecimiento (Reiswig 1973; Sebens 1987; Koopmans & Wijffels 2008), aunque las esponjas pueden utilizar materia orgánica particulada (Ribes et al. 1999a, 2012; De Caralt et al. 2008) o disuelta (de Goeij et al. 2008).

Las características mencionadas convierte a las esponjas únicas para estudiar en los Metazoos la evolución y filogenia, su taxonomía, la ecología química y sus aplicaciones en el descubrimiento de nuevos fármacos, entre otras temáticas. Pero todo ello sin olvidar los miles de acompañantes que han estado desde el comienzo (Wilkinson 1984), y que son responsables en gran medida de su éxito evolutivo (Taylor et al. 2007a).

HOLOBIONTE ESPONJA-MICROORGANISMOS

Las esponjas albergan comunidades microbianas en su interior formadas por virus, arqueas, eucariotas unicelulares y bacterias (e.g. Webster & Taylor 2012; He et al. 2014; Zhang et al. 2014). Las esponjas pueden adquirir estas comunidades microbianas horizontalmente del agua circundante, verticalmente de madre a larva, o por ambos (e.g. Sipkema et al. 2015). Las simbiosis esponja-bacterias son las más estudiadas, y hasta ahora se han identificado 20 filos bacterianos en las esponjas (e.g. Webster & Taylor 2012), muchas de ellas presentes en la mayoría de las esponjas ("núcleo bacteriano") (Schmitt et al. 2012a, b). Los simbiontes bacterianos pueden formar densas y abundantes comunidades en el mesohilo de las esponjas, que pueden alcanzar concentraciones varios órdenes de magnitud mayores a las del agua de mar (Hentschel et al. 2006). Los microsimbiontes pueden llegar a ocupar el 40% del volumen de la esponja (Vacelet & Donadey 1977), pero la concentración microbiana puede variar dependiendo de la esponja, por lo que convencionalmente se ha clasificado la esponjas, de acuerdo a la diversidad y a la abundancia de sus microbiomas, como "esponjas con alta abundancia microbiana" (HMA) o bacterioesponjas, y "esponjas con baja abundancia microbiana" (LMA) (Hentschel et al. 2003; Ribes et al. 2015).

Existen diferencias morfológicas entre las HMA y las LMA esponjas. Las primeras poseen un mesohilo más denso y un sistema acuífero más complejo que retiene el agua en el interior de la esponja durante más tiempo. Por el contrario, el mesohilo de las LMA es menos denso, y su sistema acuífero permite bombear grandes volúmenes de agua (Weisz et al. 2008). Sin embargo, en los últimos años han aparecido excepciones a esta división,

y algunas esponjas LMA presentan una alta diversidad y abundancia bacteriana (e.g. Blanquer et al. 2013).

Se han utilizado muchas técnicas para estudiar las comunidades microbianas. La microscopía electrónica ha permitido observar a las comunidades bacterianas dentro de las esponjas, estudiar sus morfotipos, diferenciar entre bacterias intra- y extracelulares, y confirmar que las esponjas son capaces de distinguir entre bacterias simbiontes y bacterias que son fuente de alimentación (Vacelet & Donadey 1977; Wilkinson et al. 1984; Santavy et al. 1990; Croué et al. 2013; Gloeckner et al. 2014). El cultivo y la secuenciación del gen ribosomal 16S han permitido la reconstrucción filogenética de estos simbiontes, y con los microanálisis de rayos X, además ayudan a identificar los productores de compuestos bioactivos (e.g. Turon et al. 2000; Hentschel et al. 2001; Webster & Hill 2001). Las técnicas de hibridación *in situ* fluorescente, FISH y CARD-FISH, se han usado sobre tejidos de esponjas para identificar y localizar taxonómicamente las bacterias, y cuantificar las comunidades microbianas (Flatt et al. 2005; Ribes et al. 2012; Croué et al. 2013). Sin embargo, gracias a las técnicas de "secuenciación de nueva generación" (NGS), como las plataformas 454 o Illumina, se ha incrementado considerablemente el número de secuencias conocidas de estos simbiontes, a menudo no cultivables, incrementando el conocimiento sobre la diversidad de las comunidades microbianas en las esponjas (e.g. Schmidt et al. 2012; Webster & Taylor 2012; Easson & Thacker 2014; Reveillaud et al. 2014).

ESPECIES ESTUDIADAS

La especie objetivo de esta tesis era *Hemimycale columella* (Bowerbank, 1874), junto con sus simbiontes. Sin embargo, otras especies se incluyeron posteriormente en para comprender mejor la particular simbiosis encontrada previamente en *H. columella* (Uriz et al. 2012). Estas especies fueron: *Cliona viridis* y *Hemimycale mediterránea* del oeste del Mare Mediterráneo y Mar Adriático (la segunda), *Hemimycale arabica* y *Crella cyatophora* del Mar Rojo y Océano Indico (la última), *Prosuberites sp.* y *Cinachyrella alloclada* del Mar Caribe.

Hemimycale columella (Bowerbank, 1874)

Order Poecilosclerida, Family Hymedesmiidae

Hemimycale columella es una demosponja incrustante fina con color variable entre el rosa y naranja con tonalidades blanquecinas. Las tonalidades marrones descritas anteriormente corresponden a la especie críptica recientemente descripta *H. mediterranea* (Uriz et al. 2017, Chapter 1). Se distribuye entre los 10 y 60 m de profundidad (Bell & Barnes 2000b) en la cuenca Atlanto-Mediterránea, en superficies rocosas con cierta cantidad de sedimento (Bell & Barnes 2001) y sistemas coralígenos profundos. Esta especie produce algunos compuestos bioactivos con capacidad antimitótica y citotóxica (Becerro et al. 1997).

Hemimycale columella tiene un esqueleto silíceo formado por estróngilos y algunos estilos, y tiene además esférulas de carbonato cálcico de 1-2 μm, que podrían ser las responsables del color blanquecino observado en algunos individuos (Vacelet et al. 1987). Uriz et al. (2012) identificaron las esférulas calcáreas como bacterias intracelulares calcificadoras, denominándolas calcibacterias, y las células de esponja donde se encontraban, similares a los arqueocitos, se denominaron calcibacteriocitos.

Hemimycale mediterranea Uriz, Garate, Agell, 2017

Order Poecilosclerida, Family Hymedesmiidae

Esta especie es morfológicamente críptica con *H. columella* (Uriz et al. 2017, Capítulo 1). *H. mediterranea* tiene color marrón claro, y su morfología ligeramente diferente a *H. columella*: tiene estróngilos menores y las áreas inhalantes menos elevadas. Se distribuye por todo el Mar Mediterráneo y por el Mar Adriático.

Hemimycale arabica Ilan, Gugel, Van Soest, 2004

Order Poecislosclerida, Family Hymedesmiidae

Es una esponja incrustante de color azul oscuro-gris cuyo esqueleto está formado por estróngilos y estilos, más cortos que los de *H. columella*. Se

distribuye por el centro y norte del Mar Rojo, y vive en colonias del coral *Millepora dichotoma*. Su reproducción tiene lugar en los meses de primavera y verano (Ilan et al. 2004).

Crella cyathophora (Carter, 1869)

Order Poecilosclerida, Family Crellidae

C. cyathophora es una esponja rosa, casi blanca, debido a la acumulación de esférulas calcáreas acumuladas en su periferia. La superficie tiene numerosos poros aerolados y elevados y ósculos esparcidos. Las espículas son acantoxas y estróngilos. Esta especie habita en aguas poco profundas del Mar Rojo y Océano Indico.

Cinachyrella alloclada (Uliczka, 1929)

Order Spirophorida, Family Tetillidae

La especie *C. alloclada* es una esponja globular de color naranja-amarillo, más claro en su interior, con ósculos pequeños (0.1-0.5 cm) y varios porocálices (cavidades con poros). Las espículas son grandes oxas, estiloides, estróngilos y subestilostilos, protrienas con el rhabdo redondeado, prodienas, anatrienas y sigmaspiras. Se distribuye de 3 a 80 m de profundidad en aguas templadas y tropicales del Océano Atlántico (Rützler 1987).

Cliona viridis (Schmidt, 1862)

Order Hadromerida, Family Suberitidae

Cliona viridis es una esponja excavadora con tres estados de crecimiento (alfa, beta y gamma). El color varía dependiendo de los individuos y el hábitat: amarillo blanquecino, marrón, verdoso o negruzco. Sus espículas son tilostilos, espirasteras y anfiasteras. Se ha observado en la cuenca atlanto-mediterránea entre 1 y 80 m de profundidad (Rosell & Uriz 2002).

Prosuberites sp.

Order Hadromerida, Family Suberitidae

Es una especie no descrita actualmente, donada amablemente por Klaus Rützler, recogida en el Mar Caribe (Florida). Es un pequeño fragmento de una esponja incrustante y muy fina, con color variable entre el amarillo y el blanco. Las espículas son tilostilos orientados perpendicularmente al sustrato.

OBJETIVOS

Esta tesis en principio iba dirigida al estudio de las características biológicas, ecológicas y moleculares del único representante del género *Hemimycale* (Demospongiae, Poecilosclerida) en la cuenca atlanto-mediterránea: la especie *Hemimycale columella.* Esta especie parecía tener diferentes patrones de crecimiento y supervivencia dependiendo de la profundidad a la que se encontrara en el noroeste del mar Mediterráneo. Además, cuando se quería comparar la genética de poblaciones entre las poblaciones someras y profundas de *H. columella* con microsatélites específicamente diseñados para la misma, la mayoría de estos no amplificaron en los individuos de poblaciones someras. Los diferentes ciclos de vida observados, junto con las ligeras diferencias morfológicas y genéticas hicieron redirigir los objetivos de la tesis. En primer lugar se quería confirmar si las supuestas diferencias biológicas entre poblaciones eran reales, y en caso positivo si se debían a que las poblaciones someras y profundas en realidad pertenecían a dos especies crípticas.

Además, *H. columella* (individuos de poblaciones profundas) alberga una bacteria intracelular capaz de calcificar, lo que hizo que se especulara sobre la relación de la simbiosis con la biología y ecología de la esponja (Uriz et al. 2012). El estudio de la simbiosis entre esponjas y bacterias calcificadoras tiene implicaciones evolutivas ya que podría aclarar el papel de las bacterias simbiontes en la formación de esqueletos en metazoos primitivos. Los individuos de poblaciones someras también presentaban

esférulas calcáreas similares a las identificadas en *H. columella,* por lo que la simbiosis no sería específica.

Se planteaban varias cuestiones relacionadas con esta particular simbiosis y sus potenciales funciones: ¿La bacteria calcificadora la de las poblaciones someras es la misma que la de las poblaciones profundas? ¿Esta simbiosis es exclusiva de *H. columella* o está presente en otras especies? ¿Está presente en otras latitudes? ¿Representa alguna ventaja para la esponja? Además, la identificación taxonómica de la bacteria era necesaria para localizarla en los tejidos de la esponja, así como determinar su presencia en otras especies para considerar posibles patrones de coevolución.

La tesis se ha dividido en cinco capítulos de acuerdo a los cinco objetivos principales.

Capítulo 1

Confirmar si las supuestas poblaciones de *H. columella* con diferentes ciclos de vida pertenecen a diferentes especies. Para ello se llevó a cabo un estudio filogenético de individuos de *H. columella* recogidos a lo largo del mar Mediterráneo con tres marcadores moleculares (fragmentos de los genes 18S rRNA, 28S rRNA y COI mtDNA). Se incluyeron además otras especies de la familia Crellidae para cocnocer las relaciones taxonómicas entre *Hemimycale* y *Crellidae.*

Capítulo 2

Determinar las estrategias biológicas de las dos especies crípticas de *Hemimycale* descubiertas en el Capítulo 1, y comprobar si estas estrategias dependen de las condiciones ambientales o son específicas de cada especie. Las características biológicas incluyen las tasas de crecimiento y la supervivencia, los procesos de fusión y fisión. Los factores ambientales analizados fueron la temperatura y la concentración de nutrientes (C y N) disueltos y particulados (DOC, POC, DON y PON).

Capítulo 3

Investigar la identidad taxonómica de la calcibacteria endosimbionte en diferentes especies con esférulas calcáreas en sus tejidos, así como los mecanismos que conllevan a la acumulación de estas bacterias en la periferia y su posible implicación en la formación de esqueletos en los metazoos primitivos.

Se utilizaron varias técnicas moleculares y microscópicas, incluyendo el diseño de una sonda especifica de calcibacterias para CARD-FISH, estudios de ultraestructura y microscopía confical, el clonaje del gen 16S ribosomal y análisis filogenéticos de los mismos.

Capítulo 4

Comparar las comunidades microbianas de las especies que albergan esférulas calcáreas similares a las identificadas en *H. columella.* Se realizaron análisis de pirosecuenciación (plataforma 454) en las siguientes especies: *Cliona viridis, Hemimycale arabica, Crella cyathophora, Cinachyrella alloclada* y *Cinachyrella sp.*, recogidas del Mar Mediterráneo, Mar Rojo y Mar Caribe, y del Océano Indo-Pacífico.

Capítulo 5

Investigar el papel anti-predatorio de las esférulas calcáreas producidas por la calcibacteria, acumuladas en el ectosoma de *H. columella*, y el de los compuestos químicos producidos por la esponja, contra los depredadores potenciales de la comunidad bentónica, y comprobar si ambos actúan de forma sinérgica. Se llevaron a cabo experimentos en el laboratorio con peces (*Parablennius incognitus*) y erizos (*Paracentrotus lividus*), y en el mar con peces de la comunidad (*Parablennius incognitus, Chromis chromis*, *Coris julis*, *Diplodus vulgaris* y *Oblada melanura*).

RESULTADOS

Capítulo 1: Filogenias moleculares confirman la presencia de dos especies crípticas de *Hemimycale* en el Mar Mediterráneo y revelan la polifilia de los géneros *Crella* y *Hemimycale* (Demospongiae, Poecilosclerida).

En este capítulo se realizaron reconstrucciones filogenéticas con tres marcadores moleculares (18S y 28S rRNA y COI mitocondrial), con muestras de individuos de los que *a priori* se consideraba *Hemimycale columella,* recogidas a lo largo del Mar Mediterráneo. Los tres marcadores moleculares confirmaron la presencia de una especie críptica con *H. columella*, a la que se denominó *Hemimycale mediterranea*. Los caracteres morfológicos que diferencian a las dos especies son sutiles pero consistentes. En primer lugar, las espículas (estróngilos asimétricos) son ligeramente menores en *H. mediterranea,* y en esta especie aparecen algunos estilos, raros en *H. columella.* El diámetro de las áreas inhalantes de *H. mediterranea* son menores (3 mm), con los bordes no tan elevados. El color también difiere entre las dos especies: varía entre el anaranjado y el rosado en *H. columella,* mientras que *H. mediterranea* presenta un color variable entre rosa pálido y marrón.

En cuanto a la taxonomía de los géneros *Crella* y *Hemimycale*, las tres filogenias realizadas soportan la polifilia de ambos géneros. *Crella cyathophora* presenta más diferencias genéticas con las *Crellas* mediterráneas que con *H. arabica,* lo que sugiere que podría pertenecer a un género diferente. Además, *H. arabica* presenta muchas diferencias genéticas con las *Hemimycales* atlanto-mediterráneas, además de las características físicas (color, presencia de estilos, ausencia de bordes en áreas inhalantes), por lo que pertenecería a otro género, que incluiría a *C. cyathophora* dada la similitud genética entre ellas (no hay diferencias en el gen COI).

Capítulo 2: Diferencias en los ciclos de vida y dinámica de crecimiento de las dos especies morfológicamente crípticas en el Mar Mediterráneo.

Se realizó un seguimiento fotográfico mensual en el litoral catalán de dos poblaciones de las dos especies crípticas *H. columella* y *H. mediterránea.* Además se recogieron muestras de agua para analizar los nutrientes disueltos y particulados (DOC, POC, DON, PON) y se midió la temperatura *in situ*

durante todo el monitoreo. Los resultados indican una dinámica de crecimiento y ciclo de vida contrarias entre las dos especies. *H. columella* presenta las tasas de crecimiento más elevadas en los meses en que la temperatura es menor y tiene un ciclo de vida multianual. Por otro lado, *H. mediterranea* crece más rápido en los meses cálidos y tiene un ciclo de vida anual, algo hasta ahora desconocido en demosponjas. En cuanto a las fusiones y fisiones también mostraron diferencias, ya que en la población de *H. mediterranea* se registraron mayor número de fisiones justo antes de la reproducción, mientras que en *H. columella* el mayor número de eventos se dio en los meses posteriores.

En lo referente a los factores ambientales, todos excepto la T y el PON resultaron significativamente diferentes entre los dos hábitats correspondientes a las especies. Las temperaturas máximas se alcanzaron en verano en ambas localidades, aunque en el hábitat de *H. mediterranea* fue 4ºC mayor que en el hábitat de *H. columella*. El DOC fue mayor en en el primer hábitat en primavera y los primeros meses de verano, pero alcanzó valores más altos en el hábitat de *H. columella* al final de verano (agosto-septiembre). Aquí, se alcanzaron los valores más elevados de la fracción particulada de C (POC), donde además se dieron dos picos en los inviernos posteriores (2013-2014). Los valores más altos de DON se dieron en el hábitat de *H. mediterranea*, y el PON no presentó diferencias significativas entre ambos hábitats, y durante los meses posteriores en el de *H. columella* se alcanzaron los valores máximos en primavera (Abril 2013 y Mayo 2104).

Sólo la T y el DON mostraron una correlación, negativa en ambos casos, con la tasa de crecimiento de *H. columella*. La población de *H. mediterranea* no presentó correlación alguna con los parámetros ambientales estudiados.

Capítulo 3: Bacterias endosimbióticas calcificadoras presentes en diferentes esponjas y océanos.

Se realizaron diversos análisis (microscópicos y filogenéticos) en las esponjas en las que se habían observado esférulas calcáreas, con el fin de identificar la calcibacteria, cuantificarla y conocer su posición filogenética. Por un lado, la

observación con microscopía óptica de diferentes especies de esponjas (*H. columella* –blanca y rosada–*, Cliona viridis, Prosuberites sp, Cinachyrella alloclada* y *Crella cyathophora*) recogidas de diferentes mares (Mar Mediterráneo, Mar Rojo y Mar Caribe) revelaron que todas poseían esférulas calcáreas similares a las identificadas previamente en *H. columella*. En los individuos más blanquecinos de esta especie, se observó que las esférulas calcáreas se acumulaban en el ectosoma de la esponja. Además, se observaron los calcibacteriocitos con filopodios y pseudópodos capaces de trasportar las calcibacterias hacia la periferia. Dentro de los calcibacteriocitos se observaron numerosas vacuolas que contenían calcibacterias, a menudo una sola, pero también aparecían vacuolas que contenían más de una calcibacteria dividiéndose. En general, una vez las calcibacterias calcificaban se degradaba el contenido interior, aunque en algunos casos se observa material que podría corresponder a sus formas de resistencia. En el mesohilo de las larvas, se observaron calcibacterias no calcificadas dividiéndose, supuestamente después de haber sido liberadas por los calcibacteriocitos provenientes de la madre.

La hibridación *in situ* (CARD-FISH) realizada con la sonda diseñada específicamente para calcibacteria, junto con los "helpers" y competidores permitió identificar la calcibacteria en el mesohilo de *H. columella, C. viridis, C. alloclada* y *Prosuberites sp.,* y dentro de calcibacteriocitos de *H. columella* y *Prosuberites sp.*. Esto confirma que la calcibacteria corresponde a la secuencia bacteriana más abundante en *H. columella,* perteneciente a la clase Alpha-proteobacteria. Los controles negativos utilizando la sonda "non-sense" y la hibridación realizada sobre *Crambe crambe* (Schmidt, 1862) no mostraron hibridación, indicando que las hibridaciones observadas no eran un artefacto sino que correspondían a la calcibacteria.

El número de calcibacterias según el tejido (ectosoma-coanosoma) y el morfotipo (blanca-rosa) de *H. columella* fue significativamente diferente: los individuos blanquecinos presentaban más calcibacterias en el ectosoma que los individuos rosados, mientras que no había diferencias significativas entre los coanosomas de ambos morfotipos. Individuos con larvas tenían menos calcibacterias que los individuos en estado no reproductivo.

Por último, con la reconstrucción filogenética realizada con el gen ribosomal 16S de las calcibacterias obtenidas de *H. columella* y *C. viridis,* junto con las secuencias más cercanas de la base de datos SILVA, se obtuvo un clado bien soportado estadísticamente, dentro del grupo SAR116. El clado está formado por secuencias bacterianas obtenidas de esponjas en las que se había observado esférulas calcáreas, dos secuencias de dos corales y dos secuencias de muestras de agua tropical. En este clado se pueden diferenciar siete OTUs bacterianos, que pertenecerían a siete especies bacterianas pertenecientes a dos géneros.

Capítulo 4: Similitudes y diferencias entre microbiomas de esponjas con bacterias calcificadoras.

Se realizó la secuenciación masiva (pirosecuenciación con la plataforma 454) de los simbiontes de diferentes esponjas en las que se habían observado esférulas calcáreas, pertenecientes a diferentes familias: *H. columella y H. mediterranea* (Fam. Hymedesmiidae) y *C. viridis* (Fam. Clionaidae) del noroeste del Mar Mediterráneo; *H. mediterranea* (Fam. Hymedesmiidae) del Mar Adriático; *C. cyathophora* (Fam. Crellidae) y *H. arabica* (Fam. Hymedesmiidae) del Mar Rojo; *C. cyathophora* del Oceano Indo-Pacífico; *C. alloclada* y *Cinachyrella sp.* (Fam. Tetillidae) del Mar Caribe.

Se obtuvieron un total de 27 filos bacterianos, de los cuales, Proteobacteria fue uno de los más abundantes, con >70 % de la abundancia relativa en *H. columella*, *H. mediterranea*, *C. viridis*, *C. cyathophora* y *H. arabica*. En las muestras de *C. alloclada* y *Cinachyrella sp.* filos como Tenericutes, Acidobacteria y Cloroflexi también eran abundantes. En las muestras donde Proteobacteria era el filo dominante, la clase Alpha-proteobacteria era la más abundante en *H. columella, C. viridis* y *C. cyathophora* del Océano Indo-Pacifico. En las muestras del Mar Rojo, las clases Alpha- y Gamma-proteobacteria presentaron una abundancia relativa similar. Por último, en *H. mediterranea* del Mar Adriático, predominaba la clase Beta-proteobacteria, mientras que en las muestras del oroeste del Mar Mediterráneo la abundancia relativa era similar entre las clases Alpha- y Beta-proteobacteria, que podría indicar una influencia medioambiental en el microbioma de estas muestras.

Los microbiomas de las diferentes especies resultaron ser específicos de cada especie, dominados por uno o dos OTUs. La especie con el índice más alto de diversidad (H´) fue *Cinachyrella sp.* y la que menor índice tenía fue *H. mediterranea* del Mar Adriático. *Cinachyrella sp.* presentó el número de OTUs específicos más elevados, mientras que *C. alloclada* fue la especie con menor porcentaje de OTUs específicos.

La alineación de las secuencias resultantes frente a las dos secuencias de calcibacteria conocidas de *C. viridis* y *H. columella,* revelaron la presencia de 30 OTUs, distribuidos en estas dos especies y además en *C. cyathophora*, *H. arabica* y *C. alloclada*, indicando la amplia distribución geográfica y taxonómica de bacterias calcificadoras. No se descarta que otras bacterias filogenéticamente distintas puedan calcificar, dado que en *H. mediterranea* se habían observado esférulas calcáreas similares a las de *H. columella*, y sin embargo no tenía ningún OTU de la clase Alpha-proteobacteria.

Capítulo 5: Las esférulas calcáreas producidas por las calcibacterias protegen la esponja *Hemimycale columella* de la depredación más que los metabolitos secundarios.

Se realizaron diferentes experimentos en mar (*Chromis chromis*, *Oblada melanura*, *Coris julis* y *Diplodus vulgaris*) y en laboratorio con peces (*Parablennius incognitus*) y erizos de mar (*Paracentrotus lividus*), a los que se les ofrecía alimento que contenía, por un lado, extracto químico obtenido de la esponja, y por otro, las esférulas calcáreas producidas por las calcibacterias. Además, a los erizos también se les ofrecía alimento que incluía los dos compuestos (extracto químico y esférulas calcáreas) para evaluar un posible efecto sinérgico entre los dos componentes.

Los resultados del experimento con erizos mostraron un efecto antidepredatorio similar en los dos compuestos, aunque no se observó efecto sinérgico.

En el experimento de laboratorio con *P. incognitus* los resultados mostraron efecto antidepredatorio sólo por parte de las esférulas calcáreas, mientras que el extracto químico no tuvo ningún efecto.

En el experimento realizado en la comunidad de peces los resultados mostraron un claro efecto antidepredatorio por parte de las esférulas calcáreas en todas las especies de peces y un efecto antidepredatorio por parte del extracto químico en dos de las cuatro especies analizadas (*O. melanura* y *D. vulgaris*).

CONCLUSIONES GENERALES

❖ Especies crípticas y filogenia de los géneros *Hemimycale* y *Crella*.

Se ha descrito una nueva especie morfológicamente críptica con *H. columella*, la especie *H. mediterranea*, distribuida por el Mar Mediterráneo a menor profundidad que *H. columella*.

Hemimycale columella, la especie tipo del género, posiblemente tiene origen atlántico, y habita sistemas profundos de coralígeno en el noroeste mediterráneo, aunque sin descartar su presencia en el este del Mar Mediterráneo.

La presencia de especies crípticas no sólo puede afectar en los estudios de biodiversidad en un sistema, sino que puede ocultar diferencias biológicas y ecológicas, llegando a invalidar las políticas de gestión y conservación de esos sistemas.

Hemimycale es un género monofilético a pesar de las diferencias entre las especies atlanto-mediterráneas y *H. arabica* del Mar Rojo.

Hemimycale forma un grupo monofilético junto con *Crella*, lo que sugiere que el primero podría situarse dentro de Crellidae más que Hymedesmiidae.

Crella cyathophora forma un nuevo género más cercano a *H. arabica* que a las *Crellas* atlanto-mediterráneas.

❖ Características ecológicas y biológicas de las especies crípticas de *Hemimycale* en el Mar Mediterráneo.

H. mediterranea y *H. columella* presentan un ciclo de vida opuesto, la primera presenta un ciclo de vida anual, la primera vez observado en Demosponjas, mientras que *H. columella* es multianual. Las tasas de crecimiento de las dos

especies también son diferentes: *H. mediterranea* crece más en verano y no se ve afectada por ninguno de los parámetros ambientales estudiados, mientras que *H. columella* crece más en los meses más fríos, y presenta correlación negativa con la temperatura.

Los factores ambientales medidos en los dos hábitats eran significativamente diferentes y podrían haber contribuido a la distribución ecológica de las dos especies.

La disponibilidad de alimento puede haber contribuido a la desaparición de *H. mediterranea* después del periodo reproductivo, por lo que no se descarta que la especie sobreviva después de la liberación de las larvas en localidades con la concentración de alimento más apropiada para afrontar el periodo post-reproductivo.

Las fuertes diferencias biológicas contrastan con las pocas diferencias fenotípicas y resaltan la necesidad de desenmascarar la diversidad críptica para garantizar la fiabilidad de estudios ecológicos.

❖ Localización y cuantificación de las calcibacterias.

La sonda diseñada para calcibacteria confirmó la presencia de calcibacterias en las esponjas *H. columella, Cliona viridis, Cinachyrella alloclada* y *Prosuberites sp.* Además, las calcibacterias se acumulan en la periferia de los individuos blanquecinos de *H. columella.* Se confirma la transmisión vertical de la calcibacteria ya que se han observado calcibacteriocitos con calcibacterias en larvas de *H. columella*, y la transmisión horizontal, por la presencia de OTUs de calcibacteria en dos muestras de agua. Se ha demostrado además la distribución circumtropical de las calcibacterias.

❖ Taxonomía y filogenia de las calcibacterias.

Al menos se han identificado siete OTUs relativos a calcibacterias ampliamente distribuidos geográfica y taxonómicamente, en esponjas, corales y agua de mar.

Las calcibacterias pertenecen al grupo SAR116 dentro de las Rickettsiales, como un grupo hermano de las Rhodospirillales, dentro de la Clase Alpha-proteobacteria, en el filo Proteobacteria.

Sin embargo, otras bacterias distantes taxonómicamente pueden producir esférulas calcáreas similares a las producidas por calcibacterias, ya que se han observado estas esférulas en los tejidos de *H. mediterranea*, la cual carece de los OTUs de Alpha-proteobacteria responsable de la calcificación en otras especies.

❖ Comunidades microbianas de esponjas que albergan calcibacterias.

Las comunidades microbianas son específicas de cada especie estudiada, y presentan poca variación entre réplicas, con la excepción de *H. mediterranea,* cuyos individuos del noroeste mediterráneo eran notablemente diferentes a los del Mar Adriático, lo que sugiere cierta influencia de bacterias presentes en el agua circundante.

El filo Proteobacteria presenta una abundancia relativa mayor en las especies identificadas como "especies con baja abundancia microbiana" que en las consideradas "especies con alta abundancia microbiana", con la excepción de *H. mediterranea,* en la que el filo Tenericutes tiene una abundancia relativa similar al filo Proteobacteria.

❖ Defensas antidepredatorias en *H. columella*.

Las esférulas calcáreas producidas por las calcibacterias protegen a la esponja de depredadores generalistas, mientras que los compuestos químicos protegen frente a depredadores específicos.

Los dos componentes (esférulas calcáreas y compuestos químicos) no tienen un efecto sinérgico, sino más bien aditivo, ya que la presencia de ambos ayuda en la defensa contra más depredadores potenciales.

La protección frente a depredadores potenciales es una de las ventajas que *H. columella* obtiene de la simbiosis con las calcibacterias, lo que ha podido ayudar a establecer la simbiosis de calcibacterias con esponjas.

Es la primera vez que se ha identificado en esponjas una bacteria simbionte productora de una defensa física antipredatoria.

❖ Implicaciones evolutivas de la simbiosis con calcibacterias.

La amplia distribución de la calcibacteria a través de los océanos y los anfitriones (esponjas y cnidarios), su acumulación en la periferia de la esponja imitando un exoesqueleto, y su papel defensivo frente a depredadores en *H. columella,* refuerza la hipótesis de la implicación bacteriana en la formación de esqueletos en los Metazoos primitivos.

General Introduction

WHY SPONGES?

Sponges are the oldest extant Metazoans in Earth (Love et al. 2009). They are among the most important members of benthic communities at all latitudes and at a wide range of depths, representing the dominant group in many temperate, tropical, and cold-water habitats (Uriz et al. 1992a; McClintock et al. 2005; Wulff 2012). Sponges are sessile, filter-feeding, aquatic animals. They are present in both freshwater and marine systems with ca. 8500 known species (van Soest et al. 2012) and ca. 29000 more are predicted to be discovered in the forthcoming years (Appeltans et al. 2012). They play decisive functions in benthic ecosystems by interacting with a wide range of organisms such as algae, plants, animals and microbes (Wulff 2006). They provide micro-habitats for other organisms, favour the stabilization of the substrate, transfer nutrients from the pelagic to the benthic system, are a source of food for other animals, and recycle organic matter, through their associate bacteria that play a major role in the biogeochemical cycles (e.g. Dunlap & Pawlik 1996; McClintock et al. 2005; Bell 2008; de Goeij et al. 2013; Zang et al. 2013). Sponges have been adapted to a wide range of environmental conditions to survive across oceans and time. They are able to obtain nutrients from seawater from both particulate and dissolved fractions, modify their shape to cope with water hydrodynamics, and reproduce sexual and asexually to ensure species persistence (Ayling 1980; Bell & Barnes 2000a; de Goeij et al. 2008).

The sessile style of life of sponges likely has favoured an evolutionary fixation of an array of chemical defences. Sponges produce bioactive metabolites to compete with neighbours for the space or to avoid being recovered by fast-growing foulers (e.g. Uriz et al. 1995), and in particular to avoid being predated by mobile animals. Predation is an important shortcoming for sponges. Among potential sponge predators are crustaceans, echinoderms, fishes, and turtles (Meylan 1988; Cerrano et al. 2000; Santos et al. 2002; Loh & Pawlik 2009). A series of bioactive molecules have been isolated from sponges and their efficacy against predators has been assayed in numerous laboratory and field experiments (Uriz et al.

1996a; Becerro et al. 2003; Ruzicka & Gleason 2009). Many of these secondary metabolites have been deeply investigated for pharmacological applications (Uriz et al. 1992b; Sipkema et al. 2005; Leys et al. 2011), what has fuelled different types of research, from basics to applied, on sponges in the last decades. Moreover, in addition to chemical compounds, sponges can also be physically defended by siliceous, and calcium carbonate-made structures, called spicules (e.g. Uriz et al. 2003; Hill et al. 2005).

Sponge taxonomy is an intriguing issue that needs from molecular (genetic) and morphological (phenotypic) approaches. Misidentification of sponges, as of any other organism, may veil biological features such as divergent reproduction patterns and growth dynamics, inter-species interactions, adaptation to particular environmental conditions (Knowlton et al. 1993; Prada et al. 2014; De Meester et al. 2016), and other characteristics that may lead to differences in the ecosystem functioning (Loreau 2004). When morphological characters are not enough to identify sponge species, as in the case of cryptic species, biological studies and molecular tools are necessary to discern them (Blanquer & Uriz 2007; Blanquer et al. 2008b; de Paula et al. 2012). The importance of identifying sponges correctly, which is particularly difficult when they are morphologically cryptic, become evident when those sponge species are considered in preservation programmes, as confounding species lead to incorrect assessment of their biological and ecological traits and this might decrease success of the *ad hoc* implemented management policies (e.g. Forsman et al. 2010).

Successive growth and regression episodes together with fusion and fission events reflect the continuous interactions of sponges with their environment (Garrabou & Zabala 2001; Tanaka et al. 2002; Blanquer et al. 2008; De Caralt et al. 2008). The sponge strategy for change "*to change every thing to avoid any drastic change*" help them to cope with competition, predation, interference facilitation, and other interaction modes (Cebrian & Uriz 2006), and determines sponge shape and size with time in a given habitat, allowing at the same time their long-term persistence.

Temperature and/or nutrient availability have been proved relevant environmental factors that influence sponge growth (e.g. De Caralt et al.

2008; Koopmans & Wijffels 2008). Temperature has been reported to affect positively or negatively to sponge growth depending on the species (Tanaka 2002; Duckworth et al. 2004; Page et al. 2005; Blanquer et al. 2008b; De Caralt et al. 2008; Koopmans & Wijffels 2008). Food availability is also decisive for growth and survival of sponges (Reiswig 1973; Sebens 1987; Koopmans & Wijffels 2008), although they can feed either on particulate (Ribes et al. 1999a, 2012; De Caralt et al. 2008) or dissolved (de Goeij et al. 2008) organic matter.

Some of the above mentioned characteristics of sponges make them unique targets for studies on Metazoa evolution and phylogeny, life histories, and chemical ecology and its applications to the discovering of new drugs. But sponge ecology and life histories cannot be analysed properly without considering the thousands of associated partners that have accompanied them since the beginning (Wilkinson 1984) and that are responsible to a great extent for their evolutionary success through multiple interactions (Taylor et al. 2007a).

THE HOLOBIONT SPONGE: a multi-partner complex world

Sponges live together with microbial communities within their tissues, which include viruses, archaea, unicellular eukaryotes, fungus, and bacteria (e.g. Webster & Taylor 2012; He et al. 2014; Zhang et al. 2014). These microorganisms can be acquired horizontally from the surrounding seawater or vertically from mother to larvae, although both mechanisms have been reported in the same species (e.g. Sipkema et al. 2015). The bacteria-sponge symbioses are the most studied so far and up to 20 of bacteria phyla have been identified from sponge species (e.g. Webster & Taylor 2012). Many of these bacteria are shared by many species, which has lead to name them as sponge core bacteria (Schmitt et al. 2012a, b). These bacteria form dense and abundant communities in the sponge mesohyl, with abundances several orders of magnitude higher than those in the surrounding seawater (Hentschel et al. 2006). Associated bacteria can occupy up to ca. 40% of the sponge volume (Vacelet & Donadey 1977) but bacteria concentration vary among sponge species and thus sponges have been conventionally classed,

according to the diversity and abundance of their microbiomes, into "high microbial abundance" (HMA) sponges or bacteriosponges and "low microbial abundance" (LMA) sponges (Hentschel et al. 2003; Ribes et al. 2015).

Some morphological and functional characteristics seem to characterise HMA and LMA sponges. HMA sponges have a dense mesohyl and a complex aquiferous system, which retains the water inside the sponge for longer than LMA sponges with a less dense mesohyl and a well-developed aquiferous system, able to pump large volumes of seawater (Weisz et al. 2008). However, microbiome profiling studies, which are proliferating in the last years, report exceptions to this, at first sight, clear division. Some previously considered LMA sponges according to their aquiferous traits, showed higher diversity indices and bacteria abundances than some HMA sponges (e.g. Blanquer et al. 2013).

The study of sponge bacterial assemblages has been approached with several techniques, which are complementary and slowly have improved the knowledge on the sponge-bacteria interactions. Electron microscopy informs on the diversity and ultrastructure of bacteria morphotypes and their location in the sponge tissues, whether intracellular or extracellular. Ultrastructure images may also help to distinguish between bacterial symbionts and bacteria that are used as a food by the sponge (Vacelet & Donadey 1977; Wilkinson et al. 1984; Santavy et al. 1990; Croué et al. 2013; Gloeckner et al. 2014). Cultivation and 16S rRNA gene cloning and sequencing facilitate phylogenetic reconstructions of bacterial symbionts, and through coupling X-ray microanalysis, also has allowed the identification of bacteria that produce some bioactive compounds (e.g. Turon et al. 2000; Hentschel et al. 2001; Webster & Hill 2001). Catalysed Reporter Deposition-Fluorescence In Situ Hybridisation (CARD-FISH) has been used on sponge tissues to identify and locate different types of bacteria and bacterial genes and to quantify microbial communities, (Flatt et al. 2005; Ribes et al. 2012; Croué et al. 2013). On the other hand, "new generation sequencing" (NGS) techniques, such as 454 and Illumina platforms, have triggered dramatically the number of bacteria and archaea sequences recovered form sponge. These

tools altogether, have allowed researchers to unveil uncultivable bacteria, improving our knowledge on the real diversity of sponge microbiomes (Webster et al. 2010; Schmidt et al. 2012; Webster & Taylor 2012; Easson & Thacker 2014; Reveillaud et al. 2014).

STUDY SPECIES

The main target sponge species of this thesis was *Hemimycale columella* and its bacterial symbionts. However, other species were posteriorly included in the research to better understand the extent of a particular symbiosis with calcifying bacteria previously found in *H. columella* (Uriz et al. 2012). The species included in this thesis were: *Cliona viridis* and *Hemimycale mediterranea* from western Mediterranean and Adriatic Sea (the latter), *Hemimycale arabica* and *Crella cyatophora* from Red Sea and Indian Ocean (the latter), *Prosuberites* sp. and *Cinachyrella alloclada* from the Caribbean Sea.

Hemimycale columella (Bowerbank, 1874)

Order Poecilosclerida, Family Hymedesmiidae

Hemimycale columella is a thick encrusting sponge (Fig. 1). Its external colour varies between orange and pink with whitish tinges. Brownish, flesh-coloured individuals that were previously described as *H. columella* are now known to belong to the new cryptic *H. mediterranea* (Uriz et al. 2017, Chapert 1). The species has been recorded from ca. 10 m to up to 60 meters of depth (Bell & Barnes 2000b), across the Atlanto-Mediterranean basin. It dwells on rocky surfaces with a certain amount of sediment (Bell & Barnes 2001) and on deep coralligenous assemblages. The species has been reported to produces some bioactive compounds with antimitotic and cytotoxic activities (Becerro et al. 1997).

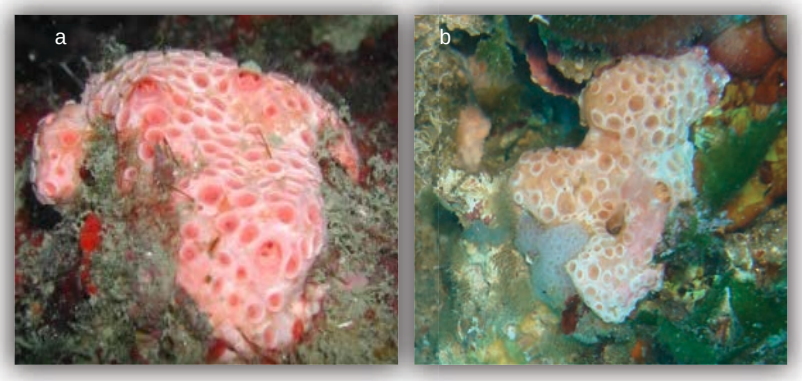

Figure 1. Two individuals of *H. columella* showing different colorations: a) pinkish, and b) whitish.

A previous study of reproduction traits of *H. columella* (Pérez-Porro et al. 2012) was instead of the new cryptic species *H. mediterranea*. It has been observed that individuals of *H. columella* release their larvae at the end of October or beginning of November.

The species has a siliceous skeleton formed by anysostrongyles with a few styles (Bowerbank, 1874) and harbours huge amounts of small calcareous spherules, which were hypothesized as responsible of the creamy colour of some individuals (Vacelet et al. 1987). Uriz et al. (2012), identified the calcareous spherules as endosymbiotic calcifying bacteria, named calcibacteria, which were located inside archeocyte-like sponge cells, called calcibacteriocytes.

Hemimycale mediterranea Uriz, Garate, Agell, 2017

Order Poecilosclerida, Family Hymedesmiidae

This species (Fig. 2) is a morphologically cryptic species with *H. columella* (Uriz et al. 2017, Chapter 1). *H. mediterranea* has brownish colour, and the encrusting morphology slightly different from *H. columella*. It presents slightly smaller strongyles and inhalant areas than *H. columella* (See description in

Chapter 1). The species is spread across western, eastern, and central Mediterranean and Adriatic Sea.

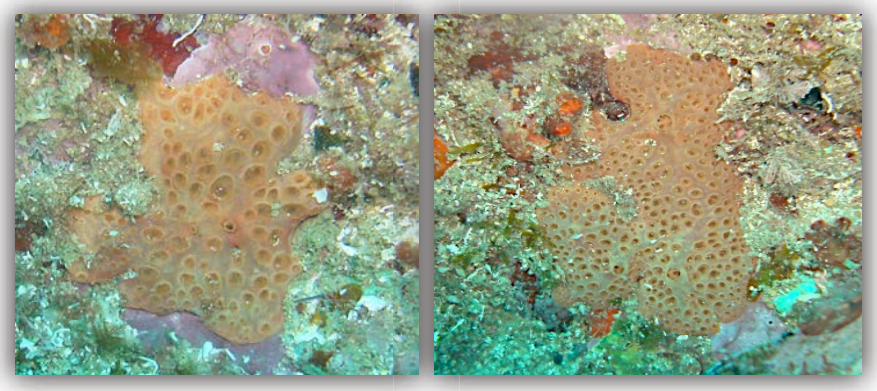

Figure 2. Two individuals of *H. mediterranea* from the western Mediterranean Sea.

Hemimycale arabica Ilan, Gugel, Van Soest, 2004

Order Poecilosclerida, Family Hymedesmiidae

It is a encrusting deep blue to grey sponge (Fig. 3) with skeleton formed by strongyles and styles, shorter than those of *H. columella*. It is distributed in central and northern Red Sea. The sponge lives on colonies of the coral *Millepora dichotoma,* and its reproduction takes place on spring and summer months (Ilan et al. 2004).

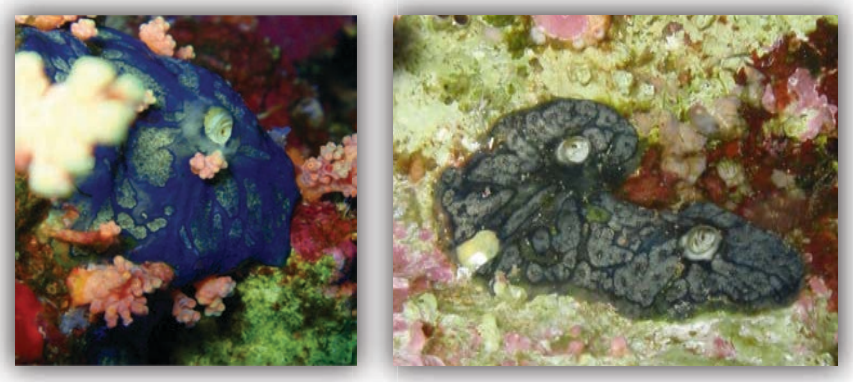

Figure 3. Two individuals of *H. arabica* with different blue and grey tinges.

Crella cyathophora (Carter, 1869)

Order Poecilosclerida, Family Crellidae

C. cyathophora (Fig. 4) is a pink to almost white sponge. The white colour is due to accumulation of calcareous spherules at the periphery. Sponge surface has numerous subcircular, areolate and elevated pore areas and sparse oscules. The spicules are acanthoxeas and strongyles. The species inhabits the Red Sea and Indian Ocean shallow waters.

Figure 4. *C. cyathhophora*

Cinachyrella alloclada (Uliczka, 1929)

Order Spirophorida, Family Tetillidae

The species *C. alloclada* (Fig. 5) is a globular sponge orange to yellow in colour outside, lighter inside with small oscules (0.1-0.5 cm) on the top and several porocalyces (pore-bearing cavities). Spicules are large bent oxeas, styloid, strongyles and substylostylotes, protriaenes with rounded rhabd tips, prodiaenes, anatriaenes and sigmaspires. It is distributed from 3 to 80 m of depth, in the temperate-tropical Atlantic Ocean (Rützler 1987).

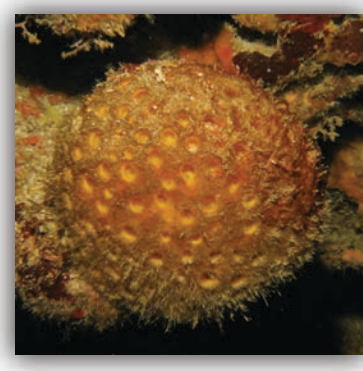

Figure 5. *C. alloclada*

Cliona viridis (Schmidt, 1862)

Order Hadromerida, Family Suberitidae

Cliona viridis (Fig. 6) is an excavating sponge with three stages of growth (alpha, beta and gamma). The colour of the sponge varies depending on specimens and habitats: from yellow-whitish, brownish, greenish or blackish. Its spicules are tylostyles, spirasters and amphiasters. It has been recorded from 1m to ~ 80m depth through the Atlanto-Mediterranean basin (Rosell & Uriz 2002).

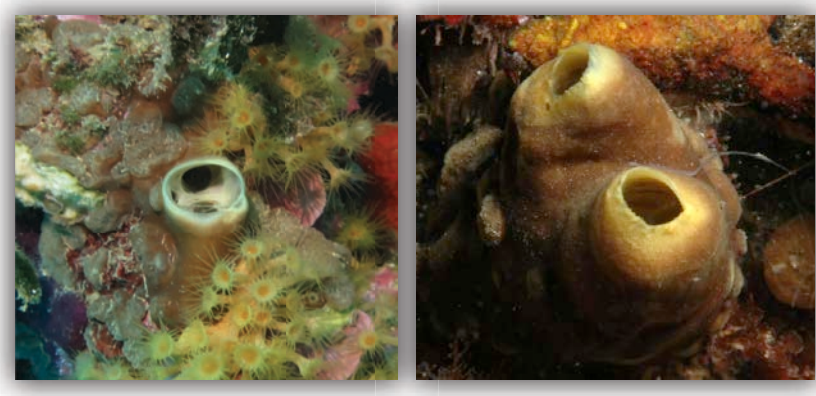

Figure 6. Two individuals of *C. viridis*.

Prosuberites sp.

Order Hadromerida, Family Suberitidae

This is an undescribed (likely new) species kindly supplied by Klaus Ruetzler collected from Florida (Caribbean Sea). It consists in a small thinly incrusting fragment, yellowish to white in colour (Fig. 7). Spicules are tylostyles arranged perpendicularly to the substrate.

Figure 7. *Prosuberites sp.*

Rationale & Objectives

This thesis was initially addressed to study the biology, population genetics, and symbiosis with a calcifying bacteria of the only representative of the genus *Hemimycale* (Demospongiae: Poecilosclerida) in the Atlanto-Mediterranean basin: the species *Hemimycale columella*. Informal observations seemed to suggest contrasting biological traits (i.e. morphology and survival) of the species in the north-western Mediterranean, depending on the depth where the populations were established. These different features suggested some local adaptation of the species populations to depth. However, when shallow and deep populations were analysed for a population genetics' study, using 20 microsatellite loci designed from massive sequencing of deep specimens (González et al. 2015), most of these loci failed to amplify in individuals from shallow populations. Contrasting life span, slight morphological differences, and genetic distances, overpassing those expected for intra-species variation, moved us to redirect the thesis objectives. First, we aimed to confirm whether the strong biological differences between deep and shallow populations, suggested from informal observations, were accurate. Then, we addressed whether deep and shallow *Hemimycale* populations may belong indeed to two morphologically cryptic species.

Moreover, *H. columella* (deep individuals) had been recently reported to harbour an abundant intracellular calcifying bacterium, and this symbiosis was hypothesized to play a relevant role in the species biology and ecology (Uriz et al. 2012). The study of the symbiosis between sponges and calcifying bacteria has evolutionary implications as it might cast light on the role of symbiotic bacteria on early skeletonization of metazoans. Shallow individuals also harboured the small calcareous spherules, which in deep specimens had been proved to be bacteria-mediated (Uriz et al. 2012) and thus, this symbiosis appeared not to be species-specific.

Several questions related to this particular symbiosis were addressed to gain insight in its potential evolutionary and functional roles: Is the calcifying bacterium of shallow populations the same as that of deep *H. columella* individuals? Is the symbiosis limited to *Hemimycale* species or it is also present in other sponges? Is the symbiosis restricted to the Mediterranean or is it spread to other latitudes? Does the symbiosis

represent any advantage for the host sponges? Moreover, the taxonomical identification of these calcifying bacteria was mandatory for locating them in the sponge tissues as well as to explore their presence in other sponges to consider possible co-evolution patterns.

There are five main objectives in this thesis that have been approached in five chapters.

Chapter 1

The aim of chapter 1 was to confirm whether the purported *H. columella* populations with contrasting life spans belonged to the same or to different species. A phylogenetic study of *H. columella* individuals collected across the Mediterranean Sea was performed, using nuclear and mitochondrial gene partitions (18S rRNA, 28S rRNA, and COI). Moreover, other *Hemimycale* species and representatives of the close family Crellidae were included in order to put the two possible *Hemimycale* species into a phylogenetic context and to unveil the taxonomical relationships between the genera *Hemimycale* and Crellidae.

Chapter 2

Chapter 2 was addressed to determine the biological strategies of the two morphologically cryptic species of *Hemimycale* discovered in Chapter 1, and whether these strategies depended on the environmental conditions or were species-specific. The biological strategies studied included sponge growth, survival rates, and fusion and fissions events.

Chapter 3

Chapter 3 aimed to investigate the taxonomic identity of the calcifying endosymbiotic bacterium across several species harbouring morphologically similar calcareous spherules in their tissues, as well as at exploring the cellular mechanisms ending in spherule accumulation at the sponge periphery, forming a kind of exoskeleton, which may support the potential implication of these symbioses in the skeleton formation of Early Metazoa.

The study required the use of an array of molecular and microscopical techniques including the design of a calcibacteria' specific probe for CARD-FISH experiments, ultrastructure and confocal studies,

cloning of the calcibacterium 16S rRNA gene of two sponge species, and phylogenetic analyses.

Chapter 4

In Chapter 4, the microbial profiling of sponge species harbouring small calcareous spherules was compared. We analysed the microbiome of *Cliona viridis, Hemimycale arabica, Crella cyathophora, Cinachyrella alloclada* and *Cinachyrella sp.* from the Mediterranean, Red and Caribbean Seas and Indian Ocean by tag-pyrosequencing (454 platform).

Chapter 5

Chapter 5 was addressed to investigate the purported defensive role of the calcareous coats produced by the calcibacteria in *H. columella versus* that of the chemical compounds produced by the sponge, against sympatric potential predators and whether both bacteria-mediated and chemical defences worked in synergistic or additive ways. Field and laboratory experiments were performed with the sea urchin *Paracentrotus lividus* and the fishes *Parablennius incognitus, Chromis chromis*, *Coris julis*, *Diplodus vulgaris* and *Oblada melanura*.

Chapter 1

Molecular phylogenies reveal a new cryptic *Hemimycale* species in the Mediterranean and show the polyphyly of the genera *Crella* and *Hemimycale* (Demospongiae: Poecilosclerida)

ABSTRACT

Background. Sponges are particularly prone to hide cryptic species as their paradigmatic plasticity often favors species phenotypic convergence as a result of adaptation to similar habitat conditions. *Hemimycale* is a sponge genus (F. Hymedesmiidae, O. Poecilosclerida) with four formally described species, from which only *Hemimycale columella* had been recorded in the Atlanto-Mediterranean basin, on shallow to 80 m deep bottoms. Contrasting biological features between shallow and deep individuals of *H. columella* suggested larger genetic differences than those expected between sponge populations. To assess whether shallow and deep populations belonged indeed in different species, we performed a phylogenetic study of *H. columella* across the Mediterranean. We also included other *Hemimycale* and *Crella* species from the Red Sea, with the additional aim of clarifying the relationships of the genus *Hemimycale*.

Methods. *Hemimycale columella* was sampled across the Mediterranean, and Adriatic Seas. *H. arabica* and *Crella cyathophora* were collected from the Red Sea and Pacific. DNA was extracted from two to three representative specimens per species and locality and amplified for COI (M1-M6 partition), 18S rRNA, and 28S rRNA (D3-D5 partition) and sequenced. Sequences were aligned using Clustal W v.1.81. Phylogenetic trees were constructed under Neighbour Joining (NJ), Bayesian Inference (BI) and Maximum Likelihood (ML) criteria as implemented in Geneious software 9.01. Moreover, spicules of the target species were observed through Scanning Electron microscopy.

Results. The several phylogenetic reconstructions retrieved both *Crella* and *Hemimycale* polyphyletic. Strong differences in COI sequences indicated that *Crella cyathophora* from the Red Sea might belong in a different genus, closer to *Hemimycale arabica* than to the Atlanto-Mediterranean *Crella* spp. Molecular and external morphological differences between *H. arabica* and the Atlanto-Mediterranean *Hemimycale* spp. also suggest that *H. arabica* fit in a separate genus. On the other hand, the Atlanto-Mediterranean Crellidae appeared in 18S and 28S phylogenies as a sister group of the Atlanto-Mediterranean *Hemimycale*. Moreover, what was known up to now as *H. columella,* is formed

by two cryptic species with contrasting bathymetric distributions. Some small but consistent morphological differences allow species distinction.

Conclusions. A new family (Hemimycalidae) including the genus *Hemimycale* and the two purported new genera receiving *Crella cyathophora* and *H. arabica*, might be proposed according to our phylogenetic results. However, the inclusion of additional OTU's appears convenient before taking definite taxonomical decisions. A new cryptic species (*Hemimycale mediterranea* sp. nov.) is described. Morphologically undifferentiated species with contrasting biological traits, as those here reported, confirm that unidentified cryptic species may confound ecological studies.

INTRODUCTION

Many cryptic, new sponge species have been discovered in the last decades thanks to the use of molecular markers (see Uriz & Turon 2012 for a review until 2012; de Paula et al. 2012; Knapp et al. 2015). However, less often, cryptic, molecularly discovered species have also been described morphologically (but see Blanquer et al. 2008a; Cárdenas & Rapp 2012; Reveillaud et al. 2011; 2012), which is necessary if phylogeny is aimed to translate into taxonomy, and the discovered species are wanted to be considered in ecological studies.

Sponge species can be morphologically (e.g. Uriz & Turon 2012) or, more rarely, molecularly (with the markers used) cryptic (Carella et al. 2016; Vargas et al. 2016) but show contrasting reproductive or biological features, which allow them to coexist in the same habitat. For instance, *Scopalina blanensis* (Blanquer & Uriz 2008a), which is sympatric with *S. lophyropoda*, mainly grows in winter. Conversely, *S. lophyropoda* regresses in winter and grows principally in summer-autumn (Blanquer et al. 2008b), thus indicating temporal niche partition.

The Order Poecilosclerida (Porifera: Demospongiae) harbours the highest number of species within the Class Demospongiae (Systema Porifera) and it is far from being resolved from a phylogenetic point of view (Morrow et al. 2012; Thacker et al. 2013). Within Poecilosclerida, the Family Hymedesmiidae represents a hotchpotch where genera of dubious adscription have been placed (Van Soest 2002). As expected, this family appears clearly polyphyletic in a phylogenetic study of the so-called G4 clade with based on the 28S gene (Morrow et al. 2012).

Hymedesmiidae currently contains then accepted genera among which, *Hemimycale* Burton 1934. The position of genus *Hemimycale,* which shares with *Hymedesmia*, and *Phorbas* (Hymedesmiidae) and with *Crella* (Crellidae) the so-called cribose areas with an inhaling function, has changed from Hymeniacidonidae in Halichondria (Lévi 1973) to Hymedesmiidae in Poecilosclerida (Van Soest 2002). More recently, in 18S phylophylogenies of

Poecilosclerida, *Hemimycale columella* was retrieved within the Crellidae clade, although with low support (Redmon et al. 2013).

Hemimycale harbours only four formally described species*:* the type species *Hemimycale columella* (Bowerbank 1874), from western Atlantic and Mediterranean, *H. rhodus* (Hentchel 1929) from the North Sea, *H. arabica* Illan et al. 2004 from the Red Sea and *H. insularis* Moraes 2011 from Brazil. However, given the simple spicule complement of the genus, which consists of diactines (strongyles) with some occasional monactines (styles) exclusively, may propitiate the existence of morphologically cryptic species.

Hemimycale columella is widely distributed across the North Atlanto-Mediterranean basin, from shallow (ca. 10 m) to deep (ca. 60 m) (Uriz et al. 1992b) waters. Assays performed with eight microsatellite loci developed from deep specimens of *H. columella* (González et al. 2015) failed to amplify a high percentage of the individuals assayed from a shallow population. Furthermore, after a reproduction study on this species most individuals disappeared after larval release (Pérez-Porro et al. 2012), although the deeper populations of *H. columella* (González et al. 2015) seemed to survive for years, which suggested larger genetic differences than those expected between intra-species sponge populations. Therefore, we performed a phylogenetic study of individuals considered as *H. columella* across the Mediterranean, using three molecular (nuclear and mitochondrial) gene partitions. Moreover, we incorporated to the analyses other species belonging to the close family Crellidae, trying to simultaneously clarify the *Hemimycale* taxonomical relationships with Crellidae.

MATERIALS & METHODS

1.1. Sampling

Fragments of what was *a priori* thought to be *Hemimycale columella* were collected by SCUBA diving across the Mediterranean (Northwestern, Central Mediterranean, Eastern Mediterranean, and Adriatic Sea), between 12 and 45 m of depth during several campaigns (Coconet, Benthomics, and MarSymbiOmics projects) (Table 1.1). Moreover,

fragments of *H. arabica* and *Crella cyatophora* from the Red Sea (Dedalos and Ephistone) and Indo-Pacific between 5 and 20 m of depth were also collected (Table 1.1). Individuals were photographed underwater before being sampled. Collected fragments were divided into two pieces, one of them was preserved in 100% ethanol and, after three alcohol changes, kept at –20°C until DNA extraction; the other fragment was fixed in 5% formalin in seawater and preserved in 70% ethanol as a voucher for morphological and spicule studies. All vouchers have been deposited at the Sponge collection of the Centre d'Estudis Avançats de Blanes (voucher numbers: CEAB.POR.GEN.001-CEAB.POR.GEN.029).

Species	Sea/Ocean	Locality	Voucher numbers	Accession numbers
***H. arabica* ind. 1**	Red Sea	Dedalos-Brother Islands	CEAB.POR.GEN.001	COI: KY002124 18S: KY002171 28S: KY002181
***H. arabica* ind. 2**	Red Sea	Elphistone-Brother Islands	CEAB.POR.GEN.002	COI: KY002125 18S: KY002172 28S: KY002182
H. columella	Northeastern Atlantic	Plymouth, Wales-UK		28S: HQ379300.1 18S: KC902127.1
***H. columella* ind. 1**	Northwestern Mediterranean	Arenys de Mar-Spain	CEAB.POR.GEN.003	28S: KY002183
***H. columella* ind. 2**	Northwestern Mediterranean	Arenys de Mar-Spain	CEAB.POR.GEN.004	28S: KY002184
***H. columella* ind. 3**	Northwestern Mediterranean	Arenys de Mar-Spain	CEAB.POR.GEN.005	COI: KY002126
***H. columella* ind.1**	Northwestern Mediterranean	Tossa de Mar-Spain	CEAB.POR.GEN.006	COI: KY002127 18S: KY002160 28S: KY002185
***H. columella* ind. 2**	Northwestern Mediterranean	Tossa de Mar-Spain	CEAB.POR.GEN.007	COI: KY002128 18S: KY002161 28S: KY002186
***H. columella* ind. 3**	Northwestern Mediterranean	Tossa de Mar-Spain	CEAB.POR.GEN.008	COI: KY002129 28S: KY002187

Species	Sea/Ocean	Locality	Voucher numbers	Accession numbers
H. columella **ind.4**	Northwestern Mediterranean	Tossa de Mar-Spain	CEAB.PO R.GEN.009	28S: KY002188
H. mediterranea **sp. nov. ind. 1**	Northwestern Mediterranean	Tossa de Mar-Spain	Holotype: CRBA-56057	COI: KY002130 18S: KY002162 28S: KY002189
H. mediterranea **sp. nov. ind. 2**	Northwestern Mediterranean	Tossa de Mar-Spain	Paratype: CRBA-56057	18S: KY002163 28S: KY002190
H. mediterranea **sp. nov. ind. 4**	Northwestern Mediterranean	Tossa de Mar-Spain	CEAB.PO R.GEN.012	COI: KY002131
H. mediterranea **sp. nov. ind. 5**	Northwestern Mediterranean	Tossa de Mar -Spain	CEAB.PO R.GEN.013	COI: KY002132
H. mediterranea **sp. nov. ind. 3**	Adriatic Sea	Koznati-Croatia	CEAB.PO R.GEN.014	COI: KY002134
H. mediterranea **sp. nov. ind. 7**	Adriatic Sea	Koznati-Croatia	CEAB.PO R.GEN.015	18S: KY002170 28S: KY002193
H. mediterranea **sp. nov. ind. 8**	Adriatic Sea	Koznati-Croatia	CEAB.PO R.GEN.016	28S: KY002194
H. mediterranea **sp. nov. ind. 2**	Adriatic Sea	Tremity-Italy	Paratype: CRBA-56060	COI: KY002133
H. mediterranea **sp. nov. ind. 11**	Adriatic Sea	Tremity-Italy	Paratype: CRBA-56059	28S: KY002199
H. mediterranea **sp. nov. ind. 8**	Central Mediterranean	Porto Cesareo-Italy	CEAB.PO R.GEN.019	18S: KY002164

Species	Sea/Ocean	Locality	Voucher numbers	Accession numbers
***H. mediterranea* sp. nov. ind. 9**	Central Mediterranean	Porto Cesareo-Italy	CEAB.PO R.GEN.020	18S: KY002165 28S: KY002197
***H. mediterranea* sp. nov. ind. 10**	Central Mediterranean	Porto Cesareo-Italy	CEAB.PO R.GEN.021	28S: KY002198
***H. mediterranea* nov. sp. ind. 5**	Adriatic Sea	Karaburum-Albania	CEAB.PO R.GEN.022	18S: KY002166 28S: KY002191
***H. mediterranea* nov. sp. ind. 6**	Adriatic Sea	Karaburum-Albania	CEAB.PO R.GEN.023	18S: KY002167 28S: KY002192
***H. mediterranea* sp. nov. ind. 3**	Eastern Mediterranean	Othonoi-Greece	CEAB.PO R.GEN.024	18S: KY002168 28S: KY002195
***H. mediterranea* sp. nov. ind. 4**	Eastern Mediterranean	Othonoi-Greece	CEAB.PO R.GEN.025	18S: KY002169 28S: KY002196
***Crella cyatophora* ind.1**	Red Sea	Dedalos-Brother Islands	CEAB.PO R.GEN.026	COI: KY002120 18S: KY002173 28S: KY002177
***Crella cyatophora* ind. 2**	Red Sea	Elphistone-Brother Islands	CEAB.PO R.GEN.027	COI: KY002121 18S: KY002174 28S: KY002178
***Crella cyatophora* ind. 3**	Indo-Pacific	Bempton Patch Reef (beween New Caledonian and Australia	CEAB.PO R.GEN.028	COI: KY002122 18S: KY002175 28S: KY002179
***Crella cyatophora* ind. 4**	Indo-Pacific	Bempton Patch Reef (beween New Caledonian and Australia	CEAB.PO R.GEN.029	COI: KY002123 18S: KY002176 28S: KY002180
Crella elegans	Mediterranean	France		18S: KC902282
Crella elegans	Mediterranean	France		18S: AY348882
Crella elegans	Mediterranean	France		28S HQ393898

Species	Sea/Ocean	Locality	Voucher numbers	Accession numbers
Crella plana	Northeastern Atlantic	Northern Ireland		18S: KC9023009
Crella rosea	Northeastern Atlantic	Northern Ireland		28S:HQ379299
Crella rosea	Northeastern Atlantic	Northern Ireland		18S:KC902282
Phorbas bihamiger	Northeastern Atlantic	English Channel		18S: KC901921.1 28S: KC869431
Phorbas punctatus	Northeastern Atlantic	Wales		18S: KC869439.1 28S: KC869439.1
Phorbas dives	Northeastern Atlantic	English Channel		28S:HQ379303
Phorbas fictitioides	North Pacific	-		COI:HE611617.1
Phorbas tenacior	Northeastern Atlantic	-		18S: AY348881
Phorbas glaberrimus	Antarctic	Ross Sea		COI:LN850216.1
Hymedesmia paupertas	Northeastern Atlantic			18S: KC902073.1 28S: KF018118.1
Hymedesmia pansa				18S: KC902027.1
Hymedesmia paupertas	Northeastern Atlantic			28S: KF018118.1
Kirkpatrickia variolosa	Antarctic	Ross Sea		COI: LN850202.1

Table 1.1. Geographical origin and ecological distribution of the individuals used in the phylogenetic study, with accession numbers. Individuals sequenced *de novo* are in bold.

1.2. DNA extraction, amplification, and sequencing

A mínimum of three specimens per species and locality (totaling 18 individuals) were extracted with DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's protocol. Standard primers were used for COI partitions M1-M6 (Folmer et al. 1994), and two nuclear genes: 18S rRNA (1F and 1795R, from Medlin et al. 1988) and the D3-D5

partition of 28S (Por28S-830F and Por28S-1520R from Morrow et al. 2012). Different amplification protocols were performed for each gene: COI M1-M6 partition (94 °C, 2 min [94°C, 1 min, 43°C, 1 min, 72°C, 1 min] x 35–40 cycles, 72°C, 5 min); 18S (94 °C, 5 min, [94 °C, 1 min, 50-55 °C, 1 min, 72 °C, 1 min] x 35-40 cycles, 72 °C, 5 min); 28S D3-D5 partition (94 °C, 5 min [94°C, 30 s, 53°C, 30 s, 72°C, 30 s] x 30 cycles, 72°C, 5 min). COI, M1-M6 partition amplifications were performed in a 50 μL volume reaction, containing 37,6 μL H_20, 5 μL buffer KCL (BIORON), 2μL BSA, 2μL dNTP (Sigma), 1 μl primers forward/reverse, 0.4 μL Taq (BIORON) and 1μL of genomic DNA. 18S rRNA amplifications were performed in a 50 μL volume reaction, containing 36,85 μL H_20, 5 μL buffer (INVITROGEN), 0,75 μL MgCl (INVITROGEN), 1,2 μL DMSO (dimethyl sulfoxide), 1 μL BSA, 1.5 μL dNTP (Sigma), 1 μl primers forward/reverse, 0.7 μL Taq (INVITROGEN) and 1 μL of genomic DNA. On the other hand, partition D3-D5 of 28S rRNA amplifications were performed in a 50 μL volume reaction, containing 36.85 μL H_20, 5 μL buffer (INVITROGEN), 0.75 μL MgCl (INVITROGEN), 2μL BSA, 2 μL dNTP (Sigma), 1 μl primers forward/reverse, 0.4 μL Taq (INVITROGEN) and 1 μL of genomic DNA. PCR product was purified and sequenced in both directions using Applied Biosystems 3730 Xl DNA analyzers in Macrogen, Korea.

1.3. Sequence alignment and phylogenetic reconstructions

Sequences of COI, 28S, and 18S were aligned using Clustal W v.1.81, once their poriferan origin was verified using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), as implemented in Genieous 9.01 (http://www.geneious.com, Kearse et al. 2012). When sequences were identical only one sequence per locality and species was included in the phylogenetic trees. After alignment, ambiguous regions were determined with Gblocks v.091 b software (Castresana 2000), which removes from 1% to 4 % of poorly aligned positions and divergent regions of an alignment of DNA. In cases where the forward and reverse reads disagreed, we kept either the better quality of the two

reads or introduced an IUPAC ambiguity code into the consensus sequence.

JModelTest 0.1.1 (Posada 2008) was used to determine the best-fitting evolutionary model for each dataset. The model GTR+I+G was used for both mitochondrial and nuclear genes. Phylogenetic trees were constructed under Neighbour Joining (NJ), Bayesian Inference (BI) and Maximum Likelihood (ML) criteria. BI analyses were performed with MrBayes 3.2.1 (Ronquist & Huelsenbeck 2003). Four Markov Chains were run with one million generations sampled every 1000 generations. The chains converged significantly and the average standard deviation of split frequencies was less than 0.01 at the end of the run. Early tree generations were discarded by default (25%) until the probabilities reached a stable plateau (burn-in) and the remaining trees were used to generate a 50% majority-rule consensus tree. ML analyses were executed with PhyML v3.0 program (Guindon & Gascuel 2003, Guindon et al. 2005). The robustness of the tree clades was determined by a nonparametric bootstrap resampling with 1000 replicates in PhyML.

Incongruence Length Difference (ILD) test (PAUP 4.0b10) was run (Swofford 2002) to verify sequence homogeneity or incongruence between the 18S and COI markers and the 18S and 28S markers. The ILD test indicated no significant conflict ($p = 0.93$ and $p = 0.91$, respectively) between the two couples of markers respectively. Thus, concatenated 18S-COI and 18S-28S datasets were constructed for the species with sequences available for both markers. The phylogeny on the three genes concatenated was not performed due to the few species/individuals for which the three genes were available in the GenBank.

To assess whether molecular differences among the target populations and species (*H. columella*, *H. arabica,* and *Crella cyatophora*) were supported by morphological and spicule traits, the target species were observed both *in situ* and on recently collected samples. Moreover, spicules of all the species were observed through light and Scanning Electron Microscopes (SEM) after removing the sponge organic matter from

small (3 mm^3) pieces of each individual by boiling them in 85% Nitric acid in a pyrex tube and then washed three times with distilled water and dehydrated with ethanol 96% (three changes). A drop of a spicule suspension in ethanol per individual was placed on 5 mm diameter stuffs, air dry, and gold-palladium metalized (Uriz et al. 2008) in a Sputtering Quourum Q150RS. Observation was performed through a Hitachi Scanning Electron Microscope Hitachi M-3000 at the Centre d'Estudis Avançats de Blanes.

RESULTS

18S phylogeny

The 18S dataset comprised 25 sequences (17 new) of 699 nt. Six nucleotides differed between *Hemimycale columella* and *H. mediterranea,* while between *H. columella* and *H. arabica* differed 10 nt., and 13 nt. did between *H. mediterranea* and *H. arabica*. There was no nucleotide difference between *Crella cyathophora* collected from the Red Sea and Indo-Pacific, and there was 15 nt., 16 nt., and 14 nt. different with *C. elegans*, *C. plana* and *C. rosea*, respectively.

The resulting phylogeny was mostly congruent under BI, NJ, and ML (Fig. 1.1). The representatives of the family Crambeidae (*Monanchora*) appeared as outgroups and the genus *Phorbas* was a sister group of the remaining species. In the BI, NJ, and ML trees, the genera *Hemimycale* and *Crella* appeared polyphyletic, with the Red Sea species *H. arabica* and *C. cyatophora*, far away from the Atlanto-Mediterranean *Hemimycale* and *Crella* species. The Atlanto-Mediterranean *Crella* formed a well-supported clade (1/ 81/98, posterior probability/bootstrapping values), which was the sister group of the Atlanto-Mediterranean *Hemimycale* (1/ 97/98). Moreover, the deep_*H. columella* clustered with an Atlantic sequence downloaded from the GeneBank (0.89/89/88) forming a separate clade from the also well-supported (1/97/98) group containing the shallow Mediterranean *Hemimycale*. No genetic differences for this partition were found among shallow individuals. In the BI and ML trees the two individuals of *H. arabica*

appeared in unresolved positions while they formed a poorly supported (75%) clade in the tree under the NJ criterion.

Figure 1.1. Phylogeny with 18S partition under BI, NJ and ML.

28S (D3-D5) phylogeny

The 28S (D3-D5) dataset comprised 31 sequences (24 new) of 730 nt. There were nine nt. different between *H. columella* and *H. mediterranea*. Between *H. arabica* and *H. columella* there were 16 nt. different and between *H. arabica* and *H. mediterrane* there were 13 nt. different. *C. cyathophora* collected from the Red Sea and from the Indo-Pacific differed in seven nt. *C. cyathophora* from Red Sea differed in 16 and 14 nt. with *C. elegans* and *C. rosea*, respectively, and

C. cyathophora from the Indo-Pacific differed in 18 and 16 nt. with *C. elegans* and *C. rosea*, respectively.

The resulting phylogenies also were mostly congruent with the three clustering criteria and matched in most cases the phylogeny based on the 18S partition, although the supporting values of some clades were in some cases slightly lower (Fig. 1.2).

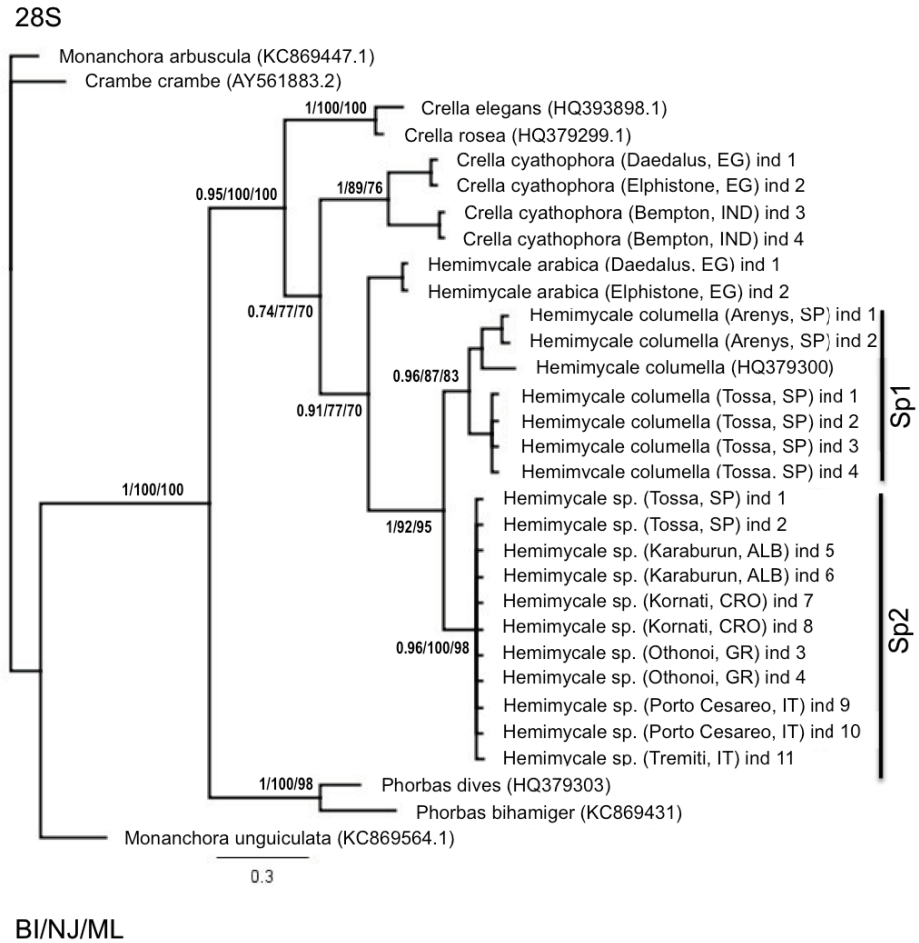

Figure 1.2. Phylogeny with 28S partition under BI, NJ and ML.

The three phylogenies retrieved the representatives of Family Crambeidae (*Monanchora* and *Crambe*) as outgroup. The monophyly of the in-group containing *Crella* spp and *Hemimycale* spp was strongly supported under

the BI, NJ, and ML criteria (1/100/100). The genus *Phorbas* was a sister group of the remaining species considered. *Crella* was polyphyletic, with *C. cyatophora* separated from the well-supported clade (1/100/100) encompassing the Atlanto-Mediterranean *Crella*. The latter appeared as a sister clade of a poorly supported group (0.7/77/70) harbouring *Crella cyatophora and Hemimycale spp*. The *Hemimycale* spp group, although monophyletic, was poorly supported under the NJ and ML criteria (77/70) while the Atlanto-Mediterranean *Hemimycale* clade was well supported under the three clustering criteria (1/92/95).

The deep and shallow Mediterranean populations of *Hemimycale* formed two well-supported monophyletic groups (0.96/87/83 and 0.96/ 100/98, for deep and shallow individuals, respectively), the former containing the Atlantic sequence of *H. columella*. No genetic differences for this partition were retrieved for shallow individuals despite their spread distribution across the Mediterranean. The individuals of *C. cyatophora* from the Red Sea clustered with those from the Indo-Pacific collected between Australia and Nouvelle Caledonie (1/89/76).

COI phylogeny

The COI dataset comprised 21 sequences (15 new) of 752 nt. There were four nt. different between *H. columella* from the two localities in western Mediterranean. Between *H. columella* from Arenys and *H. mediterranea* there were eight nt. different, and nine nt. different between *H. columella* from Tossa and *H. mediterranea*. *H. arabica* differed in 63 nt., 64 nt., 65 nt., with *H. mediterranea*, *H. columella* from Arenys and *H. columella* from Tossa, repectively. Between *C. cyathophora* collected from Red Sea and Indo-Pacific there was one nt. different, and both differed in 77 nt. with *C. elegans*.

The COI phylogeny also retrieved the representatives of Crambeidae as outgroups of the group formed by *Crella, Phorbas,* and *Hemimycale*. The genus *Phorbas* clustered with the Atlanto-Mediterranean *Crella* spp (0.98/100/86) likely because we only could include one individual/species of *Phorbas* (Fig. 1.3).

A clade containing *Hemimycale* spp. and *Crella cyatophora* was well supported (0.94/94/80). This clade was divided into two subclades, one of them with the Mediterranean *Hemimycale* (no sequence of the Atlantic *H. columella* was in the GenBank for this partition) clearly divided in two monophyletic, well-supported groups corresponding to deep and shallow individuals. No genetic differences among the shallow individuals analyzed were found. A sister, well supported, group (1/100/94) contained *C. cyatophora* and *H. arabica* representatives with almost no genetic differences (Fig. 1.3).

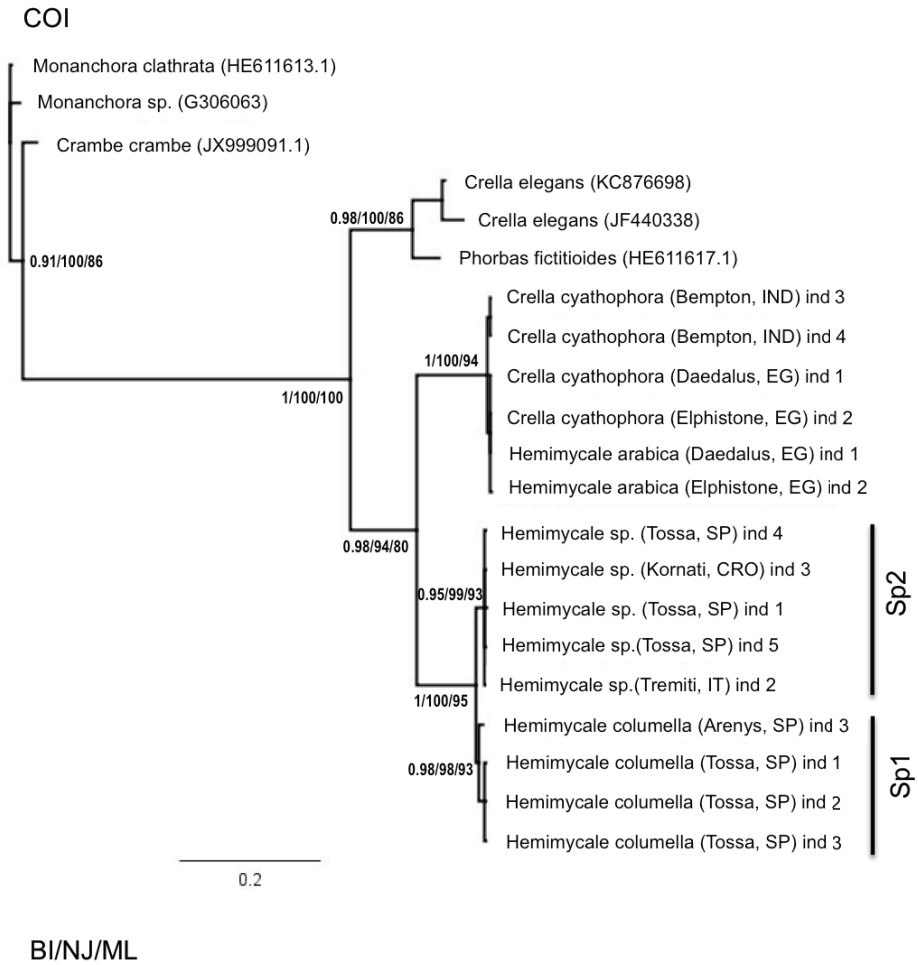

Figure 1.3. Phylogeny with COI partition under BI, NJ and ML.

Concatenated trees

The concatenated 18S+28S (Fig. 1.4) confirmed the outgroup position for the Crambeidae representative (*Monanchora*), the polyphyly of *Crella* with the Red Sea and Indo-Pacific species forming a separate clade (1/100/100) from the Atlanto-Mediterran *Crella*, which appeared in a non-resolved position. *Hemimycale* also appeared polyphyletic, but the position of *H. arabica* was unresolved. The Atlanto-Mediterranean *Hemimycale* clade was confirmed as well as its division into two subclades: one containing the deep Mediterranean individuals together with two Atlantic sequences of the species and the other one harbouring the shallow Mediterranean individuals, with did not show any genetic difference across the Mediterranean and Adriatic Sea.

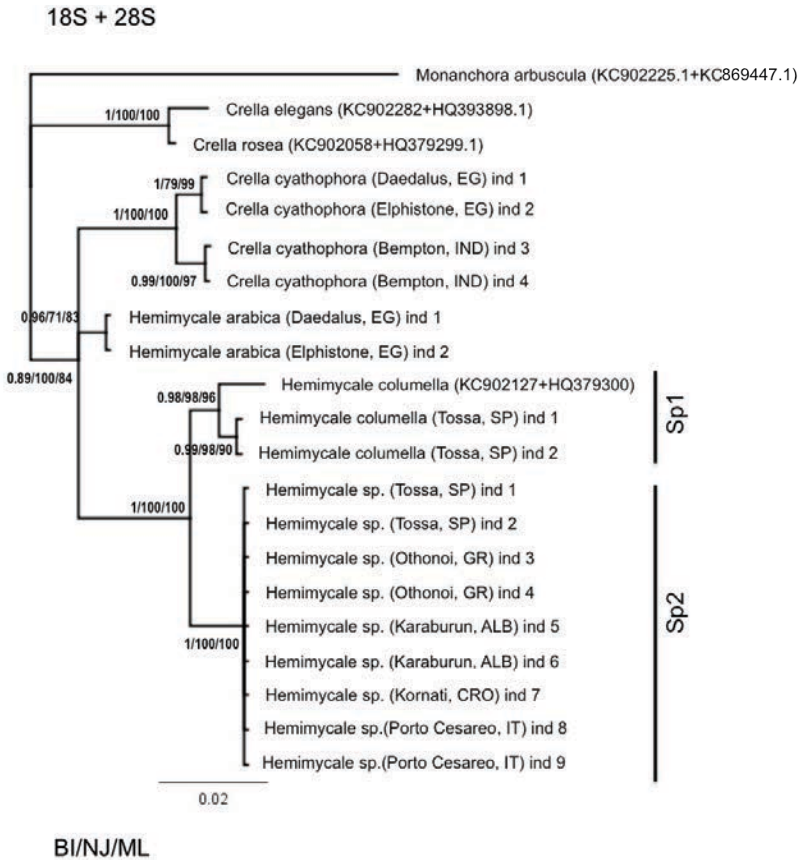

Figure 1.4. Concatenated 18S + 28S tree under BI, NJ and ML.

The concatenated 18S+COI (Fig. 1.5) tree contained only 13 sequences and no representative of Crambeidae could be included. The representatives of the Atlanto-Mediterranean *Crella* appeared as outgroup of the remaining target species, which formed two well-supported clades: one containing *C. cyatophora* and *H. arabica* representatives (1/100/100) and the other with the Atlanto-Mediterranean *Hemimycale* (1/100/100) divided in two monophyletic well-supported groups (deep and shallow individuals).

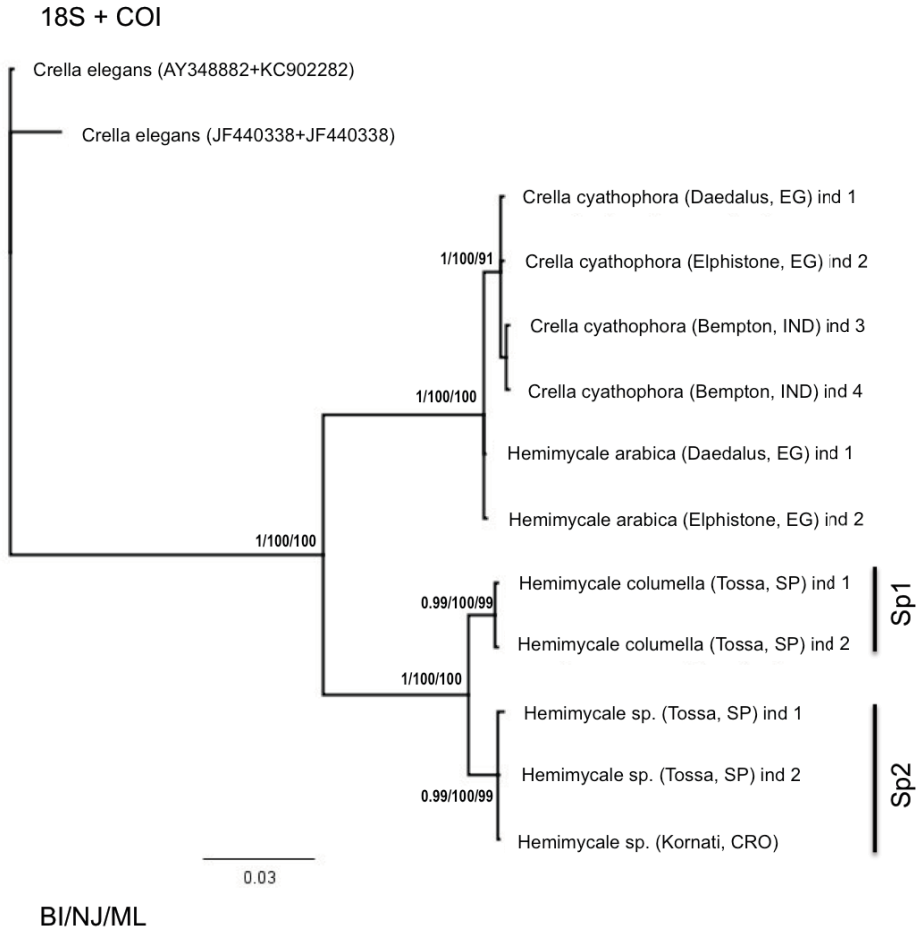

Figure 1.5. Concatenated 18S + COI tree under BI, NJ and ML.

DISCUSSION

The phylogenetic reconstructions performed with 18S and COI as well as pair of genes concatenated (18S+COI and 18S +28S) support the polyphyly of both *Crella* and *Hemimycale*, under the three clustering criteria used. However, *Hemimycale* turned to be monophyletic but with a low statistical support with the 28S (D3-D5) marker, while *Crella* also proved polyphyletic with this partition. The COI phylogeny retrieved a clade including Red Sea *Crella cyatophora* and *Hemimycale arabica*, separated from the Atlanto-Mediterranean *Crella* and *Hemimycale*. Thus COI clustered species rather as a function of their geographical proximity instead of to conventional taxonomy, which induced us to foresee a sequencing mistake. However, the sequences analyzed proved to belong to sponges (NCBI blast) and not to any particular symbiont. Even more, the microbiome profiling of the several species considered in our phylogeny (Chapter 4) showed (even for the candidatum *Calcibacterium* species) a higher similarity between *Crella cyatophora* (Red Sea) and *Hemimycale columella* (Mediterranean) than between *H. columella* and *H. mediterranean* sp. nov. On the other hand, *H. arabica* has a secondary chemistry (polycyclic guanidine alkaloid) that is present in *Crambe* and *Monanchora* but not in *H. columella* (Van Soest et al. 1996). This supports previous results showing that, at least in some cases, the secondary metabolites are not informative characters in phylogeny, as they can be produced by phylogenetically far sponges (Sfecci et al. 2016), likely thanks to similar symbiotic microbes (Croué et al., 2013).

To conclude, *Hemimycale* is polyphyletic with the OTU's set and the nuclear and mitochondrial markers used. The Red Sea- Indo-Pacific species may belong in another genus according its strong, molecular and external morphological differences with the Atlanto-Mediterranean species. *Crella* is also polyphyletic and *C. cyatophora* from the Red Sea might belong in a different genus, closer to *H. arabica* than to the Atlanto-Mediterranean *Crella* spp. Moreover, the Atlanto-Mediterranean Crellidae appeared in 18S and 28S phylogenies as a sister group of the Atlanto-Mediterranean *Hemimycale*, which suggests higher affinities of this genus with Crellidae than with Hymedesmiidae (its current family). However, more complete analyses

including more Crellidae and Hymedesmiidae OTU's are needed to take taxonomical decision to move *Hemimycale* from Hymedesmiidae to Crellidae.

Moreover, the phylogenetic trees with any of the three gene partitions either separately or concatenated confirm, under the three aggregation criteria, the presence of two cryptic *Hemimycale* species in the Mediterranean within what was considered until now *H. columella.* These species are: a new species that we named *H. mediterranea* sp. nov. (see species description) with a shallower distribution and *H. columella,* with Atlantic affinities and a general deeper distribution.. The new species has been up to now only recorded in the Mediterranean. However, the lack of genetic diversity with any of the three markers used among the individuals analyzed across the whole Mediterranean suggests its recent presence in the Mediterranean, which is compatible with a recent introduction.

Many cryptic species that were revealed by molecular markers have never been formally described owing to the difficulty of finding diagnostic phenotypic characters. Although after exhaustive observation, only slight, morphological differences have been found to differentiate *H. mediterranea* sp. nov. from *H. columella*, these phenotypic differences (diameter and rim height of the aerolate areas, color tint, and size of the anisostrongyles) are consistent across individuals and thus add to molecular markers and biological traits to consistently differentiate these two at first sight morphologically cryptic species.

Species description

Genus *Hemimycale* Burton, 1934

Type species *Hemimycale columella* (Bowerbank 1874)

Hemimycale is the only genus of Hymedesmiidae that has smooth diactines and monactines as the only spicule types (Van Soest, 2002). The genus was re-described by Burton (1934) as "reduced Mycaleae with skeleton of loose fibers of styli, sometimes modified into anisostrongyles, running vertically to

the surface; fibers tending to branch and anastomose; no special dermal skeleton, no microscleres".

This spicule complement seems different from the predominant straight anisostrongyles with rare or absent styles reported in the several redescriptions of *Hemimycale columella*. Indeed, Burton stated that the Bowerbank representation of *H. columella* spicules was wrong because it figured anisostrongyles instead of styles and was precisely the dominance of styles what induced Burton to place the species among the *Mycaleae*. The termination of the diactines either round or pointed ends may be the result of different silica concentration in the water masses, as reported for other siliceous sponge skeletons, but It cannot be totally discarded that the Burton *H. columella* belonged in another *Hemimycale* species.

Species: H. columella (Bowerbank, 1874)

Sequence accession Numbers GenBank (See Table 1.1 of Methods)

Description (Fig. 1.6): Encrusting to massive sponges. Surface smooth, covered with circular inhaling, areas up to 6mm in diameter with an up to 2 mm high rim. Consistence morbid and fleshy. Ectosome translucent to whitish, difficult to separate from the choanosome.

Thousands of calcareous spherules, 1µm in diameter formed by intracellular calcifying bacteria (Uriz et al. 2012) are spread through the sponge mesohyl and specially accumulated at the sponge periphery of whitish individuals (Chapter 3).

Color from pinkish-orange to whitish outside, dark orange inside.

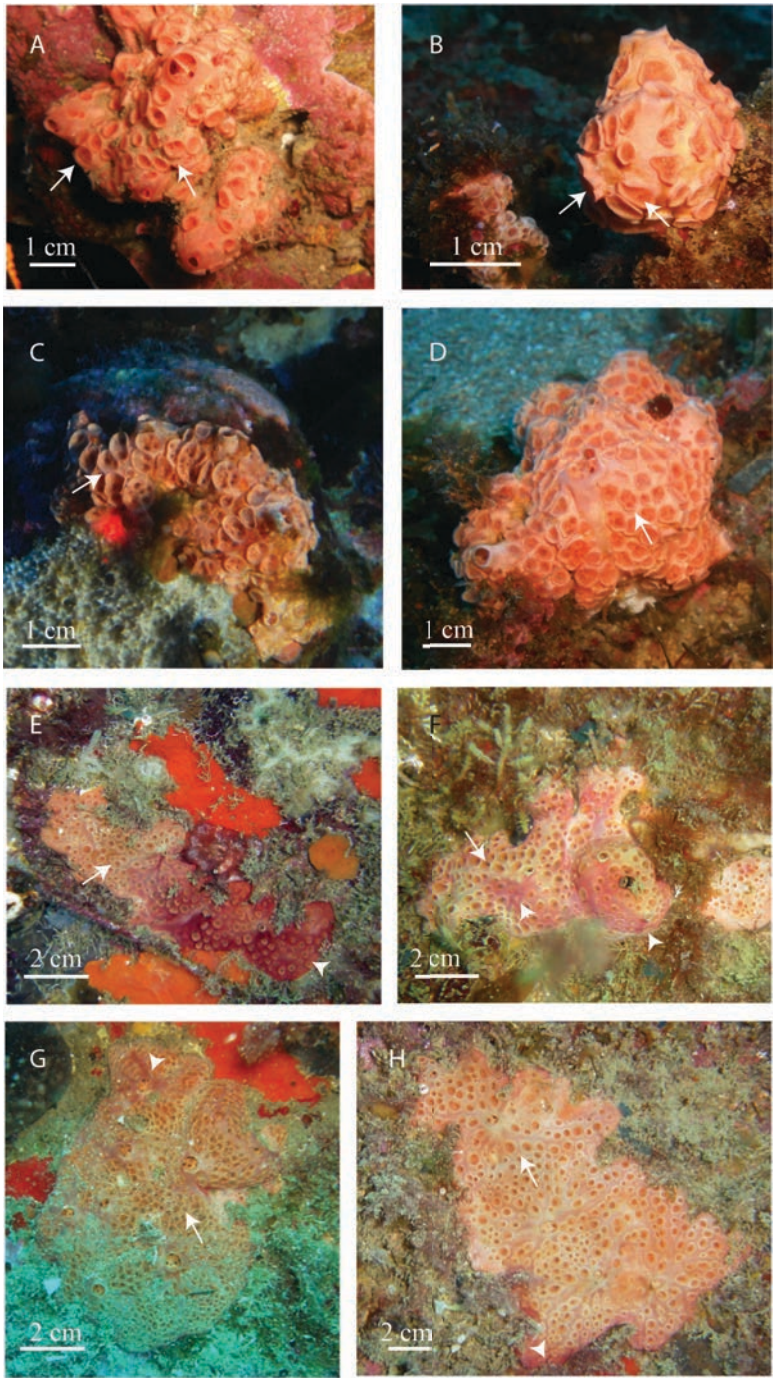

Figure 1.6. Several individuals of *H. columella* from northwestern Mediterranean Sea.

Spicules (Table 3, Fig. 1.7 E): asymmetric strongyles, straight, 302-435 µm x 3-4 µm in size. Styles rare or completely absent from the Mediterranean specimens (this study) and Canary Islands (Cruz 2002).

Distribution: Northeastern Atlantic (United Kingdom and Ireland coasts) Canarias Islands (Cruz 2002), western Mediterranean: Tossa de Mar, Arenys de Mar, from 28 to 60 m depth (this study). It is not possible to confirm whether previous Mediterranean records of the species (see Vacelet & Donadey, 1977) belonged to *H. columella* or to *H. mediterranea*. However, in some cases the size of the spicules and the external color allow identifying whether the specimens belong to one or to the other species.

Biology: multiannual life span, ca. 60% survival after two monitoring years; maximum growth in autumn-winter (Chapter 2).

Reproduction: Larval release at the beginning of November in Mediterranean deep specimens (pers. obs.).

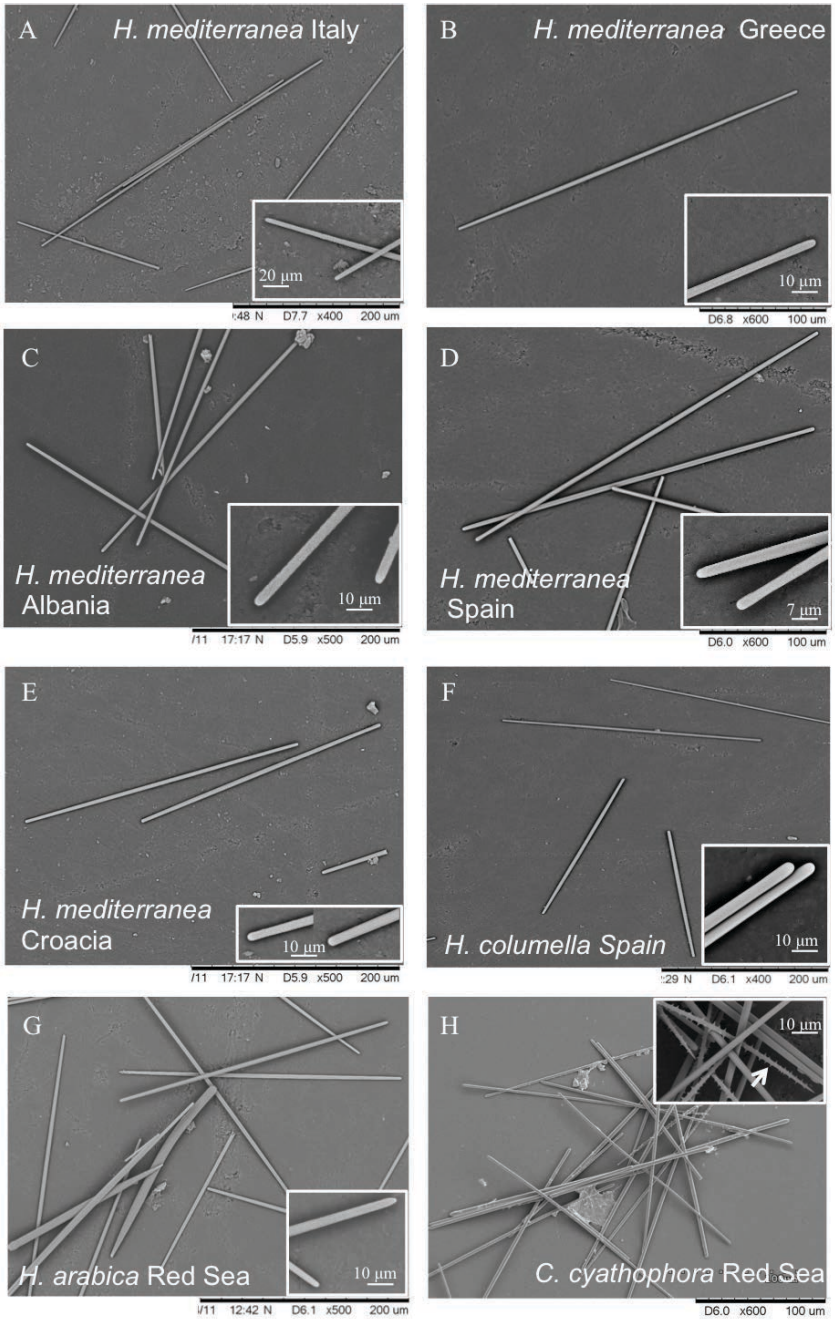

Fig. 1.7. Spicules of *Hemimycale* spp. and *Crella cyathophora* though SEM. A, B, C, D, E) Anysostrongyles (*Hemimycale mediterranea*). F) Anisostrongyles (Hemimycale columella). G) Anisostrongyles and one style (*Hemimycale arabica*). H) Anysotrongyles

and acantoxeas (*Crella cyatophora*). Inserts on each picture correspond to magnifications of the spicule ends.

Species: H. mediterranea sp. nov. (Holotype: CRBA-56057)

Sequence accession Numbers GenBank (See Table 1.1 of Methods)

Description (Fig. 1.6): thick encrusting sponges with aerolate inhaling areas up to 3mm in diameter, surrounded by an up to 2mm high rim, which in some cases barely surpasses the sponge surface.

Ectosome: firmly attached to the choanosome

Color: flesh to clear brownish externally, more o less whitish depending on calcibacteria accumulation at the surface, sometimes partially covered by an epibiotic (reddish or pinkish) cyanobacteria.

Spicules: smooth, uniform in size, straight, anysostrongyles, straight, 200-296 x 3-4 µm in size (Table 3, Fig. 1.7 A-F). Styles completely absent.

Skeletal arrangement: plumose undulating bundles of anysostrongyles together with spread spicules. A palisade of vertical strongyles forms the rim around the inhaling areas.

Known distribution: Northwestern Mediterranean Central Mediterranean, Adriatic, Eastern Mediterranean (Spain: Cap De Creus, Tossa, Blanes, Arenys, South Italy: Croatia, Tremiti, Turkey, Greece) between 3 and 17 m deep.

Biology: annual life spam, maximum growth rates in summer (Garate et al., unpublished data). Intracellular calcifying bacteria constantly present, sometimes highly abundant bacteria.

Reproduction: Larval release at the end of September beginning of October (authors unpublished obs.).

Species	Author	Locality	Depth (m)/ assemblage	Styles	Strongyles (range/mean)	Acanthoxeas
H. arabica **ind. 1**	This study	Red Sea (Egypt)	14 /coral reef	160-189 (179.6) x 7-8 (7.5)	210-290 (273)x 2.8-4.1 (3.6)	_
H. arabica	Illan et al. 2004	Red Sea (Egypt)		190-250 (218) x 3.5-5(4.7)	200-290(266) x 2.5-4 (3.5)	_
H. mediterranea **ind. 7**	This study	Adriatic (Croatia)	10-15/rocky sub-horizontal	_	233-330 (274.8) x 3-4.6 (4.0)	_
H. mediterranea **ind. 11**	This study	Adriatic (Italy)	10-15/rocky sub-horizontal	_	251-300 (276.6) x 2.1-4 (3.0)	_
H. mediterranea **ind. 5**	This study	Adriatic (Albania)	10-15/rocky sub-horizontal	_	274-317 (296.4) x 2.9-4.5 (4.0)-	_
H. mediterranea **ind. 10**	This study	Central Med. (Italy)	10-15/rocky sub-horizontal.	_	229-328 (291.3) x 2.4-5.2 (3.5)	_
H. mediterranea **ind. 3**	This study	Eastern Med. (Greece)	10-15/rocky sub-horizontal	_	242-340 (272.7) x2.6-4 (3.2)	_
H. mediterranea **ind. 1**	This study	NW Med. (Spain)	12-16/rocky wall .	_	261-320(296.3) x 3.1-3.8 (3.5)	_
H. columella **ind. 1**	This study	NW Med. (Spain)	27-29/coralligenous	_	302-435 (370) x 3-4 (3.7)	_
"H columella"	Vacelet 1987	NW Med. (France)	_	_	225-310 (285) x 2-4 (3)	_
H. columella	Vacelet 1987	NW Med. (France)	_	_	320-410(369) x 2.5-3.8(3.1)	_
"H. columella"	Vacelet 1987	NW Med. (France)	_	_	220-320(273) x 2-4(2,7)	_
H. columella	Vacelet 1987	North Atlantic (France)	_	_	290-465 (394) x 4-7(5.1)	_
H. columella	Topsent 1925	North Atlantic (France)	_	_	400 x 6	_
H. columella	Foster 1995	North Atlantic (UK)	_	_	330-420(373) x 5-6(5.85)	_
H. columella	Bowerbank1874	North Atlantic (UK)	_	_	376 x 7	_
Crella Cyatophora **ind 3**	This study	Indo-Pacific (Bemptom)	18m/coral reef	_	205-308 (263.9)x 2.2-4.3 (3.4)	92-115 (105.4)x 2-2.3(2)
C. cyatophora **ind 1**	This study	Red Sea (Egypt)	12/coral reef	_	227-293 (267.8)x 2.5-3-9 (3.4)	89-120 (109.4)x 1.8-2.5(2.47)

Table 2. Locality and spicule sizes of the studied individuals, and comparison with descriptions by other authors.

It is difficult to ascertain but in some cases whether individuals of *H. columella* recorded by other authors belong in *H. columella* or *H. mediterranea*. The redescription of *H. columella* by Van Soest (2002) based on the holotype (from the Atlantic) reported large aerolate porefields with elevated rims, which are shared with the deep Mediterranean specimens of *H. columella* (Fig. 1.6) in contrast to the small, short-rimmed porefields showed by *H. mediterranea* sp. nov. Both species have mainly straight slightly asymmetric strongyles but the spicule sizes tended to be systematically larger in *H. columella* (Table 2). However, while styles were rarely present in *H. columella* individuals, they have not found in specimens of *H. mediterranea* sp. nov. The external color also differs between the two species, being orange to pinkish in *H. columella* and flesh color to brownish *H. mediterranea* sp. nov. (Fig. 1.6). Vacelet & Donadey (1977) reported two different color forms occurring side by side on the littoral of Provence (France), one pink cream and the other one brownish. Likely the second color morph, which had smaller strongyles, corresponded to the *H. mediterranea* sp. nov.

Color has not received much attention as a diagnostic character in sponges because it has been generally attributed to a higher or lower light irradiance at the sponge habitat, or to the presence of epibiotic or symbiotic cyanobacteria. However, color has proven to be taxonomically relevant to distinguish other invertebrates such as shrimp species (Knowlton & Mills, 1992) and also sponge species of the genus *Scopalina* (Blanquer & Uriz, 2008), and thus it seems worthy to be taken into account in sponge taxonomy.

The slight phenotypic differences found between the two species appear, however, consistent across individuals and localities within the Atlanto-Mediterranean basin. Moreover, their ecological distribution and bacterial symbionts, strongly differentiate these two cryptic species. For instance, calcareous spherules produced by intracellular bacteria are present in the two species (Chapter 3), but the taxonomy of the calcifying bacterium, as well as the whole respective microbial communities totally differ between them (Chapter 3). Symbionts, as predators do (e. g. Wulff, 2006), often lucidly distinguish their target sponge preys or hosts while the species remain morphologically cryptic to taxonomists. Moreover, *H. mediterranea* sp. nov.

shows an annual life spam, with individuals disappearing after larval release, while *H. columella* has a multiannual life span (Chapter 2) and growth dynamics also differs between the two species, as *H. mediterranea* sp. nov. grows more in summer, while d *H. columella* grows preferentially in autumn-winter (Chapter 2).

The contrasting ecological distribution in the Mediterranean of these two cryptic species helps to predict their identity in the field. *H. mediterranean* sp. nov. preferentially inhabits shallower zones than *H. columella* individuals. However, it is not unlike that both species may share occasionally habitat, as the occasional record of the two color morphs side by side (Vacelet & Donadey, 1977), seems to indicate. *H. mediterranea* sp. nov. seems to be more abundant and widespread in the Mediterranean than *H. columella*, and the presence of additional cryptic species among the deep Mediterranean *Hemimycale* cannot be discarded.

The presence of two morphologically cryptic *Hemimycale* species in the Mediterranean, which show contrasting biological traits, reinforces the idea that cryptic species represent something more that wrong taxonomic identifications or biodiversity underestimates. They may feature contrasting biological cycles and life spans, and puzzle biological studies, which may invalidate conservation policies based on those studies.

ACKNOWLEDGEMENTS

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Chapter 2

Contrasting biological features in morphologically cryptic Mediterranean sponges.

ABSTRACT

Sponges play essential roles in ecological processes in the marine benthos, such as creating new niches, competition for resources, and organic matter recycling. Despite the increasing number of taxonomic studies, many sponge species remain hidden, whether unnoticed or cryptic. The occurrence of cryptic species may confound ecological studies by underestimating biodiversity. In this study, we photographically monitored growth, fusions, fissions, and survival of two morphologically cryptic species, *Hemimycale mediterranea* Uriz, Garate & Agell, 2017, and *H. columella* (Bowerbank, 1874). Additionally, we characterized the main environmental factors of the corresponding species habitats. Sponge monitoring was performed monthly. Seawater samples were collected the same monitoring days in the vicinity of the target sponges. Results showed contrasting growth and survival patterns for each species: *H. mediterranea* totally disappeared after larval release while 64% of individuals of *H. columella* survived the entire two years we monitored. The species also differed in the number of fissions and fusions. These events were evenly distributed throughout the year in the *H. mediterranea* population but concentrated in cold months in *H. columella*. No measured environmental factor correlated with *H. mediterranea* growth rates, while temperature and dissolved organic nitrogen were negatively correlated with *H. columella* growth rates. The strong differences in depth distribution, survival, growth, fusions, and fissions found between these two cryptic species, highlights the importance of untangling cryptic species before ecological studies are performed in particular when these species share geographical distribution.

INTRODUCTION

Sponges are worldwide-distributed invertebrates, inhabiting shallow to abyssal benthic habitats, at all latitudes (Reiswig 1973; Uriz et al. 1992a; Hooper & van Soest 2002). They play a primary role in benthic assemblages by interacting in several ways with vegetal and animal neighbours (Wulff 2006). Competition for the space, provision of macro- and microhabitats for other organisms (Diaz & Rützler 2001), organic matter recycling (de Goeij et al. 2013), and energy transfer from the pelagic to the benthic compartments (Gili & Coma 1998) are some of the key functions that the sponges perform in marine ecosystems.

Sponge growth is still intriguing in many cases. Some species maintain the same size for decades (Teixido et al. 2009), and this trait depicts them as long-lived, slow growth organisms. However, when studied over shorter temporal scales (months to years), species that did not increase in size for years (Teixido et al. 2009) proved to be highly dynamic, with fast successive increases and decreases in size (Turon et al. 1998). In fact, waxing and waning growth rates throughout the year have been documented for several encrusting Mediterranean sponges (Garrabou & Zabala 2001; Blanquer et al. 2008; De Caralt et al. 2008), such that their ecological success appears to rely on keeping a colonized niche rather than on getting large.

Hemimycale mediterranea Uriz, Garate & Agell, 2017 was recently described as a new morphologically cryptic species with *H. columella* (Bowerbank 1874) (Uriz et al. 2017). Genetic differences of what firstly though to be two *H. columella* populations, were higher than those expected to be considered intra-species variation. Phylogenetic and morphological analyses, subsequently performed, revealed that these two populations indeed belonged to two cryptic species (Uriz et al. 2017). An *a posteriori* in deep search for phenotypic differences allowed us to record color tinge, diameter and rim high of the aerolate areas, and spicule size as the only species-specific traits. The type species of the genus, *H. columella,* seemed to remain stable for years (authors obs.), while the newly described, *H. mediterranea,* appeared to be an annual species, with population demise after release of larvae (Pérez-Porro et al. 2012). Both species harbour abundant

calcifying bacteria (Uriz et al. 2012; Garate et al. 2017), which are through to protect the sponges from predation (Garate et al. 2015). There were no signs of predation in the many sponges examined, which point to causes other than predation for explaining the contrasting species mortality patterns observed.

The two cryptic *Hemimycale* species (Uriz et al. 2017) share a geographical distribution thorough the Mediterranean but showed contrasting depth preferences. *H. mediterranea* dwells on shallow waters (4-17 m deep) while *H. columella* is preferentially found from 25 to 60 m depth (Uriz et al. 2017). Thus, these species represented suitable targets to determine whether some depth-related environmental factors might account for contrasting ecological distribution of sponges. Temperature (Tanaka 2002; Page et al. 2005; De Caralt et al. 2008; Koopmans & Wijffels, 2008) and food availability, either particulate (Reiswig 1973; Sebens 1987; Riisgard et al. 1993; Ribes et al. 1999a; Ribes et al. 2005; Lesser 2006; Koopmans & Wijffels 2008; De Caralt et al. 2008) or dissolved (Yahel et al. 2003; de Goeij et al. 2008; Mueller et al. 2014) are two of the main factors determining sponge growth. These two factors undergo notable seasonal variations in temperate seas such as the Mediterranean with potential limiting values for growth and survival of some filter-feeding species in summer (Coma et al. 2000).

The main objectives of this study were to assess the growth and survival patterns of these two genetically different but morphologically cryptic species, which differ in habitat preferences (Uriz et al. 2017) and to characterize the environmental factors of their respective habitats.

MATERIALS & METHODS

2.1. Study sites

Hemimycale columella and *Hemimycalle mediterranea* were monitored in the NW Mediterranean: Iberian Peninsula, Catalan coasts (41º 34´N, 2º 33´E-41º 42´N, 2º 54´E). *H. mediterranea* dwelt on vertical shallow (12-17 m deep) rocky walls (hereafter, shallow habitats). *H. columella* grew

on horizontal coralligenous assemblages (Casas-Güell et al. 2015) at 28-30 m of depth (deep habitats).

2.2. Growth dynamics and survival

Both sponge populations were monthly monitored by SCUBA diving. A total of 24 randomly selected individuals of *H. mediteranea* and 27 individuals of *H. columella* were tagged using labels fixed with a two-component, water resistant resin (IVEGOR, SA) and photographed monthly. A SONY Cybershot digital camera was mounted on a custom-made structure consisting of a 20 x 14 cm frame fixed by a 30 cm long metallic support, to ensure the same focal distance and position during the entire monitoring period (Blanquer et al. 2008). Estimates of survival, growth, regression, fissions, and fusions were derived from pictures taken monthly. The monitoring of *H. mediterranea* started in February 2012, when the individuals began to be conspicuous (i.e. image area greater than 1 cm^2), and lasted until September 2012 when the species disappeared after release of larvae. Conversely, most labeled individuals of *H. columella* remained and were monitored from May 2012 to June 2014.

Monthly pictures of each individual were outlined and the area was calculated by using ImageJ software (Schneider et al. 2012). Both species mainly grow in two dimensions in the study sites (thinly encrusting growth shape) so that changes in area can be correlated to growth (increases) or shrinkage (decreases) (Garrabou & Zabala 2001; Blanquer et al. 2008). Monthly growth rates were derived from formula:

$$G = \frac{\frac{Am - Am - 1}{Am - 1}}{t}$$

where A_m is the sponge area at month m, A_{m-1} the sponge area of the previous month and t the months between two recorded data (Turon et al. 1998); t equaled 1 in general (monthly growth data) but was 2 in the few cases (i.e. January and March 2013) when sea conditions prevented sampling in a given month. In these cases, growth rates were calculated between two consecutive data recording.

Survival curves were derived from the number of monitoring months that an individual was recorded. For calculations, when two sponges fused, the resulting individual was considered as a new one and the preceding two that fused were considered as dead, so that the final individual pool decreased in one individual. When one sponge split in two or more clones, the individual was considered as dead and the resulting individuals were counted as new ones (De Caral et al. 2008).

2.3. Fusions and fissions events

The percentage of individuals of each species undergoing fissions or fusions along the monitoring period was also recorded. Whether the events were single, double, triple, or quadruple was noted. A single fusion event was the fusion of two sponge fragments between two observations, a double fusion event was recorded where three sponges fused, and so on. Similarly, when an individual split in two fragments, one fission event was recorded and when it was divided in three fragments between two observations, a double fission event was recorded (Blanquer et al. 2008). When two or more sponges fused, they were treated as one individual for the following monitoring month.

2.4. Environmental factors

Analyses of abiotic factors were performed monthly during the entire monitoring period (i.e. 7 months for *H. mediterranea* location and ca. 24 months for *H. columella* location). Water samples (three 500 ml replicates) were collected in glass bottles by SCUBA diving in the vicinity of the tagged sponges and taken in the dark in a cooler (ca. 4ºC) to the laboratory. Once in the laboratory, 400ml of water of each replicate were filtered through pre-combusted GF/F filters (450ºC for 4h), using a baked glass filtration system, previously cleaned with a 10% HCl solution for 24 h. The filters were stored at -80ºC until the monitoring period finished and then they were analysed for particulate organic carbon and nitrogen (POC and PON). The filtrate was used for analyses of dissolved nutrients.

For POC and PON analysis, the frozen filters were dried at 60ºC during 24h and then analyzed at Scientific and Technological Services of the

University of Barcelona, using an elemental organic analyzer Thermo EA 1108 (Thermo Scientific, Milan, Italy) working in standard conditions as recommended by the supplier (i.e., helium flow at 120 ml/min, combustion furnace at 1000ºC, chromatographic column oven at 60ºC, oxygen loop 10 ml at 100 kPa).

For dissolved organic (DOC) analyses, 10 ml of filtrate were collected in pre-combusted glass ampoules (450 ºC for 24h), heat-sealed and stored at 4ºC until the analyses were performed (every three months). For TN analyses, 15 ml of filtrate per replicate was collected in a Falcon tube, previously cleaned in an acid bath (10% HCl for 24h). Dissolved inorganic nitrogen (DIN) was recorded at Operational Observatory of the Catalan Sea (CEAB-CSIC). Dissolved organic nitrogen (DON) was estimated by subtracting DIN from TN. DOC and TN were determined using high temperature catalytic oxidation method by a Shimadzu TOC-VCSH + ASI-V (ICM-CSIC).

Seawater temperature (T) was recorded every 6 h at the study sites using a StowAway TidbiT Temperature Data Logger, placed on the rocky bottom of the respective habitats, close to the monitored sponges.

2.5. Data analyses

Data did not comply with the normality (Shapiro-Wilk W test) and homoscedasticity (Cochran C test) assumptions of parametric tests and were rank-transformed. Comparisons of monthly sponge area and growth rates between the two sponge species were performed by multivariate analysis of variance (MANOVA). MANOVA does no require the dependent variables to be equally correlated as repeated-measures ANOVA does. The total number of replicates of both species used (N=51) and the number of observation times (K= 25 months or K=4 seasons) was adequate to warrant a high-test power for the analyses (Potvin et al. 1990). Post-hoc comparisons were performed by Newman-Keuls tests (Shesking 1989). Mean growth rates and mean area changes during the entire period when both populations coexisted were compared by Newman-Keuls test. Monthly comparisons between each environmental factor at the two species sites were performed by

two-way ANOVA.

Cross-correlation is a measure of similarity of two series as a function of the displacement of one relative to the other across time. Cross-correlation was performed between the growth rates and each measured environmental factor at a time window of one month. These analyses allow to determine the effect of the factor on the variable at the time lag=0, as a simple Pearson's correlation, or a different time lags (Weisstein, 2009). The analyses generate a histogram where two groups of bins are differentiated. Positive and negative correlation values are indicated on the X-axis. Negative and positive time lags of any correlation are represented on the Y-axis. Negative time-lag bins (months) account the number of instances when values in the first series (sponge growth rates) relate to previous values in the second one (A, temperature; B, DON). Positive time-lag bins indicate the number of instances when values in the first series correlate to values afterwards in the second series.

Differences in the number and type (whether simple, double or multiple events) of fissions and fusions between sponge species did not require statistical analysis since no fissions or fusions occurred in *H. columella* during the months both species coexisted. Survival curves between the two species were compared using Wilcoxon-type test (Fox 1993).

Survival curves between the two species were compared using Wilcoxon-type test (Fox 1993).

All the statistics analyses were performed with STATISTICA 6.0 (StatSoft, Inc. 2004).

RESULTS

Growth and survival

Seasonal growth rates were significantly different (MANOVA $F=4.5$, $p<0.001$) for *H. columella*, showing higher values (post-hoc multiple comparisons $p<0.001$) in winter (January-March) than in summer (July-September) (Fig. 2.1A). Conversely, no significant differences in seasonal growth rates were found for the *H. mediterranea* population (MANOVA $F=0.97$, $p=0.422$) (Fig. 1B). Mean areas showed no significant differences among seasons, although followed the same trend as growth rates (MANOVA; $F=0.34$, $p=0.996$ and $F=0.35$, $p=0.998$ for *H. columella* and *H. mediterranea*, respectively) (Fig. 2.2).

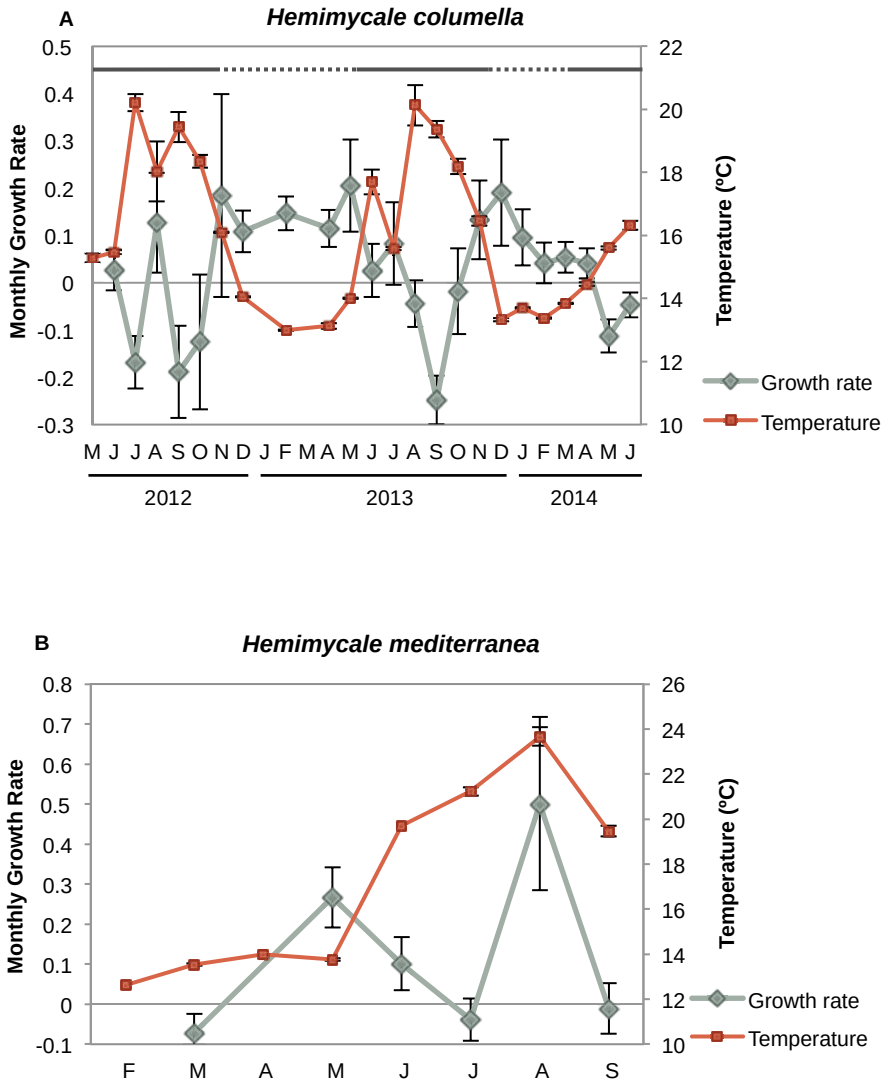


Figure 2.1. Mean growth rates of *H. columella* and *H. mediterranea*. A) Monthly mean (±SE) growth rates of *H. columella*. B) Monthly mean (±SE) growth rates of *H. mediterranea*. Continuous horizontal bars on the top of graphic A join months with no significant differences in growth rate ($p<0.0001$).

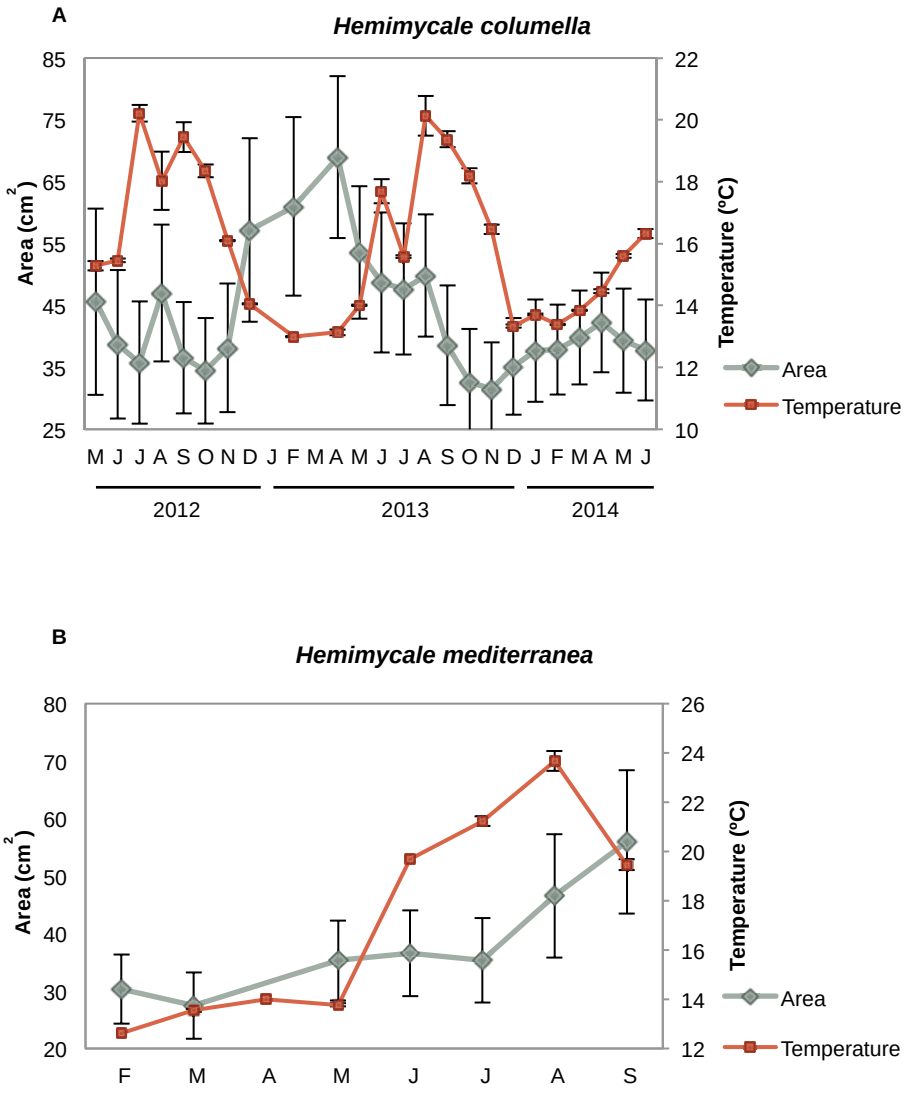


Figure 2.2. Mean areas of *H. columella* and *H. mediterranea*. A) Monthly mean (±SE) area of *H. columella*. B) Monthly mean (±SE) area of *H. mediterranea*.

When we considered the entire period when both species coexisted, the final mean growth rates were significantly higher for *H. mediterranea* than for *H. columella* (Newman-Keuls test, $F=13.94$, $p<0.001$), which approached 0, as growth (increase in area) and shrinkage (decrease in area) were compensated along study months. However, differences in mean area between species during the same period were not significant (Newman-Keuls $F=0.21$, $p=0.989$). Survival curves were significantly different for both species (Wilcoxon test $p<0.005$). While no one individual of *H. mediterranea* survived in shallow environments after seven months (i.e. after larval release), ca. 70% of the monitored individuals of *H. columella* survived in the deep environments during the same period and 64% survived at the end of two years of monitoring (Fig. 2.3).

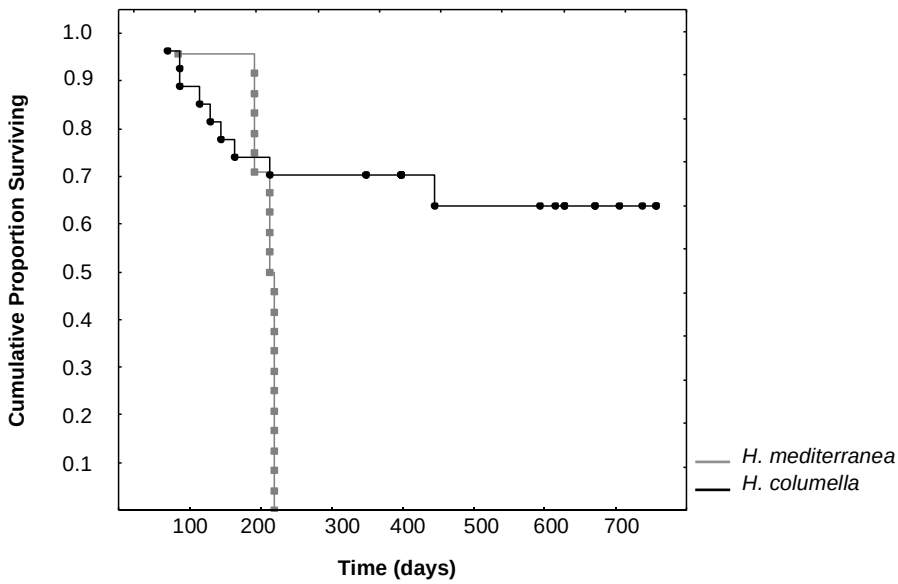


Figure 2.3. Survival curves of *H. columella* and *H. mediterranea* (Wilcoxon test, $p<0.005$).

Fission & Fusion events

There were significant differences in the number and type of fissions and fusions between the two species during the months both species coexisted (Fig. 2.4). Fusions of *H. mediterranea* increased from May to July and then slightly decreased in August-September (Fig. 2.4 B). Fissions of *H. mediterranea* were mainly recorded in May and September after the end of the reproduction and prior to the population's death (Fig. 2.4 B). Conversely, in *H. columella*, no fissions were recorded at the end of the reproduction period (October) but they occurred preferentially in winter (Fig. 2.4 A). The number of fusions increased in autumn-winter of the second monitoring year to decrease in the following spring months (Fig. 2.4 A). Moreover, fusion and fission events were single or double in *H. mediterranea*, while also triple fusions and quadruple fissions occurred in *H. columella* (Fig. 2.5).

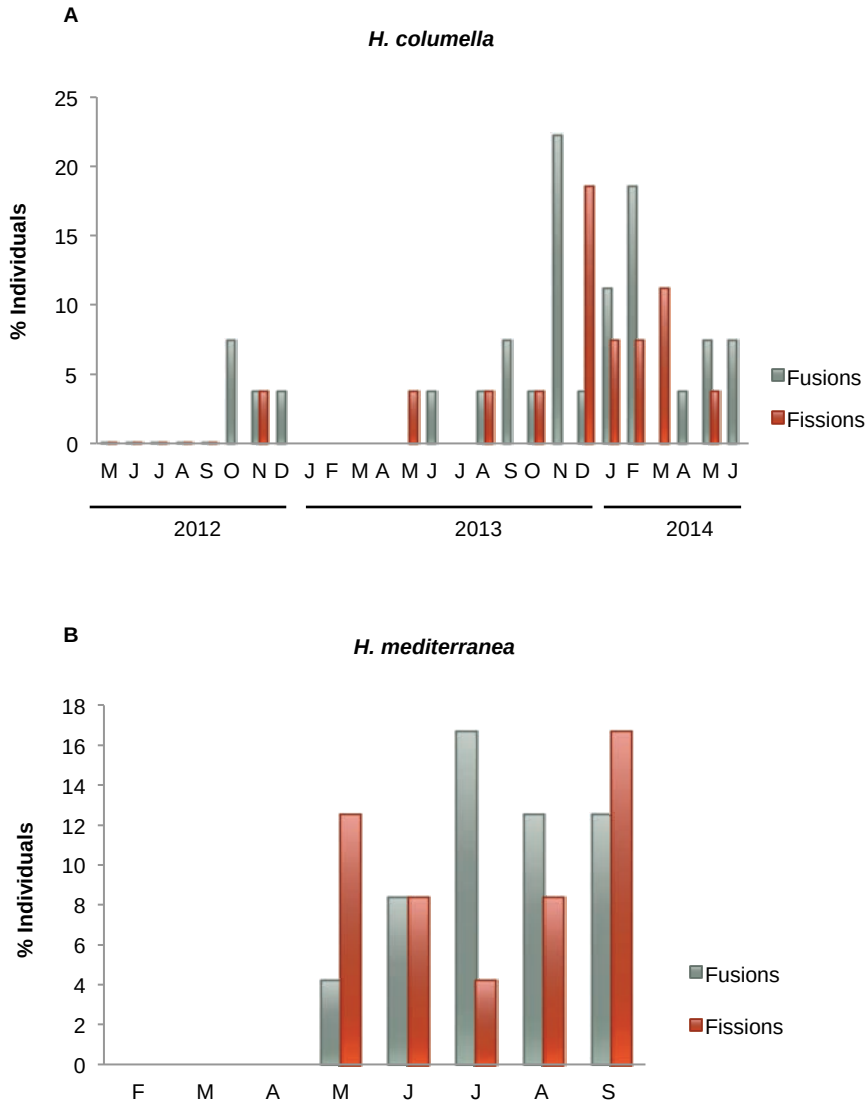


Figure 2.4. A) Percentage of *H. columella* individuals experiencing either fission or fusion events. B) Percentage of *H. mediterranea* individuals experiencing either fission or fusion events.

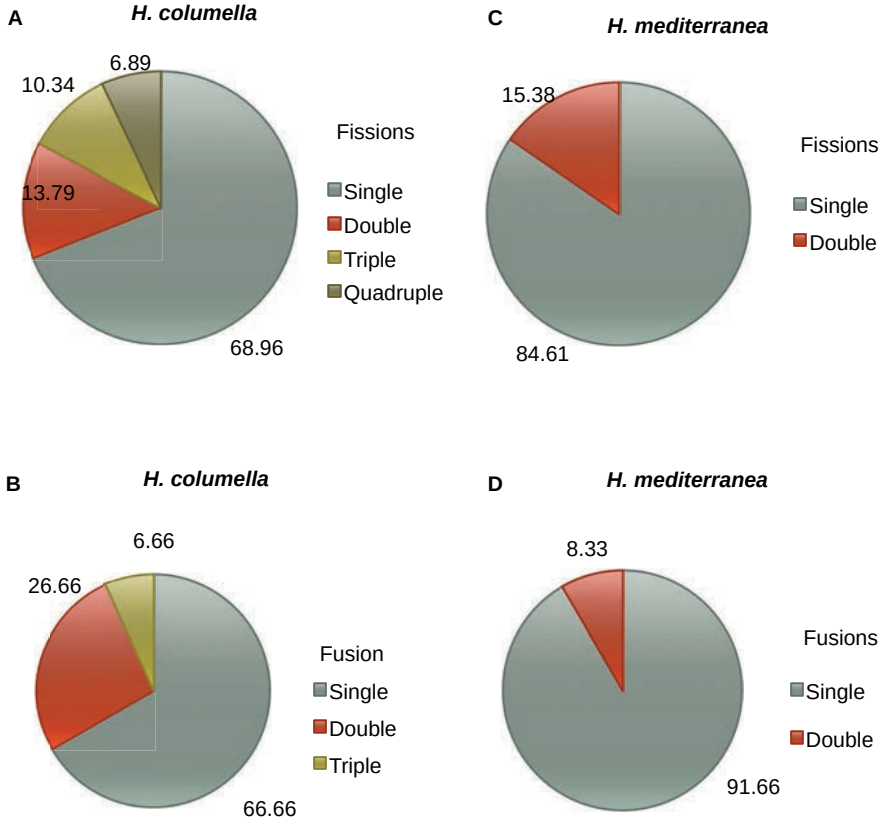


Figure 2.5. A, B) Percentage of *H. columella* individuals showing from one to four fusions or fissions. C, D) Percentage of *H. mediterranea* individuals showing single or double fusions and fissions. Single, double and triple fission and fusion events are counted at each observation time.

Environmental factors

All the environmental factors analyzed varied significantly (ANOVA, $p<0.05$) throughout the year in the habitats of both species, and all of them but PON (ANOVA, $F=0.016$, $p=0.90$) and T (ANOVA, $F=0.64$, $p=0.59$) were significantly different between shallow and deep habitats.

Temperature (T)

The highest T values were detected from June to September in both habitats, corresponding to the Mediterranean summer. However, temperature reached up to 24ºC in shallows habitats but peaked 20 ºC in deep habitats. The minimum T was similar (ca. 12.5 ºC) at both depths in winter (February) (Fig. 2.1).

Dissolved Organic Carbon (DOC)

DOC showed a strong monthly variation at both depths. In the months when both species coexisted, significant differences in DOC concentration were found between depths ($p<0.05$). DOC values were higher in shallow habitats during May and June (80 to 115 μM) than in the deep habitats, but the trend changed the following months. DOC concentrations (80 to 100 μM) in August and September were higher in deep habitats than in shallow habitats (Fig. 2.6 A).

Particulate Organic Carbon (POC)

In the months when both populations coexisted, the deep-water habitats were richer in POC than the shallow-water habitats (Two-way ANOVA, $p<0.05$) (Fig. 2.6 B). In this period, POC ranged from 0.1 mg/L in July to 0.35 mg/L in September (shallow habitats) and from 0.2 mg/L in May to 0.95 mg/L in July (deep habitats). In *H. columella* habitats, during the 21 months after *H. mediterranea* disappeared, POC showed two peaks in winter (one of 0.5 mg/L in January 2014, and the other of 0.75 mg/L in March 2014).

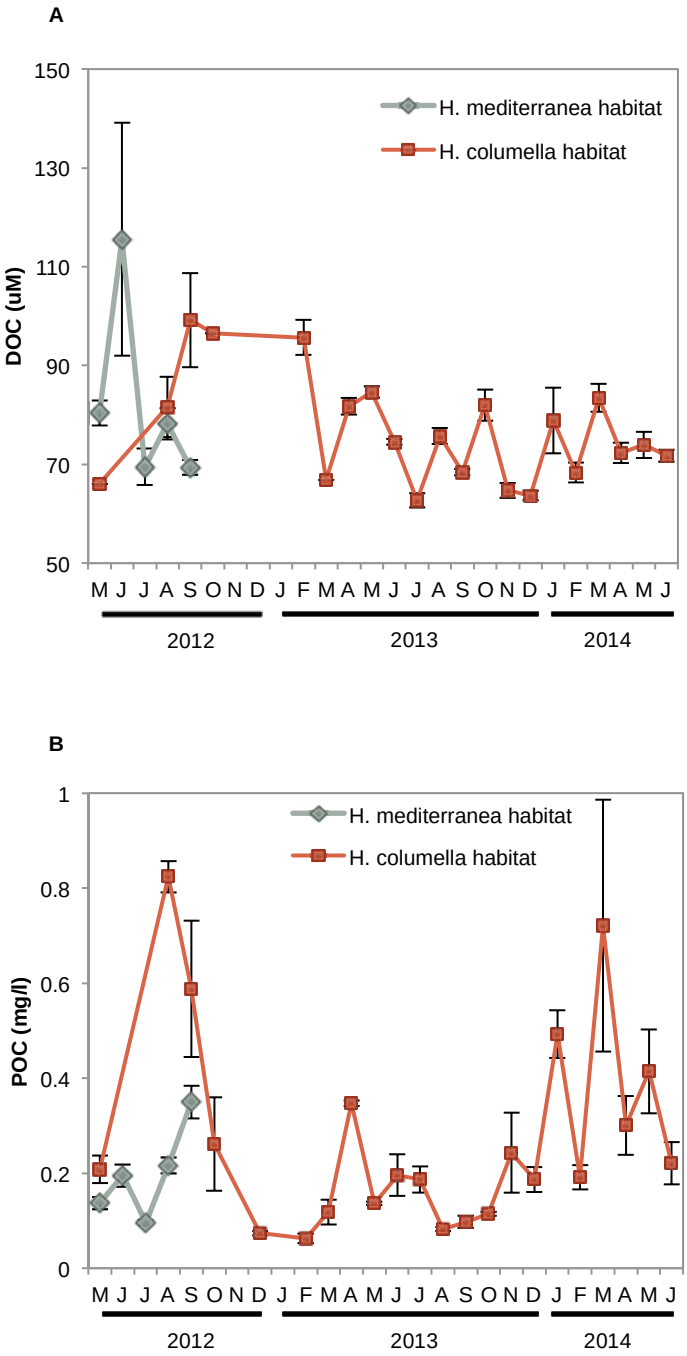


Figure 2.6. A) Mean (± SE) concentration of Dissolved Organic Carbon (DOC). B) Mean (± SE) concentration of Particulate Organic Carbon (POC) in the *H. columella* and *H. mediterranea* habitats, during the entire study period.

Dissolved Organic Nitrogen (DON)

Monthly DON values were significantly higher (ANOVA $p<0.001$) in *H. mediterranea* habitats than in *H. columella* habitats during the period both populations coexisted (2012), with a maximum of ca. 25 µM in July and ca. 11 µM in July to September, respectively (Fig. 7A). During the 21 monitoring months after the *H. mediterranea* population died, DON increased to 17 µM and ca. 12 µM (summer and winter of the second study year, respectively) in the *H. columella* habitat (Fig. 2.7 A).

Particulate Organic Nitrogen (PON)

Monthly PON values were similar between habitats during the period both species coexisted (ANOVA, $p=0.9$), ranging from 0.015 µM to 0.022 µM in *H. mediterranea* habitats and from 0.010 µM to 0.030 µM in *H. columella* habitats (Fig.2.7 B). During the 21 monitoring months after the *H. meditterranea* population died, the highest PON values were 0.035 µM in spring months (April-2013 and May-2014).

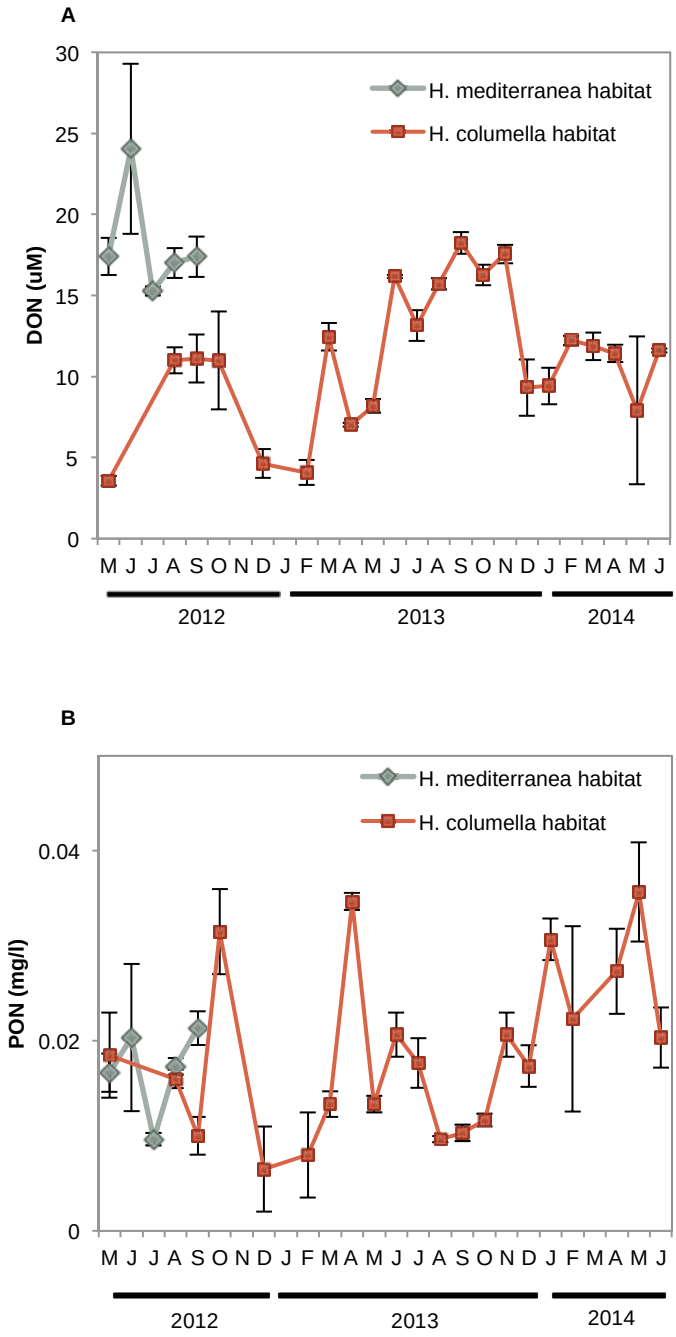
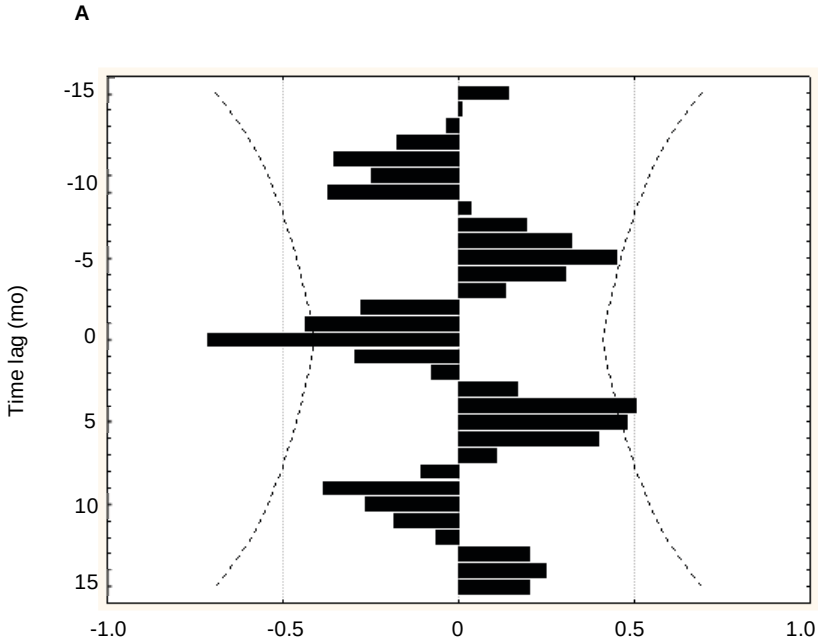


Figure 2.7. A) Mean (± SE) concentration of Dissolved Organic Nitrogen (DON). B) Mean (± SE) concentration of Particulate Organic Nitrogen (PON) *H. columella* and *H. mediterranea* habitats during the entire study period.

Cross-correlation

Growth rates of *H. columella* were only correlated, but negatively, with temperature and DON concentration (Figs. 2.8 A, B). Growth rates were negatively correlated with the temperature of the same and previous month (time-lag=0 and -1) (Fig. 2.8 A), and with DON concentration of the same month (time-lag=0) (Fig. 2.8 B). Conversely, growth rates of *H. mediterranea* were not correlated with any environmental factor analyzed.



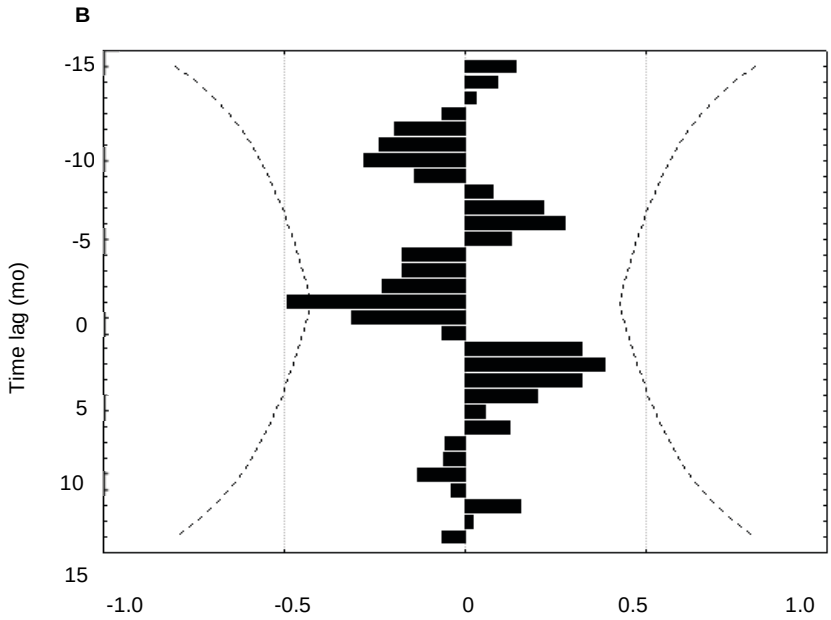


Figure 2.8. A) Cross-correlation between the growth rate of *H. columella* and temperature. B) Cross-correlation between the growth rate of *H. columella* and Dissolved Organic Nitrogen (DON). Positive and negative correlation values are indicated on the X-axis. Negative and positive time lags of any correlation are represented on the Y-axis. Correlation at time lag 0 corresponds to the usual Pearson's correlation. Negative time-lags (months) account the number of instances when values in the first series (sponge growth rates) relate to previous values in the second one (A, temperature; B, DON). Positive time-lags indicate the number of instances when values in the first series correlate to values afterwards in the second series. Curved lines denote expected 95% confidence intervals.

DISCUSSION

Hemimycale columella and *H. mediterranea* strongly differed in growth rates during the year. The highest growth rates of *H. columella* occurred in the coldest months and, consequently, the correlation between temperature and the sponge monthly growth rates was negative. Conversely, no correlation between growth rate and temperature was found for *H. mediterranea*. Previous studies have reported contrasting effects of temperature on sponge growth, depending on the species. For example, in the temperate Pacific, the highest growth rate was recorded in winter (10.6 ºC in average) for *Latrunculia wellingtonensis,* and in spring (14.5 ºC) for *Polymastia croceous,* (Duckworth et al. 2004). Positive and negative correlations between temperature and growth have also been reported in both Mediterranean (e.g. Turon et al. 1998; Blanquer et al. 2008; De Caralt et al. 2008; Ferretti et al. 2009) and North Atlantic (Koopmans & Wijffels 2008) sponge species. Consequently, the potential effect of temperature on sponge growth appears to be species-specific.

Food availability is key for animal growth. Sponges can take dissolved (Yahel et al. 2003; de Goeij et al. 2008) and particulate matter from the surrounding water, and have been reported to be particularly efficient in retaining small particles (Ribes et al. 1999a; Ribes et al. 2005; Lesser 2006; Jiménez & Ribes 2007; De Caralt et al. 2008; Koopmans & Wijffels 2008; Riisgard & Larsen 2010). Dissolved organic matter was significantly higher in the *H. mediterranea* habitat than in *H. columella* habitat, in spring for DON and DOC, and in summer for DON, likely as a result of a higher phytoplankton excretion and decomposer activity in shallow habitats (Ribes et al. 1999b; Pujo-Pay & Conan 2003). During the period the species coexisted (i.e. spring to autumn), growth rates of *H. mediterranea* were significantly higher than those of *H. columella*. In this period, DOC and DON reached the highest values in *H. mediterranea* habitats, alongside with the higher values of growth rates. However, between-species differences in growth rates could not be compared in winter, as *H. mediterranea* was not present.

H. mediterranea does not seem to suffer from the Mediterranean aestivation reported for other filter feeders (Coma & Ribes 2003) and

behaves more alike to other Mediterranean endemisms (e.g. *Scopalina lophyropoda*) that reach their maximum growth rates in summer (Blanquer et al. 2008). Conversely, growth rates of *H. columella* decreased in summer, and thus the species experiences aestivation, as reported for other sponges, such as *S. blanensis*, with a purported Atlantic origin (Blanquer et al. 2008).

Fissions and fusions are frequent in encrusting sponge species (Turon et al. 1998; Tanaka 2002; Blanquer et al. 2008; De Caralt et al. 2008) and have been interpreted as the result of stressing interactions with other organisms such as those involved in competition for space (Pawlik 1997; 1998; Wulff 1997; Cebrian & Uriz 2006), predation, or partial mortality (e.g. Tanaka 2002). Fusions and fissions were similarly moderate for both species (20% in *H. mediterranea* individuals and ca. 25% in *H. columella*), and lower than those reported for other Mediterranean sponge species: up to 40% and 60% individuals experienced fissions and fusions, respectively in *S. blanensis* (Blanquer et al. 2008). The highest number of fissions in *H. mediterranea* was recorded after release of larvae, immediately before species demise, possibly as a result of post-reproduction stress (Ereskovsky 2000). Conversely, no fissions occurred after larval release in *H. columella* (authors obs.), which did not experience mortality.

The most striking difference between the two species was their contrasting life spans. The mass death of *H. mediterranea* occurred in early autumn after larval release (Pérez-Porro et al. 2012; authors obs.), while 70% of the monitored individuals of *H. columella* persisted after the reproduction period and 64% were still alive after two years. Demise of *H. mediterranea* occurred after release of larvae when sponges have been reported to stop filtering by closing their inhalant orificies (Turon et al. 1999), and to devote their energy to the rearrangement of the aquiferous system.

Water flow can also influence sponge success not only by facilitating filtering and thus food availability and excretion of waste materials, but also by either promoting or preventing larval settlement (Maldonado & Uriz 1998; Uriz et al. 2008). Settlement of the poorly swimming, sponge larvae has been reported to be more successful on horizontal structurally complex surfaces (Maldonado & Uriz 1998), such as those at the *H. columella* habitat,

than on less complex, shallow rocky boulders, inhabited by *H. mediterranea*. The high individual survival rates recorded, together with a high potential recruitment rates on the coralligenous assemblages (Maldonado & Uriz 1998) may have contributed to *H. columella* persistence as a consequence of the overlapping of generations.

To summarize, we confirm that the two cryptic sponge species *H. columella* and *H. mediterranea* show contrasting life histories, being *H. columella* multiannual and *H. mediterranea* annual, as it has also been reported to disappear after reproduction in other shallow locations (Pérez-Porro et al. 2012). Annual life spans are common in calcareous sponges (Guardiola et al. 2012) while they are rare among Demosponges. Sponge life histories appear to be more diverse than currently though. The strong biological differences showed by these sponge species contrast with their slight differences in phenotypic characters and highlight the need of untangling the cryptic diversity of ecosystems to guarantee the reliability of ecological studies.

ACKNOWLEDGEMENTS

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Chapter 3

Endosymbiotic calcifying bacteria across sponge species and oceans

ABSTRACT

From an evolutionary point of view, sponges are ideal targets to study marine symbioses as they are the most ancient living metazoans and harbour highly diverse microbial communities. A recently discovered association between the sponge *Hemimycale columella* and an intracellular bacterium that generates large amounts of calcite spherules has prompted speculation on the possible role of intracellular bacteria in the evolution of the skeleton in early animals. To gain insight into this purportedly ancestral symbiosis, we investigated the presence of symbiotic bacteria in Mediterranean and Caribbean sponges. We found four new calcibacteria OTUs belonging to the SAR116 in two orders (Poecilosclerida and Clionaida) and three families of Demospongiae, two additional OTUs in cnidarians and one more in seawater (at 98.5% similarity). Using a calcibacteria targeted probe and CARD-FISH, we also found calcibacteria in Spirophorida and Suberitida and proved that the calcifying bacteria accumulated at the sponge periphery, forming a skeletal cortex, analogous to that of siliceous microscleres in other demosponges. Bacteria-mediated skeletonization is spread in a range of phylogenetically distant species and thus the purported implication of bacteria in skeleton formation and evolution of early animals gains relevance.

INTRODUCTION

Symbiosis, whereby different biological species live together in intimate, long-term interactions, is regarded as a major source of evolutionary innovation (Margulis & Fester 1991). Symbiotic associations of animals and microbes are widespread in marine ecosystems and play key ecological roles by contributing in an important, and often cryptic, way to the ecosystem biodiversity and stability. For example, shifts in symbiotic bacterial communities have been attributed to recurrent mass mortalities of corals and sponges (Lesser et al. 2007; Webster et al. 2008; Cebrian et al. 2011; Blanquer et al. 2016).

From an evolutionary point of view, sponges are ideal targets to study marine microbial symbioses, as they are the most ancient living metazoans on Earth and harbour highly diverse microbial communities (Thacker & Freeman 2012). The establishment of symbiotic relationships between sponges and prokaryotes has been traced back to the pre-Cambrian period (Wilkinson 1984). However, despite the abundance of available sequences from sponge-associated bacteria and their long history, the evolutionary origins of these associations and the adaptive traits of the species involved are only beginning to be understood (Thomas et al. 2010; Liu et al. 2011; Hentschel et al. 2012; Gao et al. 2014).

Recently, a symbiotic association between the Atlanto-Mediterranean sponge *Hemimycale columella* (Bowerbank, 1874), and an unidentified calcifying bacterium has been shown to produce thousands of calcite spherules (Uriz et al. 2012). The calcifying bacteria are contained within vacuoles in amoeboid, archaeocyte-like cells (Boury-Esnault & Rützler 1997), or 'calcibacteriocytes', where they divide by bipartition before becoming enclosed within a 100-nm thick calcite envelope. The calcifying bacterium is vertically transmitted to the sponge progeny (Uriz et al. 2012) via phagocytosis of maternal calcibacteriocytes as embryos grow (Simpson 1984). Uriz et al. (2012) speculated that this type of eukaryote-prokaryote symbiosis might represent a relict mechanism involved in the evolution of skeletons in Lower Metazoa. Later, Blanquer et al. (2013) used pyrosequencing to retrieve a dominant

alpha-proteobacterium in *H. columella* that represented up to 67% of the total sequences in the sponge and was similar to that obtained from the sponge *Cliona viridis* (Schmidt, 1862).

To gain insight into this newly identified symbiosis and its possible role in the evolution of skeletons in Lower Metazoa, we investigated whether the dominant alpha-proteobacteria reported by Blanquer et al. (2013) corresponded to the extremely abundant calcifying bacteria that form calcareous spherules in the sponge tissues (Uriz et al. 2012). We also explored the molecular diversity of the symbiotic calcibacteria, and their presence in other sponge species, to assess the occurrence of this symbiosis across sponges and oceans. Moreover, we explored whether the calcibacterium of *H. columella* is evenly distributed within the sponge tissues or accumulate in particular sponge zones, thus, fulfilling a potential skeletal function, and analyse the purported costs and benefits for the symbiotic partners.

To achieve these objectives, we performed ultrastructure studies using transmission electron microscopy (TEM) and scanning electron microscopy (SEM), and designed a molecular probe based on the dominant bacterial species of *H. columella*. We also conducted CARD-FISH experiments and cloned the 16S rRNA gene of the calcibacterium from *H. columella* and *C. viridis* to examine their phylogenetic relationships.

MATERIAL & METHODS

3.1. Sampling and sample preservation and treatment

The sponge species studied all harboured 1 μm in diameter calcareous spherules, similar to those reported for *Hemimycale columella* (Uriz et al. 2012) (Figure 3.1). From one to three samples per species (depending on the species availability) were collected by SCUBA diving between 10 and 30 m depth in several seas: *H. columella* (whitish and pinkish morphs) and *Cliona viridis* from the Northwestern Mediterranean (Arenys de Mar, Spain); *Protosuberites* sp., and *Chinachyrella alloclada* (Uliczka, 1929) from the Caribbean Sea (Florida, USA), and *Crella cyatophora* (Carter, 1869) from the Red Sea (Sharm el Sheikh, Egypt).

Samples were preserved and treated according to the study purposes:

3.1.1. For light microscopy, 50 mm^3 samples were fixed in 4% paraformaldehyde, embedded in paraffin, and cut with an Autocat 2030 microtome (Reichert-Jung) to obtain 5 μm thick sections.

3.1.2. For Scanning Electron Microscope (SEM), ca. 4 mm3 sponge samples were fixed, critical point dried, and coated with gold-palladium (Uriz et al. 2008). Samples were observed using a Hitachi SEM at the Institute of Marine Sciences (ICM-CSIC).

3.1.3. For Transmission Electron Microscope (TEM) observation, ca. 3 mm^3 samples were fixed, rinsed with buffer, dehydrated, and embedded in a plastic resin (Leys & Reiswig 1998). Ultrathin sections were cut using an ultramicrotome (Ultracut Leica), and stained with uranyl acetate and lead citrate. Samples were observed using a JEOL 1010 TEM, implemented with Bioscan (Gatan) for image digitalization (Microscopy Unit of the Scientific and Technical Services of the University of Barcelona).

3.1.4. For CARD-FISH experiments, ca. 50 mm^3 samples were fixed in 4% paraformaldehyde for 4 h and then transferred to ethanol 70% and embedded in paraffin. Histological sections were performed according to the study needs (Table 3.2).

3.1.5. For bacterial cloning, samples of *C. viridis* and *H. columella* were submersed in absolute ethanol immediately after collection and taken to the laboratory in a cooled container.

Different analyses were performed on different sub-sets of sponge species.

3.2. Calcibacteria location and quantification

We quantified the calcibacteria in the same set of *H. columella* individuals by two procedures to confirm that the calcibacteria were inside the calcareous spherules and to avoid possible biases in bacteria quantification in hybridized sponge sections due to signal overlapping in Epiflourescence Microscopy (EM):

i) Hybridization of a filtered aliquot of the extracted calcibacteria (see procedure below) and observation using EM.

ii) Direct tissue hybridization and observation though Confocal Laser-Scanning Microscopy (CLSM).

3.3. Calcibacteria extraction

Calcibacteria spherules were exhaustively extracted from fresh *H. columella* pieces. Pieces of ca. 1 g of fresh sponge (three individuals per colour morph and two regions per individual) were disaggregated and homogenized. Siliceous spicules were precipitated and then the pellet was discarded. The spicule-free homogenates were subjected to a series of centrifugations and re-suspensions (Garate et al. 2015–Chapter 5). One aliquot of the each final spherule suspensions (three per individual) was filtered through 0.2 µm pore filters and filters were CARD-FISH treated (Table 3.1).

3.4. Oligonucleotide probe design

An oligonucleotide probe targeting the prevalent alpha-proteobacteria sequence in the sponge *H. columella* was designed using ARB software (http://www.arb-home.de/). The target sequence matched with two alpha-proteobacteria from the water column (GenBank ID: KC425597.1; GenBank ID: EF471706) and another from the cnidarian *Erytropodium caribaeorum* (GenBank ID:889934.1). The best probe was checked in silico with the online software MathFish (http://mathfish.cee.wisc.edu/) and its efficacy confirmed using the probe match tool in ARB. We also designed two 'competitor sequences' to avoid non-specific hybridizations, and two 'helper sequences' (Table 1) to hybridize the flanking regions of the specific probe. A non-sense probe (Non-EUB 338-I 5′-ACTCCTACGGGAGGCAGC-3′, Wallner et al. 1993) was used as a negative control (Table 1). All the probes were synthesized using Biomers (http://www.biomers.net/).

3.5. Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH)

We used CARD-FISH with the designed probe to verify whether the dominant sequence in the species *H. columella* corresponded to the abundant intracellular calcifying bacteria, observed using TEM and SEM, and to detect its presence in other sponge species harbouring 1 µm diameter calcareous spherules: *H. columella* (Poecilosclerida, Hymedesmiidae), *C. viridis* (Hadromerida, Clionaidae), *Protosuberites* sp. (Hadromerida, Suberitidae), and *C. alloclada* (Spirophorida, Tetillidae). CARD-FISH was also used to quantify calcibacteria within the main sponge regions (ectosome and choanosome) of whitish and pinkish morphs of *H. columella* (N = 3) and in two stages of the sponge life cycle: reproductive individuals incubating larvae and non-reproductive individuals.

Tissue samples that were fixed in 4% paraformaldehyde were dehydrated, embedded in paraffin, cut, deparaffined, and subjected to membrane permeabilization and inactivation of endogeneous peroxidases following procedures listed in Table 3.1 (modified from Pernthaler & Pernthaler 2004). Filters containing the extracted calcareous spherules were directly subjected to bacteria membrane permeabilization and inhibition of endogenous peroxidases. The optimum formamide concentration for the specific probe (i.e. 45%) was determined from assays at concentrations of 55%, 45%, and 35%. Following CARD-FISH, the sponge sections and the isolated spherules were DAPI stained to observe DNA. Dehydrated samples were mounted using Citifluor.

To discount self-fluorescence from the hybridized tissue, three sponge sections were treated according to the CARD-FISH protocol without adding the probe. Moreover, hybridization was assayed in tissues of the sponge *Crambe crambe*, a species that does not harbour the target calcibacteria (Croué et al. 2013), to confirm that the hybridization signal observed was not an artefact.

Step	Description
1. Sample fixation	1.Incubate sponge samples within 4% paraformaldehyde solution for 4h 2. Incubate samples in ethanol 70% during 18-24h at 4˚C 3. Keep samples in ethanol 70% at -20˚C
2. Sample dehydration	Sequential incubation of sponge samples in ethanol 96% and 100%, ethanol:toluene (1:1) 30 min each, and absolute toluene 15min
3. Embedding in paraffin and tissue sectioning	1. Include samples in paraffin at 50˚C for 24-48h 2. Cut thick (6µm) sections with an Autocut 2030 (Reichert-Jung) microtome and dry for 3h at 40˚C
4. Deparaffinization	1. Incubate sections within Xylene for 10min 2. Rehydratation by sequential incubation in ethanol 100%, 96%, 70%, 10min each 3. Three baths in Milli-Q water 5min each, air dry
5. Membrane permeabilization	1. Incubate sections in 10mg/ml Lysozime solution (Sigma USA), 0.05M EDTA, 0.1M Tris-HCl for 1h at 37˚C 2. Wash with Milli-Q water for 2min, air dry
6. Endogenous peroxidases inactivation	1. Incubate sections in 0.1M HCl solution for 30-60sec 2. Wash with 1X PBS for 2min 3. Incubate in 3% H_2O_2 solution for 10min 4. Wash with Mlili-Q water and ethanol 96% 2min each, air dry
7. Hybridization	1. Cover sections with hybridization buffer (1) solution together with the probe, helpers and competitors in a 3:100 volumetric ratio. 2. Incubate at 46˚C for 5h with a solution of 45% formamide in humid chambers. 3. Wash the sections with pre-warmed washing buffer (2) at 48˚C for 10min
8. CARD	1. Wash sections in 1X PBS (pH=8) for 15min 2. Cover sections with primary CARD substrate mix (3) 3. Incubate at 46˚C for 20min 4. Wash twice in 1X PBS for 6min at 46˚C and RT 5. Wash twice in Milli-Q water for 2min each and air dry.

(1) The hybridization buffer was made by mixing 5M NaCl, 1M Tris-HCl (pH7.5), 20% sodium dodecyl sulfate (SDS), 10% (w/v) dextran sulfate, 10% Blocking Reagent and 45% formamide (Sigma).
(2) Fresh whashing buffer was prepared by mixing 0.5M EDTA, 1M Tris-HCl, NaCl and 20% SDS in steryle MilliQ water and warmed at 48ºC previously to the wash step.
(3) The primary CARD substrate was made by mixing amplification buffer (10% (w/v) dextran sulfate, 2 M NaCl, 0.1% (w/v) blocking reagent, in 1X PBS (pH=8)) with a freshly prepared H_2O_2 solution (0.15% in 1X PBS) at a ratio of 100:1. The needed volume of that primary CARD mix solution was mixed with 1mg dye ml-1 tiramide-Alexa488 solution (Molecular Probes, Inc., Eugene, OR, USA) at a ratio of 500:5.

Table 3.1. Different steps of the CARD-FISH protocol.

3.6. Quantification using Epifluorescence Microscope

Hybridization was conducted on extracted calcibacteria from the sponge ectosome and choanosome of whitish and pinkish morphs. The resulting calcibacteria were re-suspended in sterilized seawater and an aliquot of this suspension was filtered through a 0.2 μm polycarbonate filter. Filters containing the calcibacteria were hybridized using CARD-FISH and observed using an epifluorescence microscope (EM) (Axioimager, Zeiss). Pictures were captured from 10 randomly selected fields at 100× magnification using an Axiocam MR3 (Zeiss) digital camera (820 ms exposure time) attached to the microscope. We divided each field into 16 quadrats and counted the number of calcibacteria (hybridization points) in four randomly selected quadrats using Adobe Photoshop. The average number of calcibacteria per quadrat was multiplied by a factor related to the spherule concentration in the initial suspension.

3.7. Quantification using confocal laser-scanning microscopy

We quantified calcibacteria in whitish and pinkish morphs of *H. columella* (N = 3) using a Leica TCS-SP5 confocal spectral microscope (Leica Microsystems Heidelberg GmbH, Universitat Autònoma of Barcelona) with a Plan-Apochromatic 63 ¥ 1.4 (oil HC ¥ PL APO lambda blue objective). A series of images (three fields per section) were taken every 1 μm (axis-z) across 6 μm thick histological sections to observe the emission signals of Alexa 488 and DAPI. The images were processed using Metamorph Imaging software (Universal Imaging Corporation, West Chester, PA, USA). We measured the integrated fluorescence intensity of the signal emitted by hybridized bacteria after removing the background fluorescence from the control samples.

3.8. DNA extraction, amplification and cloning

Samples of *C. viridis* and *H. columella* were submersed in absolut ethanol immediately after collection and taken to the laboratory in a cooler container. *H. columella* was extracted with Qiamp DNA stool kit (Qiagen) and *C. viridis* with DNeasy blood and tissue kit (Qiagen). One 16S rRNA gene fragment, ca. 1450 nt. in size, was amplified using

universal primers 26F and 1492R (Lane 1991). PCR conditions were as described previously (Kjeldsen et al. 2010). The PCR products were purified using QIAquick PCR Purification kit (Qiagen) and cloned using TOPO® TA Cloning® Kit for Sequencing, with One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen), according to the manufacturer´s instructions. Following colony growth, correct-size inserts were identified by PCR using T3-T7 primers, and purified and sequenced using the Sanger method (Macrogen Europe). Sequences containing the 273 nt. calcibacteria fragment (Blanquer et al. 2013), were selected and aligned with the SILVA database using SINA web aligner. The alignment was merged into ARB software and improved with Fast Aligner tool according to the secondary structure.

3.9. Statistical analyses

Normality and homoscedasticity of data was verified (Potvin et al. 1990). Differences in calcibacteria abundance between sponge zones in different colour morphs were analysed using two-way ANOVA, with sponge colour (whitish or pinkish) and zone (ectosome or choanosome) as fixed orthogonal factors. Integrated fluorescence intensities obtained using CLSM were analysed using the Mann Whitney U-test, since data did not meet the assumptions of normality and/or homoscedasticity. Cloned sequences containing the 273 nt. calcibacterium fragment (Blanquer et al. 2013), and the closest sequences were exported from ARB and used to construct a Bayesian phylogenetic tree using MrBayes 3.2 software. The GTR evolutionary model was used. Four Markov Chains were run with ten million generations sampled every 1000 generations. The chains converged significantly and the average standard deviation of split frequencies was less than 0.01 at the end of the run. Early tree generations were discarded by default (25%) until the probabilities reached a stable plateau (burn-in) and the remaining trees were used to generate a 50% majority-rule consensus tree.

The threshold used for considering a group of sequences belonging to the same "species" (now OTU) was a sequence similarity higher than

98.5%16 while similarities > 95% suggest same genus (Schmitt et al. 2012).

RESULTS

Light microscope observation

Morphologically similar calcareous spherules, 1 μm in size, were observed in the sponges *Hemimycale columella, Cliona viridis*, *Prosuberites* sp., *Crella cyathophora* (Fig. 4.1), and *Cinachyrella alloclada* (not shown). Profuse numbers of spherules were released from the squeezed tissues of all the sponge species examined, except in *C. viridis* and *C. alloclada*, where they were less abundant.

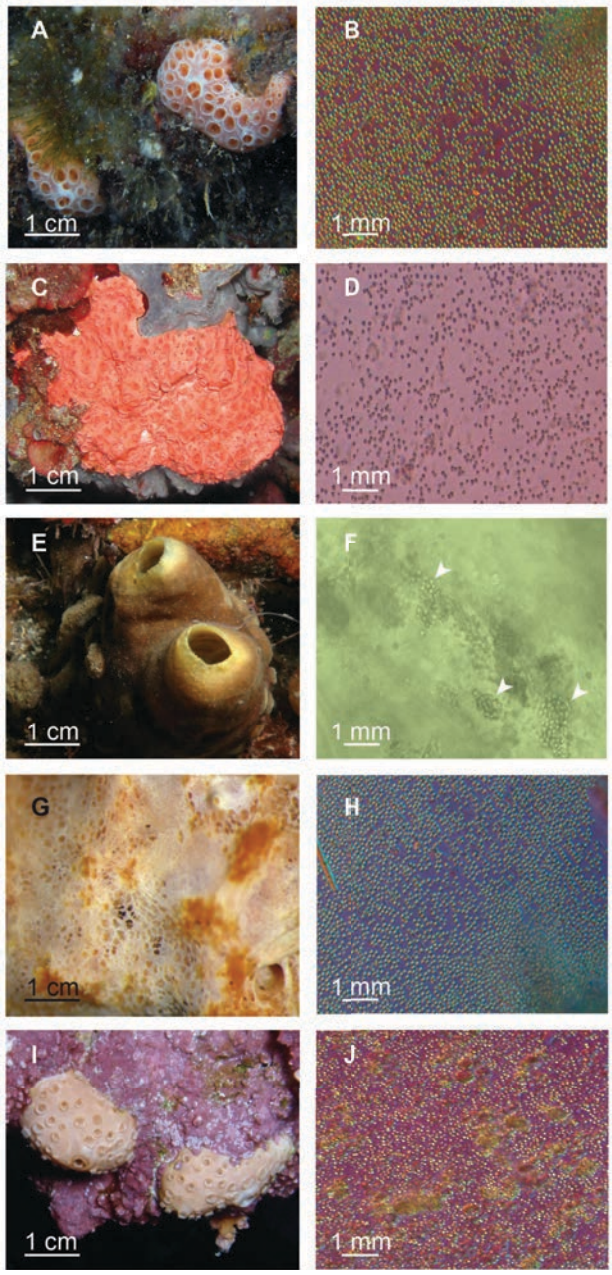

Figure 3.1. Living target sponge species and their calcareous spherules after fresh tissue squeeze. A,B) Whitish morph of *Hemimycale columella*. C,D) Pinkish morph of *H. columella*. E,F) *Cliona viridis*. G,H) *Prosuberites* sp. I,J) *Crella cyatophora*.

The calcareous spherules were concentrated at the sponge periphery of whitish individuals of *H. columella* (Fig. 3.2 A, B). Moving sponge cells (calcibacteriocytes) full of calcibacteria were recorded immediately after sponge disaggregation (Fig. 3.2 C), which confirmed the amoeboid-like properties of these cells and their capacity to transport calcibacteria across the sponge tissues.

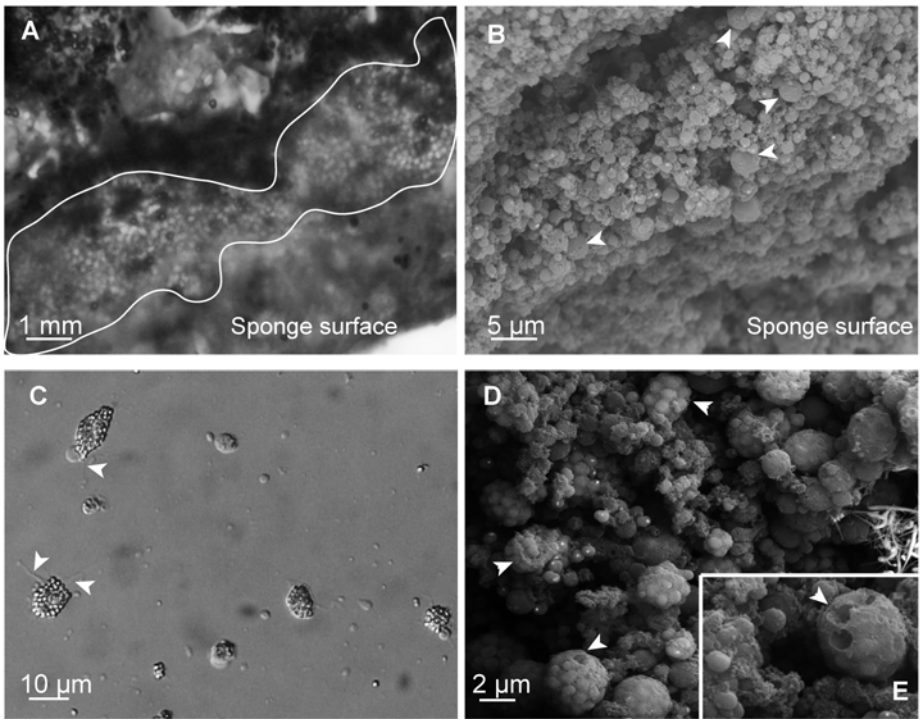

Figure 3.2. Calcibacteria and calcibacteriocytes from *Hemimycale columella* observed using light and SE microscopes. A) Light microscope and (B) SEM pictures of calcareous spherules accumulated at the sponge periphery forming a kind of cortex. A few sponge non-granulose cells (arrowheads) are shown (B) among the dense layer of calcareous spherules. C) Calcibacteriocytes showing pseudopodia and phyllopodia (arrowheads) while creeping across a solid surface (light microscopy). D) Calcibacteriocytes full of calcified calcibacteria. E) Close view of calcibacteriocytes (SEM). Hemispherical holes (arrowheads) correspond to the space previously occupied by calcibacteria.

Ultrastructure

SEM images of *H. columella* (whitish morph) showed huge numbers of calcareous spherules that had been released into the sponge mesohyl (Fig. 3.2 B), as well as abundant, 10–15 µm calcibacteria-full calcibacteriocytes (Fig. 3.2 D). The calcareous spherules were accumulated at the sponge periphery (Fig. 3.2 D) forming a 2–3 mm thick layer, which concurred with light microscope observations (Fig. 3.2 A). Some calcibacteriocytes showed hemispherical, ca. 1 µm in diameter holes, which corresponded to the space previously occupied by released calcibacteria (Fig. 3.2 D). There were frequent images of calcibacteria enclosed within the calcareous coat as they divided (Fig. 3.3 C).

TEM pictures of calcibacteriocytes showed abundant cellular vacuoles containing single or dividing calcibacteria (Fig. 3.3 A). Most vacuoles contained a single bacterium, but several bacteria were also observed enclosed within a common calcareous coat after successive incomplete divisions (Fig. 3.3 C). Calcified calcibacteria degraded in most cases, as indicated by the scarce or absent organic content within the calcareous crusts, but also formed condensed bodies, ca. 200 nm in size, that might correspond to starved forms (Fig. 3.3 B). Uncalcified calcibacteria were spherical and relatively small (ca. 0.2–0.8 µm in cross-section), showed a thin bacterial wall and were found abundantly in the mesohyl of sponge larvae, after being released from engulfed maternal cells (Fig. 3.3 F). There, they divided profusely by bipartition (Fig. 3.3 G). Archaeocyte-like embryo cells (newly differentiated calcibacteriocytes) contained calcified calcibacteria (Fig. 3.3 E). The calcareous crust that surrounded calcibacteria was clearly visible in SEM images of samples that were fixed for just 2 h (see methods) (Fig. 3.3 H).

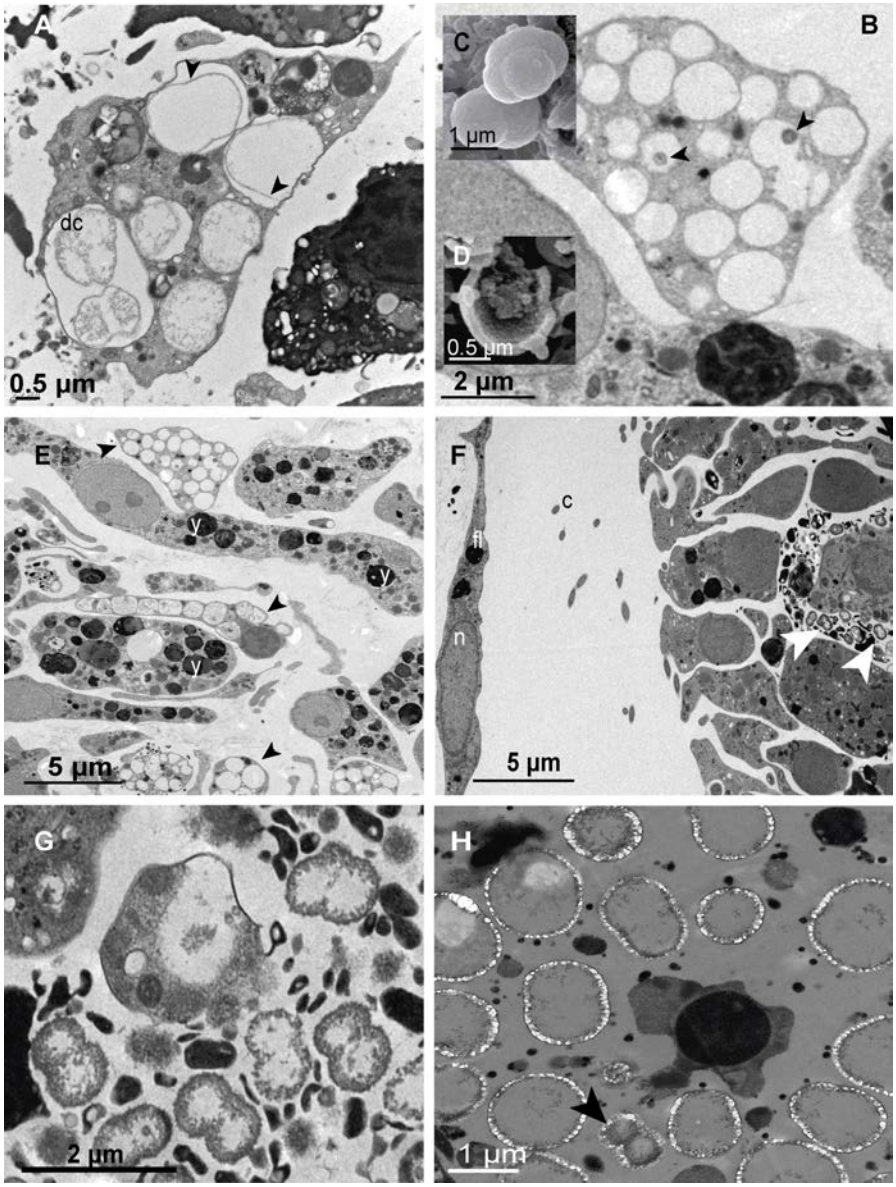

Figure 3.3. Ultrastructure images of *Hemimycale columella* tissues from adults and larvae. A) Calcibacteria in adult tissue while dividing (dc) within a calcibacteriocyte (TEM). B) Calcibacteriocyte showing the empty vacuoles previously occupied by calcified calcibacteria: arrowheads point to condensed bacterial remains (TEM). C) SEM images of calcified calcibacteria undergoing division. D) SEM image of a broken calcified calcibacterium showing a 100 nm thick calcareous crust and reduced organic matter inside. E) Larval inner cells with yolk reserves (y) and larval calcibacteriocytes (arrowheads) full of remains of calcified calcibacteria (shown in the image as light to electron vacuoles with scarce organic material) (TEM). F)

Section of a larva within the follicle showing a calcibacteriocyte of maternal origin surrounded by non-calcified calcibacteria (arrowheads) released into the larval mesohyl: fl, follicular cell; c, cilia of the larval peripheral cells; n, nucleus (TEM). G) Free calcibacteria within the larva mesohyl showing profuse cell division by bipartition (TEM). H) Pictures of free calcibacteria undergoing calcification in the sponge mesohyl (TEM). Calcification in the form of nanospherules can be observed. Arrowhead points to a calcibacterium enclosed within the calcareous crust while dividing.

Calcibacteria-specific probe

Table 1 shows the best 18 nt. long probe that targeted the prevalent alpha-proteobacteria sequence in *H. columella* (CAL32), as well as the 5´- 3´ helpers, and competitors.

The "non-sense" probe, which was used as a negative control, did not hybridize in the samples (images not shown), and no hybridization occurred when the specific probe was assayed in the species *Crambe crambe* (Schmidt, 1862) (negative sponge control, images not shown), which confirmed that the hybridization signal observed was not an artefact and corresponded to the target calcibacteria.

Catalysed reporter deposition fluorescence in situ hybridization (CARD-FISH).

CARD-FISH using the designed probe (CAL32L), helpers, and competitors allowed us to detect the presence of the target calcibacterium at high densities within the tissues of *H. columella,* (adult and larvae), and *Prosuberites sp.*, and at lower densities in *C. alloclada* and *C. viridis* (Fig. 4.4). There were numerous hybridized points in the sponge mesohyl and within sponge cells (calcybacteriocytes) (Fig. 3.4 G, H, I). Similarly, isolated calcibacteria from *H. columella* tissue on filters also hybridized in high numbers (Fig. 3.4 A, B). This confirmed that the bacteria, which form the calcareous spherules within calcibacteriocytes, corresponded to the dominant alpha-proteobacteria sequence of *H. columella.*

The DAPI staining showed two discrete cell sizes, corresponding to DNA material of sponge cells and calcibacteria. Strong hybridization signals that overlapped with the majority of the DAPI-stained small points were observed with the CAL32L specific probe (Fig. 3.4 A, B) in both tissue sections and extracted calcified calcibacteria, which proved the correspondence of the target bacteria with the calcareous spherules and the abundance of calcibacteria in the sponge tissues and cells. Negative controls including the non-sense probe, a confirmed calcibacteria-free sponge, and a non-probe control yielded no hybridization signals confirming the precision of the hybridization and a lack of endogenous peroxidases in the sponge tissue. However a few other non-hybridized bacteria also were DAPI stained in the sponge tissues. Moreover, although targeting cyanobacteria CARD-FISH was not performed, sporadically red/orange fluorescence was observed under green excitation (Fig. 3.4), indicating the occasional presence of photosynthetic bacteria in the sponge tissues.

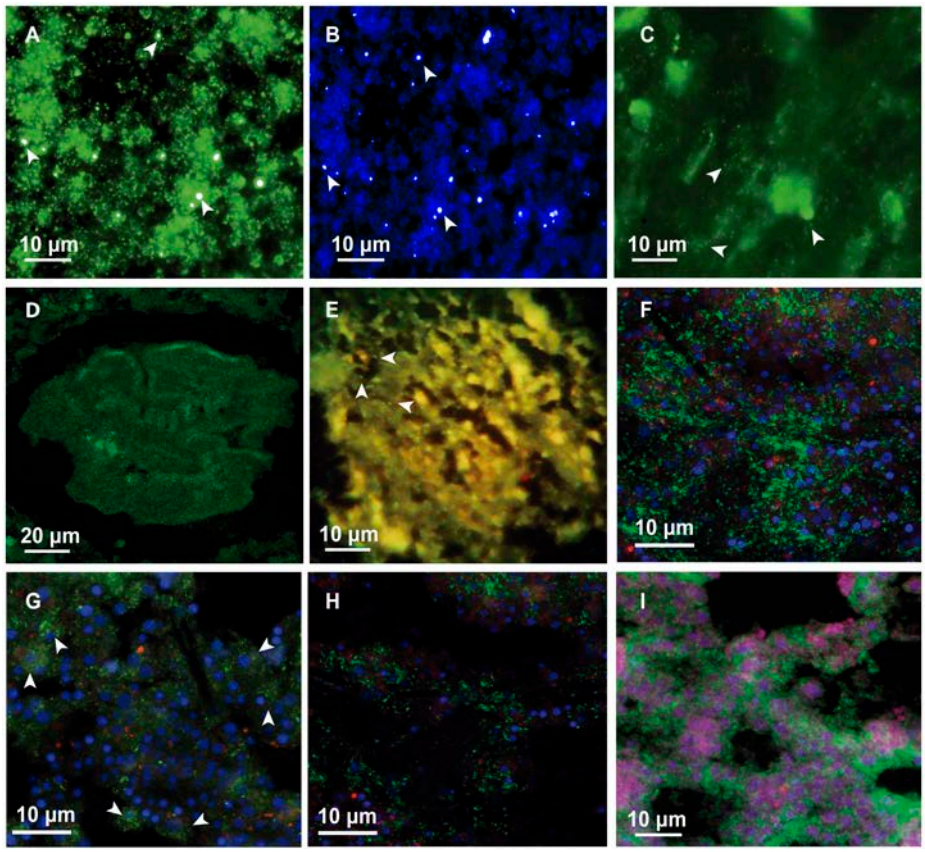

Figure 3.4. Study species hybridized with the CAL32L probe targeting the calcibacterium. A, B) Filters containing isolated calcibacteria from *Hemimycale columella* (EM). C) Filters containing isolated calcibacteria from *Cliona viridis* (EM). D) *H. columella* choanosome harbouring larvae (CM). E) Tissue section of *Cinachyrella alloclada* (EM). F) Ectosome of *H. columella* whitish morph (CM); note that most calcibacteria are released into the sponge mesohyl forming dense aggregates. G) Choanosome of *H. columella* whitish morph (CM); note that most calcibacteria are contained within calcibacteriocytes, surrounding the cell nucleus (arrowheads). H) Ectosome section of *H. columella,* pinkish morph (CM). I) Hybridized ectosome section of *Protosuberites* sp. (CM). Blue colour corresponds to sponge nuclei and bacteria nucleoid; green colour represents hybridization points; reddish colour results from self-fluorescence of cyanobacteria and pinkish colour results from overlapping DAPI blue-stained nuclei and reddish self-fluorescence of cyanobacteria. EM, Epifluorescence Microscopy; CM, Confocal Microscopy.

Two-way ANOVA with colour morph and sponge zone as orthogonal factors (5 replicates per zone and colour morph) showed significant differences ($N = 5$, $F = 12.14$, $p < 0.05$) in the number of calcibacteria extracted from *H. columella* tissues and recorded through epifluorescence microscopy (EM) (Fig. 3.5 A). However, the interaction between colour morph and sponge zone was also significant ($p < 0.05$), so that the abundance of calcibacteria in the two colour morphs depended on the sponge zone considered. T-tests on colour morph and sponge zone separately showed that whitish morphs had significantly higher numbers of calcibacteria (i.e. hybridized points) in the ectosomal region than pinkish morphs (t-value = -3.98, $p < 0.001$), whereas there were no differences in calcibacteria density between the choanosomes of both colour-morphs (t-value = 0.52, $p = 0.67$). Confocal microscopy of sponge sections confirmed the significant differences in calcibacteria density in the extracted spherules observed using EM ($N = 3$ each colour, $Z = -1.96$, $p < 0.05$, Mann-Whitney test) (Fig. 3.5 B). Together, these results confirmed that calcified calcibacteria accumulated in large numbers at the sponge ectosome, conferring a whitish tinge to some individuals of *H. columella*, and that bacterial DNA remained within the calcareous spherules for an undetermined time after bacteria became calcified.

Larvae contained lower numbers of calcibacteria than adult tissue as non-reproductive individuals showed higher fluorescence values per tissue unit ($Z = 7.32$, $p < 0.001$, Mann-Whitney test) than sponges harbouring abundant larvae (Fig. 3.5 C). The presence of larvae in reproductive individuals decreased the total integrated fluorescence of the sponge sections.

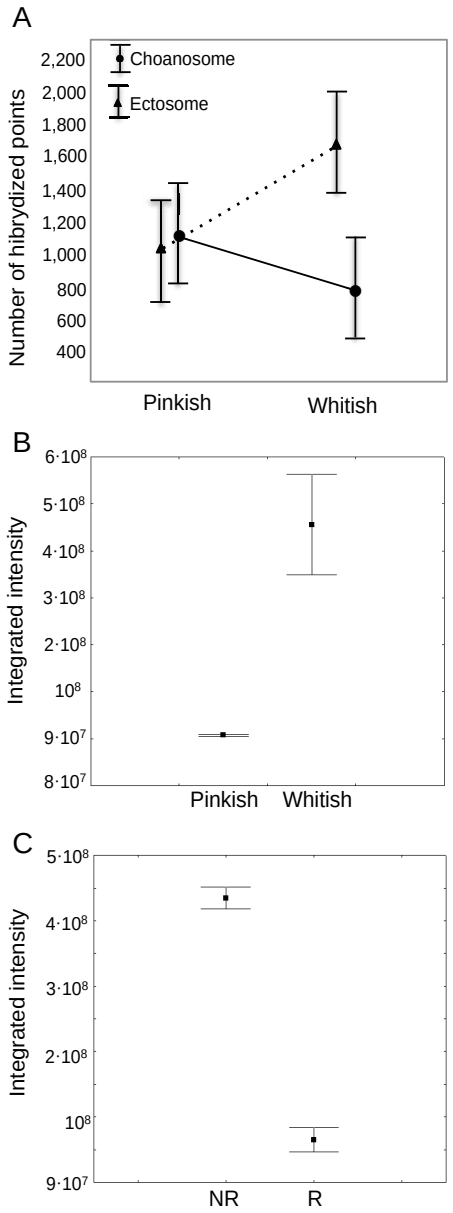

Figure 3.5. Average abundance of calcibacteria in *Hemimycale columella*. A) Direct hybridization of calcibacteria extracted from the sponge tissue and quantified using epifluorescence microscopy. Vertical bars represented ± 95% confidence intervals. B) Integrated intensity of the hybridization signal in the two colour morphs. Vertical bars represent ± standard errors. C) Integrated intensity of the hybridization signal in two lifecycle stages (NR, non-reproductive; R, reproductive). Vertical bars represent ± standard errors.

Calcibacteria phylogeny

Cloning 16S rRNA gene of the bacterial symbionts present in *H. columella* and *C. viridis* allowed us to recover two sequences, ca. 1,400 nt. long, that contained the 273 nt. calcibacterium fragment. Bayesian Phylogenetic reconstruction using the cloned sequences plus their closest neighbouring sequences in the 16S SILVA database and several outgroup sequences, retrieved a well-supported calcibacteria clade (1, posterior probability) that clustered bacteria from sponges harbouring calcareous spherules similar to those of *H. columella* along with sequences from two corals and two tropical seawater bacteria (Fig. 3.6). The calcibacteria clade contained 17 sequences, belonging to seven distinct calcibacteria OTUs with >98.5% intra-OTU similarity, which would correspond to seven calcibacteria species according to Kim et al. (2014). The calcifying bacterium of *C. cyathophora* differed from our cloned sequences of *C. viridis* and *H. columella*, in >5% and likely belonged to a different genus (Schmitt et al. 2012).

According to the Bayesian phylogeny, the calcibacteria clade belonged to SAR116 (Alpha-proteobacteria), which appeared as a sister clade of some Rhodospirillales. It was split in two well supported subclades (1, posterior probability): subclade A contained the clone of *H. columella* (GenBank ID:KU985279), and that of the Alcyonacea coral *Erythropodium caribaeorum* (Duchassaing & Michelotti, 1860), and another clade with two bacterial sequences from tropical marine waters; subclade B clustered the *C. viridis* clone (GenBank ID: KU985280) with a clone from the Alcyonacea coral *Scleronephthya gracillimum* (Kükenthal, 1906) (previously *Alcyonium gracillimum*) from the North Pacific. This subclade was a sister clade of the *C. cyathophora* clone from the Indo-Pacific.

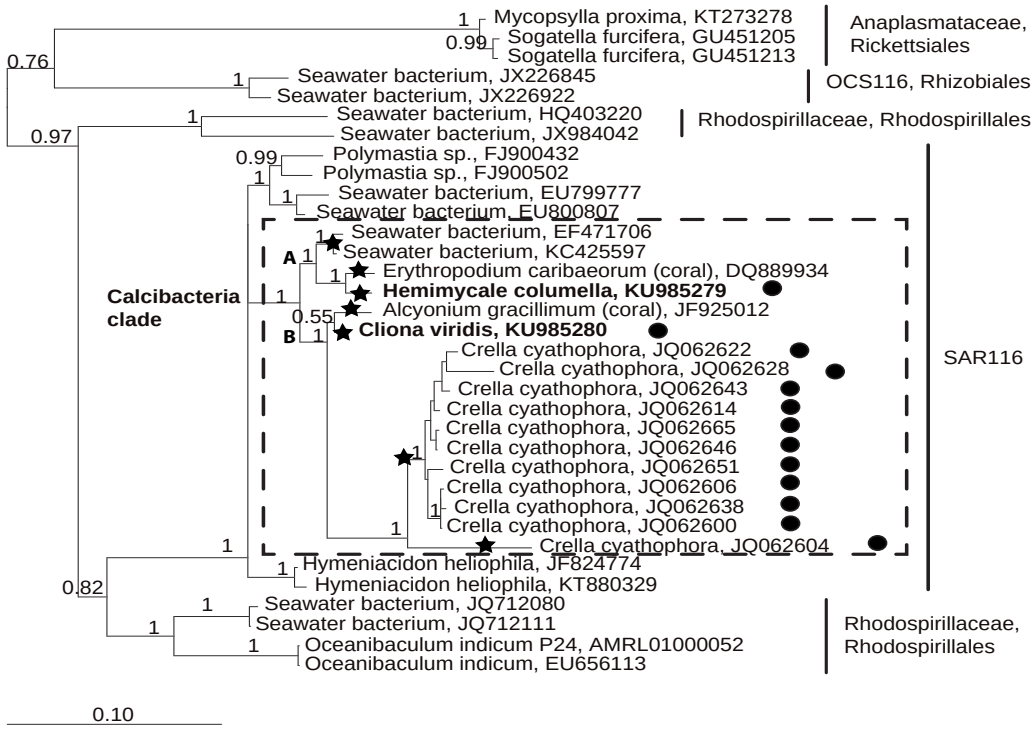

Figure 3.6. Bayesian phylogeny of the 16 S rRNA clones of calcibacteria from *Cliona viridis* and *Hemimycale columella*, and their closest sequences from the SILVA database. Sequence sources are included. The calcibacteria clade is shown within the dashed frame. Posterior probability values are indicated at each node. Bullets on the right indicate presence of ca. 1 µ m calcareous spherules (the presence of spherules in cnidarians has not been explored). Seven calcibacteria OTU's at > 98.5% similarity (stars), are identified.

DISCUSSION

Ultrastructure images combined with molecular analyses have significantly improved our understanding of the symbiosis first discovered between *Hemimycale columella* sponge and calcibacteria. The specific probe designed enabled us to confirm the identity of the endosymbiont calcifying bacteria, which was surrounded by a calcareous crust, to quantify their abundance within the sponge tissues of *H. columella*, and to prove their accumulation at the sponge periphery, which suggest an exoskeleton function. A thin bacterial wall was made evident through TEM in healthy bacteria, which contradicts the lack of bacterial wall suggested by Uriz et al. (2012). The phylogenetic reconstruction of near-complete 16S sequences from cloned *H. columella* and *Cliona viridis* classified the calcibacteria within the SAR116, close to one of the three Rhodospirillales clades (Luo 2015).

Altogether, the results from CARD-FISH and bacterial phylogeny indicate a temperate-warm geographical distribution of this symbiosis, which comprises at least seven bacteria OTUs and two potential genera, and appears to include demosponges and cnidarians host. The inclusion of two bacteria sequences from tropical waters within the calcibacteria clade suggests the presence of calcibacteria stocks in the water from which sponges and cnidarians might acquire them. Indeed, the symbiosis may be propagated by two mechanisms: vertical transmission from maternal tissue to the progeny, as evident in our study, and horizontal transmission from the environment, as suggested by the presence of free calcibacteria in seawater. Redundant mechanisms for assuring a relevant biological function are frequent en nature (Nowak et al. 1997) and two acquisition modes of symbiotic microbes have also been reported for other sponges (Sipkema et al. 2015).

In marine environments, calcium carbonates and calcium phosphates are the most commonly precipitated minerals and have formed most invertebrate skeletons since the Cambrian explosion (Wilt et al. 2003). Particular metabolisms of both autotrophic and heterotrophic bacteria are known to induce mineralization (Reid et al. 2000; Arp et al. 2001; van Lith et

al. 2003; Dupraz et al. 2004; Benzerara et al. 2011; Miot et al. 2009; Rivadeneyra et al. 2010; Couradeau et al. 2012). However, only a few cases of calcium precipitation mediated by endosymbiotic microorganisms have been reported so far in marine eukaryotes: calcification is facilitated by symbiotic microalgae (*Phaeocystis*) in the radiolarian *Acantharia* (Decelle et al. 2012), and by bacteria in foraminifera (West 1995).

Increased pH, which may result from bacterial metabolisms, could promote calcium precipitation. Significant increases in pH have been recorded during the growth of *Escherichia coli* (Sutterlin et al. 2016), and an increased pH of at least one unit above seawater pH fostered calcification in Foraminifera vacuoles, even at high Mg^{2+} and low Ca^{2+} concentrations and low temperature (De Nooijer et al. 2009).

In our target symbiosis, calcification occurs within sponge cell vacuoles. We propose that the vacuole microenvironment changed over the course of bacterial growth, as nutrients are removed from the medium and bacteria expel waste products into the medium (Fig. 3.7). As a consequence, the pH of the vacuole may increase, and calcium carbonate nucleation and precipitation on the bacteria membrane is biologically induced (Weiner & Dove 2003). A similar process of calcification has been observed in experimental studies of *Chromohalobacter marismortui* (Rivadeneyra et al. 2010).

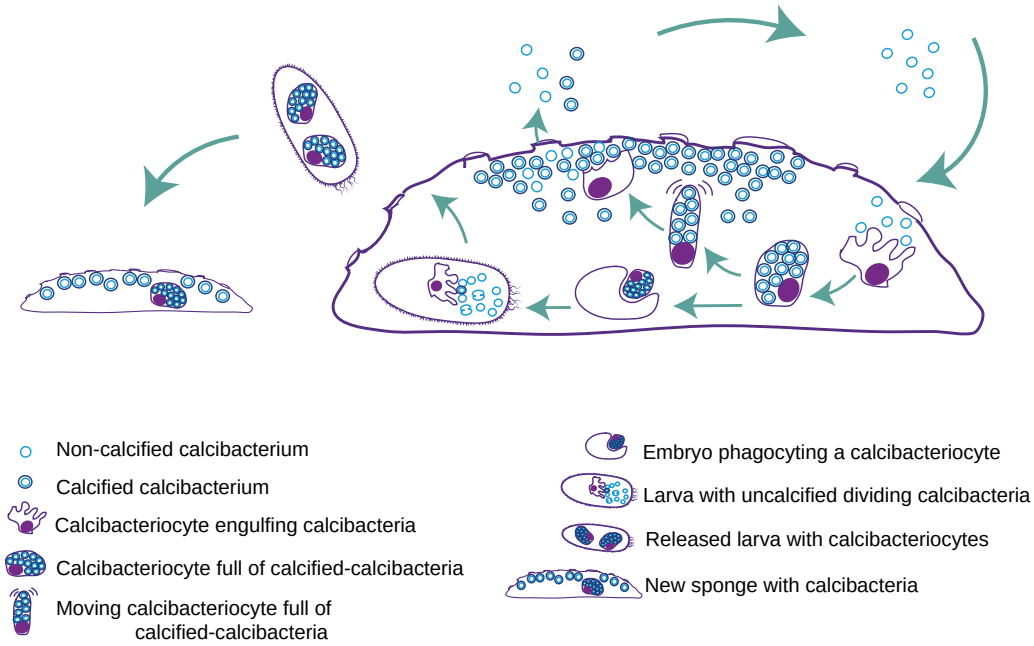

Fig. 3.7. Proposed cycle of calcibacteria acquisition and vertical transmission to sponge settlers. Uncalcified calcibacteria enter the sponge with the inhaled water, are engulfed by the sponge ameboid archeocyte-like cells (calcibacteriocytes), which place them in cell vacuoles where bacteria calcification follows. Calcibacteria-full calcibacteriocytes move to the sponge periphery, where they disintegrate releasing the calcified calcibacteria, which accumulate forming a cortical calcareous layer. Embryos engulf maternal calcibacteriocytes during the maturation process. Maternal calcibacteriocytes disintegrate, releasing the calcibacteria to the larva mesohyl, where the extracellular pH conditions would prevent their calcification. Uncalcified calcibacteria divide in the larval mesohyl until they are captured by larval calcibacteriocytes where they calcified. Free larvae carry out calcibacteriocytes, and the cycle resume after larval settlement.

In general, sponge skeletons are either siliceous or calcareous (Uriz 2006). Only a few relict sponges (the sclerosponges), which formed sponge reefs during the late Palaeozoic and Mesozoic eras (Reitner 1992), have a double mineralization system. Some parallelism can be drawn between sclerosponges and the sponge species in this study. Both have siliceous spicules and a complementary calcareous skeleton layer. In both cases, spherules (spherulites in sclerosponges) are produced within vacuoles of sponge cells (sclerocytes in sclerosponges or calcibacteriocytes in sponges in

this study), which are then excreted and accumulate at the sponge periphery. However, some differences between the calcareous bodies produced by the two sponge types should be noted. In the sclerosponge *Astroclera willeyana* Lister, 1900, calcareous bodies are solid, and 5 μm in diameter, and become cemented and form a mass skeleton at the sponge base (e.g. Wörheide 1998), whereas those of the sponges in this study are hollow and 1 μm in size, and remain free forming a cortical layer analogous to the siliceous, microsclere-constructed cortex of some Astrophorida demosponges (Uriz 2006).

Studies on the formation of intracellular spherulites in the sclerosponge *A. willeyana* also report parallel traits with the bacteria-mediated calcareous spherules. Jackson et al. (2011) proposed that sponge genes of bacterial origin promoted calcification. Later, Jackson & Wörheide (2014) suggested that sponge cells would use the remains of intracellular bacteria as a framework on which to initiate calcification. In both cases, the bacterial wall may act as nucleation centre for the precipitation of calcium carbonate. Calcification, resulting in either 5 μm spherulites or 1μm spherules, appears to be caused by the particular conditions (e.g. host enzymes or increased pH) in the sponge vacuoles, as it occurs within cell vacuoles.

It has been reported that many symbiotic microorganisms do not grow unconstrained in hosts (García & Gerardo 2014). Conversely, it is generally accepted that for mutualistic symbioses to become evolutionarily fixed, benefits at species level should compensate for the costs to the associated partners (Roughgarden 1975). The most obvious benefit to sponges from their association with calcifying bacteria is the "low cost" construction of an exoskeleton, which may serve as structural purpose and deter potential sponge predators better than the species secondary metabolites (Garate et al. 2015–Chapter 5). Protection against an increase in predators has been proposed as an evolutionary driver of exoskeletons in ancient animals during the Cambrian explosion (Bengtson & Zhao 1992). By assuming that calcium precipitation around the bacteria is spontaneously triggered by increases in pH within the vacuole, the only cost of formation of the sponge exoskeleton would be the transport of calcified calcibacteria to the sponge peripheral zone.

However, the sponge mechanism of particle capture and transport may have not evolved primarily for bacteria. Calcibacteriocytes do not differ significantly from archaeocytes, which are moving cells that are genetically programmed to remove debris and undesired substances from the sponge mesohyl (Simpson 1984). Archeocyte-like cells pack bioactive metabolites in the form of spherules to prevent sponge self-toxicity (Uriz et al. 1996a, b). These spherule-containing cells, or spherulous cells from their ultrastructure aspect (Turon et al. 2000), have been observed to migrate to the sponge surface where toxics are released to the sponge boundaries to function in deterrent and/or allelochemical roles (Uriz et al. 1996a).

In contrast, the benefits for calcibacteria are more difficult to ascertain. It has been experimentally demonstrated that some symbiotic microorganisms have an increased reproductive capacity and higher fitness within hosts relative to non-host environments (Wollenberg & Ruby 2012). Sponge tissues might offer protection from pathogens and predators, which are abundant in non-host environments (García & Gerardo 2014), and buffer nutrient ocean fluctuations that prevent the steady growth of bacteria over long periods (Navarro-Llorens et al. 2010). However, considering the detrimental consequence of calcification for the bacteria, calcibacteria may also be more akin to 'prisoners' or 'farmed crops' than equal partners as in other bacteria-invertebrate symbioses (Garcia & Gerardo 2014). Benefits might therefore be related to the propagation of the species. Symbiosis ensures that calcibacteria persist across sponge generations via the vertical transmission to sponge progeny. Moreover, the presence of free calcibacteria in seawater also suggests that viable calcibacteria are released back into the environment, which would allow the bacteria to form a species reservoir to facilitate dispersal and colonization of new invertebrate hosts.

Symbiotic relationships have been observed to be mostly stable over the lifetime of an individual host, from generation to generation, and over evolutionary time (Nyholm & McFall-Ngai 2004). Thus, mechanisms must have evolved to correct potential deviations from the necessary holobiont homeostasis. In the calcibacteria-sponge association, the host should predominantly maintain proliferation of the dominant bacteria to a level

compatible with host survival. Calcification appears to be the cost to bacteria for living in a more stable, predator-free, nutrient-rich environment. Once calcification prevents metabolic exchange between the bacteria and the vacuole medium, the calcibacteria may degrade or become starved. However, according to our TEM images, calcified calcibacteria recovered a steady growth phase as calcibacteriocytes broke and released purported resistant forms into the nutrient-rich mesohyl of sponge larvae. Then larval archaeocyte-like cells may engulf and transport them into cell vacuoles where calcification occurred (Fig. 3.7). This interpretation is based on *H. columella* observations but it can be safety extrapolated to other calcibacteria-bearing sponge species, as the cellular types involved in the process are similar in all of them (images not shown).

The current-day animal-bacteria symbioses, which likely existed when animals first appeared (McFall-Ngai et al. 2013), can provide key insights into Metazoa evolution. The reporting of bacteria-mediated calcification mechanisms in phylogenetically apart sponges suggests the implication of bacteria in early evolution of the skeleton in the pre-Cambrian metazoans (e.g. Ayala & Andrey 1998). Although several molecular mechanisms are responsible for calcium precipitation and skeleton formation in Lower Metazoa (Taylor et al. 2007b), those involving bacteria might be evolutionarily older and, thus, acquire relevance in the light of these results. Paleogenomics approaches may help in the near future to confirm the presence of calcifying bacteria in early animals.

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Chapter 4

Contrasting microbiomes in sponges harbouring calcifying bacteria.

ABSTRACT

As they are the oldest extant Metazoa and show a close relationship with its microbial symbionts, sponges represent a suitable model to study marine symbiosis. Their microbial communities differ in abundance and diversity but in most cases represent a notable proportion of the holobiont volume. Pervious studies have reported that the sponge microbiomes are in most cases species-specific, although they can be acquired both vertically and horizontally from the surrounding water. Among the bacteria associated to sponges, calcifying intracellular bacteria surrounded by a calcareous spherical coat were firstly described in *Hemimycale columella* from the Mediterranean, but similar calcified spherules have been recorded in other sponge species from several latitudes. The aim of this study was to explore the microbiomes of phylogenetic apart sponge species harbouring calcifying bacteria across seas/oceans. The sponges studied were *H. columella*, *H. mediterranea*, *H. arabica*, *Cliona viridis*, *Crella cyathophora*, *Cinachyrella alloclada* and *Cinachyrella sp* (the latter has not calcareous spherules and was used as outgroup). These species were collected by SCUBA diving from temperate and tropical oceans, tag-pyrosequenced (454 Roche platform), and their sequences analysed using QIIME 1.4.0 pipeline. The sponge species showed in general species-specific microbiomes but *H. mediterranea* from western Mediterranean, which shared bacteria with both *H. columella* and *H. mediterranean* from the Adriatic Sea, pointing to some significant environment-mediated influence. Proteobacteria was the dominant phylum in *H. columella*, *H. mediterranea*, *H. arabica*, *C. viridis,* and *C. cyathophora*, with a relative abundance > 70%. Other phyla as Tenericutes, Acidobacteria and Cloroflexi, besides Proteobacteria, were also abundant in *Cinachyrella sp*., *C. alloclada* and *H. mediterranea* from western Mediterranean. *Cinachyrella sp.* and *C. alloclada* were the species with the highest Diversity indexes (H') and *H. mediterranea* the one with the lowest H'. The calcifying bacterium previously reported in *H. columella* was also identified in *C. cyathophora*, *C. alloclada*, *H. arabica* and *C. viridis*, but not in *H. mediterranea*. This indicates that taxonomically distant bacteria are involved in calcification of sponges belonging to different Orders and Families. These results revealed that bacteria-mediated calcification is a spread mechanism,

adding support to the hypothesis on the role of bacteria in calcification processes of Early Metazoans.

INTRODUCTION

Sponges represent a suitable model to study marine symbiosis, as they are the oldest extant Metazoans and live in intimate association with microorganisms (Schmitt et al. 2012; Reveillaud et al. 2014) since the Precambriam (Wilkinson 1984). The sponge-associated microbes represent contrasting amounts of the sponge volume reaching up to 40-50% of the holobiont volume in some cases. Associated microbes are intra-or extracellular (Vacelet & Donadey 1977; Hentschel et al. 2006; Webster & Taylor 2012) and can be acquired by the sponge from the seawater or transmitted directly from the progenitors (Webster et al. 2010; Ribes et al. 2016; Garate et al. 2017). However, both vertical and horizontal transmission modes have been documented for the same bacteria species (Sipkema et al. 2015).

Most studies report a high sponge species specificity of many microbial OTUs (Taylor et al. 2007; Erwin et al. 2012a; Taylor et al. 2013) but examples of the presence of sponge-associate bacteria in seawater have also be documented (Schmitt et al. 2012; Taylor et al. 2013; Alex & Antunes 2015). Moreover, although a high resilience of the sponge microbial assemblages has been reported in many cases (Erwin et al. 2012b; 2015), microbiomes may also vary with time and locality in other species (White et al. 2012; Luter et al. 2015; Weigel & Erwin 2016; Marino et al. 2017) and these variations have been considered to contribute to the holobiont of fitness and resilience (Weigel & Erwin 2017). On the other hand, taxonomically different bacteria may play similar metabolic functions in the sponge and thus the essential roles of the microbiomes could be maintained despite taxonomic changes in their composition. In this sense, it has been reported that sympatric sponges may host-specific microbiomes with similar metabolisms and thus potential roles in the nutrient cycles of the holobiont (e.g. Ribes et al. 2012).

Co-evolution of sponges and their microbiomes has been proposed in some cases (e.g. Matcher et al. 2016), since species belonging to the same genera may harbour similar microbial communities (e. g. Lee et al. 2011; Erwin et al. 2012a). However, this is hard to demonstrate in most sponge

species, as non-phylogenetically related sponges may also share similar bacterial communities (Schmitt et al. 2012; Alex & Antunes 2015). Thus, as for co-evolution issues, no general conclusions can be established for these complex multi-partner sponge bacteria associations, and more specific cases must be studied in order to fully understand sponge-microbe symbioses.

Endosymbiotic calcifying bacteria, which were named calcibacteria (Uriz et al. 2012), were found for the first time in the sponge *Hemimycale columella* (Bowerbank, 1874). Later, in a recent study, calcibacteria from *H. columella* and *Cliona viridis* were cloned and the sequences obtained formed a well-supported monophyletic clade with those of the sponge *Crella cyatophora*, two sequences from corals, and two sequences from tropical seawater samples (Garate et al. 2017, Chapter 3). The calcibacteria cluster, which appeared to be a sister clade of some Rhodospirillales (Garate et al. 2017, Chapter 3), belonged to the SAR116 (Alpha-proteobacteria), and consisted in seven species and two genera.

Calcibacteria produce 1μm in diameter calcareous spherules in high abundance, which are easily observable through light microscope after disaggregation of the sponge tissues (Garate et al. 2017, Chapter 3). Other demosponge species from temperate and warm latitudes have also recorded to produce carbonate spherules similar to those observed in *H. columella* (authors' obs.). These species were *H. columella* and *H. mediterranea* Uriz, Garate & Agell, 2017 (Fam. Hymedesmiidae) and *Cliona viridis* (Schmidt, 1862) (Fam. Clionaidae) from the Mediterranean Sea; *Crella cyathohora* (Carter, 1869) (Fam. Crellidae) and *H. arabica* Ilan, Gugel & van Soest, 2004 (Fam. Hymedesmiidae) from the Red Sea; *C. cyathophora* from the Indian Ocean; *Cinachyrella sp.* and *Cinachyrella alloclada* (Uliczka, 1929) (Fam. Tetillidae) from the Caribbean Sea. Thus, symbiotic calcifying bacteria seemed to be much more widespread in sponges that previously found.

The aims of the current study were i) to confirm the presence of calcibacteria in those species harbouring calcareous spherules, ii) to examine and compare the whole microbial communities of those sponge species, and iii) to focus on possible differences or similarities between the microbiomes of these species and in particular of *H. columella* and *H. mediterranea*, the latter,

a cryptic species recently discovered (Uriz et al. 2017, Chapter 1). The two cryptic species showed contrasting biological and ecological traits (Garate et al. in press, Chapter 2) but shared geographical distribution. The purported differences of the microbiomes of these close species, besides helping to differentiate them, may cast light on host-symbiont co-evolution issues.

We used tag-pyrosequencing instead of other platforms such as Illumina, because the former method allows retrieving longer sequences, which facilitates taxonomic identification and phylogenetic reconstructions, and it is currently used for analysis of sponge microbiomes (Webster & Taylor 2012; Gao et al. 2014, 2015; Ribes et al. 2016; Steinert et al. 2016; Cleary et al. 2017).

MATERIALS & METHODS

4.1. Sampling

Sponge samples were collected by SCUBA diving from several temperate and tropical localities. *Cinachyrella alloclada* and *Cinachyrella sp.* were collected from Caribbean Sea (Florida bay). *Hemimycale columella*, *Cliona viridis* and *Hemimycale mediterranea* were collected from western Mediterranean (Arenys de Mar and Tossa de Mar, Spain), and *H. mediterranea* was collected from Adriatic Sea (Tremiti, Italia). *Hemimycale arabica* and *Crella cyathophora* were collected from the Red Sea (Sharm el Sheikh, Egypt) and *C. cyathophora* from the Indo-Pacific Ocean (Bempton Patch Reef). The samples were placed in 50 ml Falcon tubes underwater and immediately after diving fixed with absolute ethanol. Once there, the ethanol was changed three times and samples kept at -20 ºC until the DNA extraction.

4.2. DNA extraction, Polymerase Chain Reaction, and Sequencing

According to previous assays for optimizing extraction protocols, DNA extraction of *Hemimycale* species was carried out using the QIAamp Fast DNA Stool Mini Kit (Qiagen) while DNA from samples of *Cinachyrella sp.*, *C. alloclada* and *C. cyathophora* was extracted with DNeasy Blood and Tissue Kit (Qiagen). Concentration of DNA was measured using Qubit

fluorometer (Invitrogen) and DNA quality was avaluated with Biomate spectrophotometer (Thermo Scientific). A 16S rRNA gene fragment was amplify with primers 28F TTTGATCNTGGCTCAG and 519R GTNTTACNGCGGCKGCTG, using the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA). The PCR conditions were: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, and finally a final elongation step at 72°C for 5 min. PCR products were then purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA) and pyrosequencing was carried out in a Roche 454 FLX platform using commercially prepared Titanium reagents by a commercial laboratory (Research and Testing Laboratory, Lubbock, TX).

4.3. Sequence analyses

Sequences resulting from pyrosequencing were analyzed with QIIME 1.4.0 pipeline (Caporaso et al. 2010). Sequences of low quality were removed from the total using default parameters (i.e. minimum quality score = 25; minimum and maximum length of 200-1000 nt. respectively; no more than 6 ambiguous bases allowed per read; no mismatches allowed in primer sequence; homopolymers exceeding 6 bases). Chimeric sequences were identified and removed with ChimeraSlayer algoritm of QIIME. From the remaining set of high quality sequences, representative sequences of each OTU were aligned by Uclust method. OTUs' taxonomy was determined by RDP classifier with a level of threshold of 80% (Wang et al. 2007) against Greengenes core set, and an OTU table was generated. From the table of OTUs it was estimated their relative abundance and only OTUs with > 1% abundance were selected for phylogenetic analyses, excepting when the analyses purpose was to detect calcibacteria sequences. In the latter case all the OTUs that previously had been aligned with the already known sequence of calcibacteria were considered. The OTUs selected for taxonomic identification were aligned with the online alignment SINA of SILVA, rejecting the sequences with less than 70% of identity (Pruesse et al. 2007). The resulting alignment was imported to ARB software package (Ludwig et al. 2004) and was manually improved. Closest neighbour

sequences were selected and exported to construct phylogenetic tree. Ambiguous regions of the alignment were removed with Gblocks v.0.91b (Castresana 2000) with default parameters, except the minimum block length that was set in 5 and allowing gaps in 50% of positions. A Nexus file was then generated with free server bioportal (www.bioportal.uio.no) and Bayesian phylogenetic tree performed with MrBayes v3.0b4 software (Huelsenbeck & Ronquist 2001). Bayesian analyses started with random trees and run for 10^7 generations. A set of four Markov chains were sampled every 1000 iterations. The default parameter (25%) was used to discard early tree generations until the probabilities reached convergence, and a 50% majority-rule consensus tree was generated with the remaining trees.

4.4. Calcibacteria detection

To confirm that sponges harbouring calcareous spherules contained calcibacteria, the total pool of OTUs of each species was blasted with the two clone sequences of calcibacteria obtained from *H. columella* and *C. viridis* (Garate et al. 2017, Chapter 3) using the installed version of Blast 2.4.0.

4.5. Statistical analyses

A Bray Curtis cluster of similarity was generated for the OTUs with a least 1% of relative abundance, with PRIMER-6.1.11 and PERMANOVA 1.0.1 software, and analyses of similarities (ANOSIM) were performed to compare microbiomes among sponge species, families and location (Seas/Oceans). Multi-dimensional scaling analysis (MDS) using Bray-Curtis similarity index was performed to compare microbiomes among sponge species with vegan and ggplot2 packages of R software, after Hellinger transformation (Legendre & Gallagher 2001).

All the OTUs that matched up with the 16S rRNA gene sequence of the calcibacterium were used to generate a Bray Curtis cluster of similarity of the sponges that contained those OTUS, with PRIMER-6.1.11 & PERMANOVA 1.0.1 software.

RESULTS & DISCUSSION

Bacterial community composition

A total of 27 bacterial phyla were recorded in all the species studied here (total number of OTUs = 3519). Moreover, 196 additional OTUs were classified only as bacteria, and 12 more appeared as unclassified. Proteobacteria was the most abundant phylum (>70%) in all the species but *Cinachyrella alloclada* and *Cinachyrella sp.* and *Hemimycale mediterranea* from the Adriatic Sea*,* as it has been reported for other LMA sponges (Erwin et al. 2011; Schmitt et al. 2012; Giles et al. 2013; Naim et al. 2014). In *C. alloclada* and *H. mediterranea* from western Mediterranean the phylum Tenericutes was also abundant (~30%), and phyla Acidobacteria and Cloroflexi in *Cinachyrella sp.* reached ~10% of relative abundance. These results may indicate that these species fit within the HMA sponges (e.g. Gloeckner et al. 2014; Cleary et al. 2017) (Fig. 4.1 A), although microscope observations should be performed to confirm the membership of these species to the HMA group. Alpha-proteobacteria prevailed (95%, 70%, and 92%) in those species dominated by Proteobacteria such as *Cliona viridis*, *Hemimycale columella* and *Crella cyathophora*, respectively. Conversely, Alpha-proteobacteria only represented 43% and 52% of the total sequences in *Hemimycale arabica* and *C. cyathophora* from the Red Sea and Gamma-proteobacteria represented 53% and 45%, respectively in these two species. Beta-proteobacteria class dominated the microbiome of Adriatic individuals of *H. mediterranean* with 92% of relative abundance (Fig. 4.1 B). Amazingly, strong intra-species differences were found between the microbiomes of *H. mediterranea* from western Mediterranean and from the Adriatic Sea, at both phylum and class levels. Intra-species variation also occurred in *C. cyathophora,* mainly at class level. Individuals of *C. cyathophora* from the Red Sea presented similar relative abundance of Alpha- and Gamma-proteobacteria, while in individuals from the Indian Ocean, Alpha-proteobacteria predominated. Intraspecific variation of the symbiotic assemblages has been also reported in other sponge species (e.g. Burgsdorf et al. 2014; Thomas et al. 2016). Thus, it cannot be totally discarded that a greater intra-species variation be recorded when individuals from far enough locations will be examined.

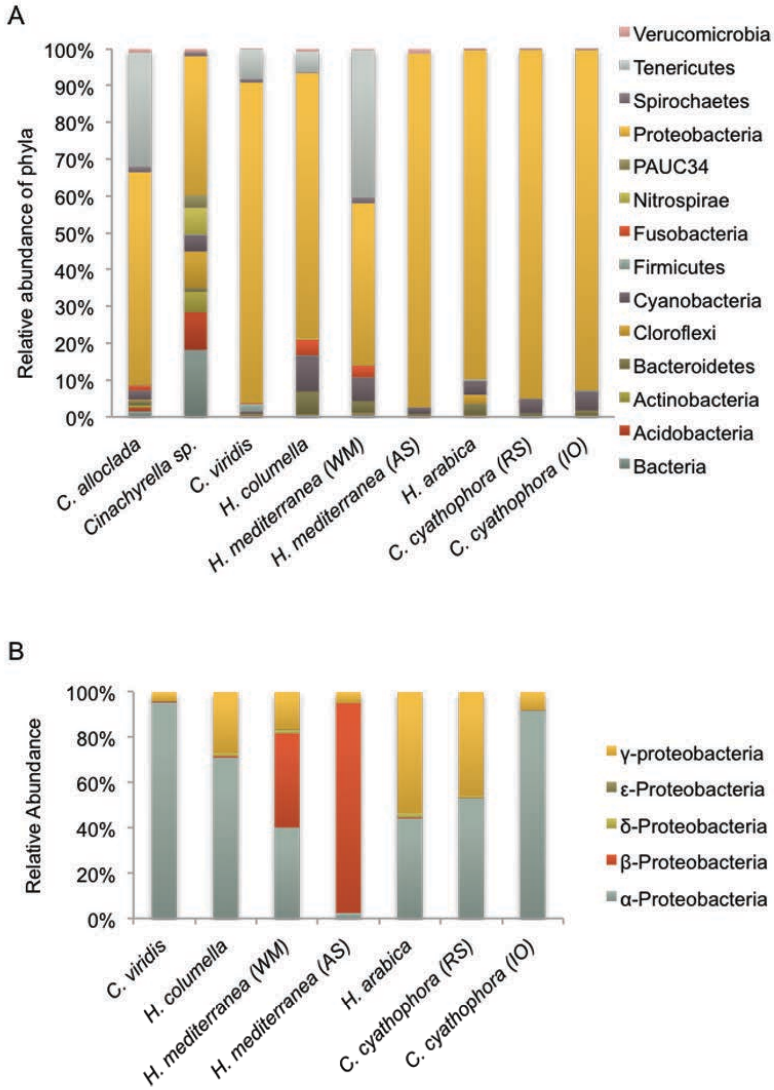

Figure 4.1. A) Taxonomic distribution of OTUs in the microbiomes of the several sponge species examined using RDP classifier at 80% confident threshold. Only the phyla with at least 1% of relative abundance are shown. B) Proteobacteria classes in those sponges with at least 70% of relative abundance of Proteobacteria and in *H. mediterranea* from western Mediterranean, which was included for comparison with *H. columella* and *H. mediterranea* from AS, although Proteobacteria represented less than 70% of relative abundance. *IO:* Indic Ocean; *RS,* Red Sea; *WM,* western Mediterranean; *AS,* Adriatic Sea.

Our results showed that the microbial communities of the sponges, harbouring calcareous spherules are mainly species-specific, as it has been reported for other sponge species (Erwin et al. 2012b; Pita et al. 2013; Easson & Thacker 2014; Reveillaud et al. 2014). However, an exception was found for *H. mediterranea*, which showed a high inter-individual variation in western Mediterranean. For example, the phylum Tenericutes was abundant in individuals of *H. mediterranea* from western Mediterranean, but it lacked from Adriatic individuals. Moreover, although with a lower abundance, this phylum was also present in *H. columella* and *C. viridis,* which shared geographical distribution with *H. mediterranea*. Environmental factors related with particular locations and depths have been reported to cause microbiome variation in some sponge species (e.g. Pita et al. 2013; Steinert et al. 2016) and seawater bacteria, which strongly vary with space and time, may also contribute to the observed variations in species with horizontal bacteria acquisition (Giles et al. 2013; Sipkema et al. 2015).

Presence and abundance of bacterial phyla through sponge samples is represented in Fig. 4.2. Most species showed a low number of OTUs, with one or two OTU clearly dominating over the rest, as it has been reported for other LMA sponges (e.g. Giles et al. 2013).

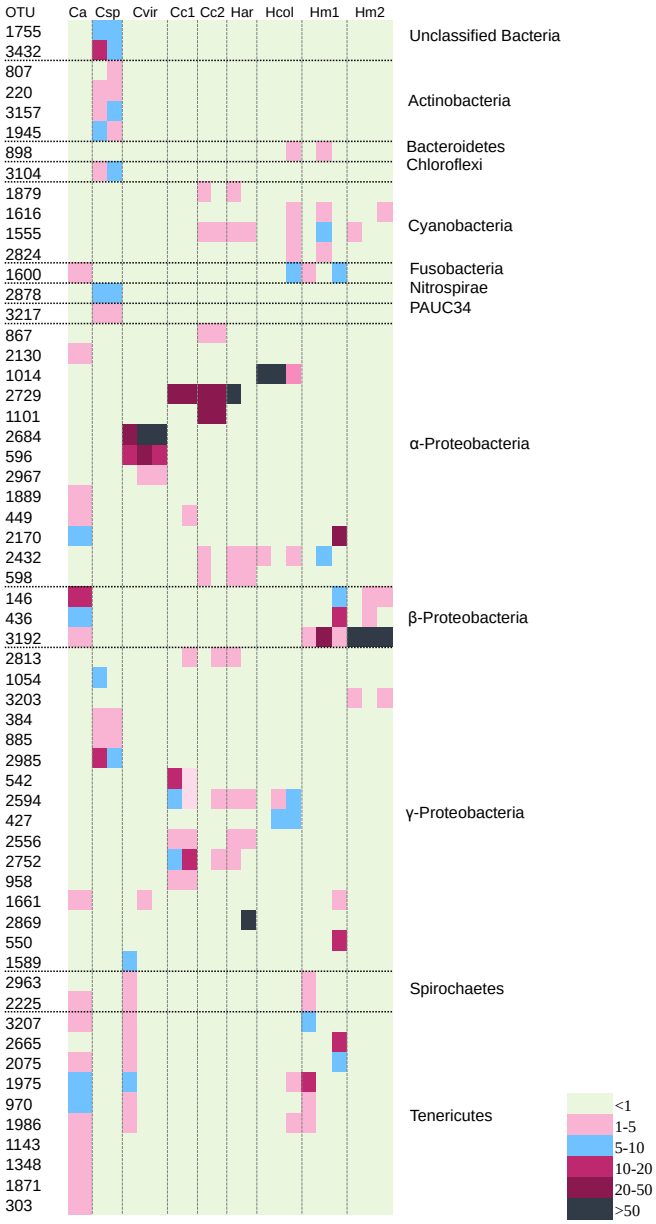

Figure 4.2. Heatmap of OTUs with at least 1% of relative abundance in at least two sponge replicates, except for *C. alloclada*, of which only one individual could be examined. Taxonomic affiliation of the OTUs is presented at a phylum level, except for Proteobacteria, which is represented at a class level. Ca: *C. alloclada*; Csp.: *Cinachrella sp*; Cvir: *C. viridis*; Cc1: *C. cyathophora* from the Red Sea; Cc2: *C. cyathophora* from the Indian Ocean; Har: *H. arabica*; Hcol: *H. columella*; Hm1: *H. mediterranea* from western Mediterranean; Hm1: *H. mediterranea* from the Adriatic Sea [2]).

Alpha diversity

The total number of raw reads obtained from pyrosequencing of the 22 sponges samples, corresponding to seven species, was 292,419. After removing chimeric and low quality sequences, the number of reads was 243,959. The total number of OTUs identified at least at a phylum level was 3519 and only 12 OTUs, which corresponded to 33 reads, were unclassified. *Cliona viridis* was the species with the lowest number of bacterial OTUs (292) while *Crella cyatophora* from the Indian Ocean harboured the highest number of OTUs (734). The species with the highest number of species-specific OTUs was *Cinachyrella sp.* with 76.34% of specific OTUs, while *C. alloclada* had the lowest percentage of specific OTUs (33.45%) (Table 4.1).

Alpha-diversity (H') and Dominance (D) indexes of the microbiomes of the target sponge species are shown in Table 4.1. The species with the highest H´ and lowest D was *Cinachyrella sp.* (H´=5.52; D=0.19, respectively), while the lowest H´ value (H´=1.26) and the highest D (D=0.91) corresponded to *H. mediterranea* from the Adriatic Sea, what agrees with the H' and D values at a phylum level reported for other LMA sponges also dominated by Proteobacteria (Kamke et al. 2010; Giles et al. 2013). The differences in diversity and dominance of particular taxonomic groups, which have been reported to characterise HMA and LMA sponges, are shown in our studied sponges. *Cinachyrella sp.* and *C. alloclada* are traditionally considered HMA sponges, what is supported by their microbiome traits. These two species show the highest diversity values (H' = 5.52 and 5.24, respectively) and lowest dominance indexes (D = 0.19 and 0.43, respectively) among the calcibacteria bearing sponge species. However, our results failed to detect a clear division between HMA and LMA sponges, as *C. cyatophora, H. mediterranea* (WM) and *H. arabica*, which are considered to be LMA, sponges, showed high H' indices. Similar examples of species considered LMA sponges with high bacterial diversity have been reported previously (Blanquer et al. 2013), what prevent to propose a categorical division between the two types of sponges, HMA and LMA (Gloeckner et al. 2014).

Sponge species	Total OTUs	Species-specific OTUs (%)	Sequences	H'	D
Cinachyrella alloclada	281	94 (33.45)	6290	5.24	0.43
Cinachyrella sp.	596	455 (76.34)	13978	5.52	0.19
Cliona viridis	297	152 (51.17)	22252	2.23	0.76
Crella cyathophora (RS)	603	327 (54.22)	20211	3.98	0.89
Crella Cythophora (IO)	734	455 (61.98)	32214	3.25	0.86
Hemimycale arabica	660	343 (51.96)	21692	3.70	0.79
Hemimycale columella	701	382 (54.49)	45946	2.85	0.52
Hemimycale mediterranea (WM)	702	346 (49.28)	18798	4.09	0.35
Hemimycale mediterranea (AS)	350	184 (52.57)	62578	1.26	0.91

Table 4.1. Shannon Diversity Index (H'), Simpson Dominance Index (D), number of total of OTUs, number (and percentage) of species-specific OTUs and number of sequences for each species in each location. *IO,* Indian Ocean*; RS*, Red Sea, *WM,* western Mediterranean and *AS,* Adriatic Se. It was considered as species-specific OTUs those that were identified only in one targeted species, and all its replicates, of this study.

Beta diversity

Multi-Dimensional Scaling (MDS) representation of the Bray-Curtis Index of similarity among OTUs of the bacterial assemblages of the studied sponges from several localities showed a clear species-specificity of the microbiomes (Fig. 4.3). The species *Cinachyrella sp.* presented the most differentiated bacterial community. *C. viridis* and *H. mediterranea* from western Mediterranean presented the highest inter-individual variability and *Cinachyrella sp. C. cyathophora* the lowest, which agrees with a previous study (Gao et al. 2015) reporting that individuals of *C. cyathophora* from the Red Sea hosted a low microbial diversity at a phylum-level. The inter-individual variability of the sponge microbiomes can be used as a proxy of their specificity (e.g. Thomas et al. 2016). In this sense, *C. viridis* and *H. mediterranea* showed the lowest microbiome specificity, while *Cinachyrella sp.* harboured the most species-specific microbiome. Sponges from the Red Sea and the Indian Ocean (*C. cyathophora* and *H. arabica*), despite taxonomically apart, were placed close to each other, while *Cinachyrella sp.* from the Caribbean Sea was located distant from the other sponge species, which altogether points to some geographical influence on their microbiomes. However, the only individual of *C. alloclada* from the Caribbean was placed close to *H. mediterranea* and *H. columella* appeared clearly separated from *H. mediterranean* and *C. viridis,* although the three species inhabited the Mediterranean Sea, proving that the geographic location of the sponges not always affect their microbiomes.

Cuvelier et al. (2014) has shown that sponge individuals of *C. alloclada* collected at different seasons grouped separately according their microbiomes. Intra-species variation and the lack of replicates may have influenced the presence of *C. alloclada* with the Mediterranean *Hemimycale spp.*

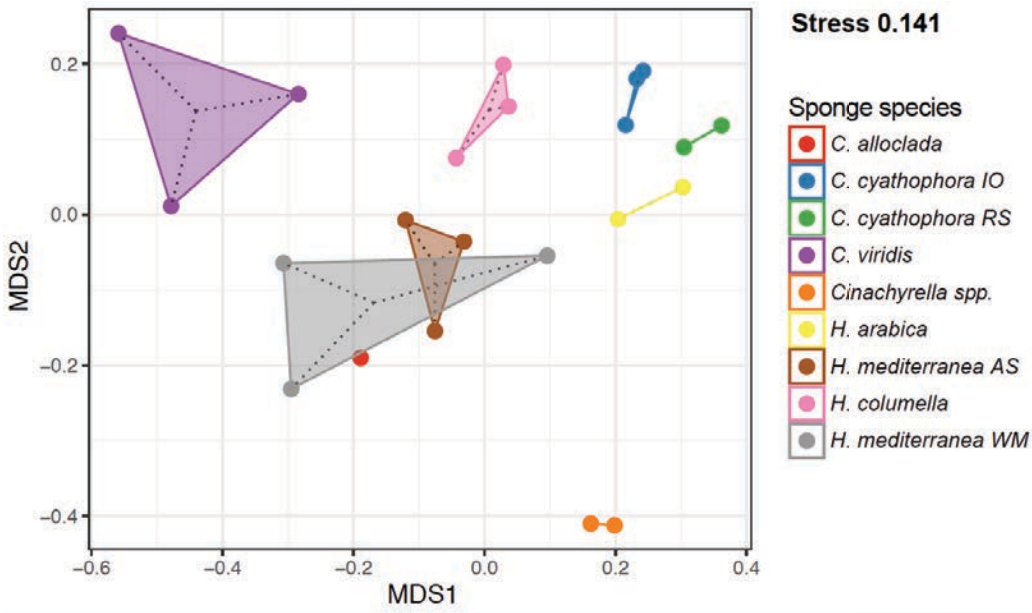

Figure 4.3. Multidimensional Scaling based on Bray-Curtis Index of similarity among sponge microbiomes. *IO:* Indic Ocean; *RS,* Red Sea; *WM,* western Mediterranean; *AS,* Adriatic Sea.

The statistic significance of the comparisons among sponge microbiomes, as a function of sponge species and location (Sea/Ocean), was calculated by one-way analyses of similarity (ANOSIM). The ANOSIM R statistic was higher among sponge species (R=0.868) than among families (R=0.671) and locations (R=0.438), indicating that microbiomes were better differentiated by sponge species than by families or geographical locations, which confirm their species-specificity in general, already reported for other sponge species (e.g. Schöttner et al. 2013; Naim et al. 2014).

Presence of calcibacteria in the target sponges

All the species analysed harboured calcareous spherules except *Cinachyrella sp.* according to light microscopy observation. Tag-pyrosequencing analyses recovered 30 OTUs that aligned with the calcibacteria sequences cloned from *H. columella* and *C. viridis* (Garate et al. 2017, Chapter 3). However, these OTUs were found in individuals of *H. columella, H. arabica*, *C. viridis*, *C. alloclada* and *C. cyathophora,* but no in *H. mediterranea*. The dendrogram

clustering the sponge species based on Bray-Curtis similarities of these calcibacteria OTUs and using *Cinachyrella sp.* as outgroup, is showed in Fig. 4.4. The dendrogram shows two main clades of calcibacteria, one clustering two subclades with sequences from *C. cyatophora* at ca. 65 % of similarity and *C. viridis*, at ca. 90% of similarity, and the other one formed by calcibacteria from the sponges *H. columella*, *H. arabica* at ca. 85% of similarity, and *C. alloclada* with ca. 78% of similarity.

The species *H. mediterranea* was observed to harbour high amount of calcareous spherules, albeit none of the sequences retrieved from the tag-pyrosequencing matched up with the two cloned sequences of the already known calcibacteria (belonging to class Alpha-proteobacteria).

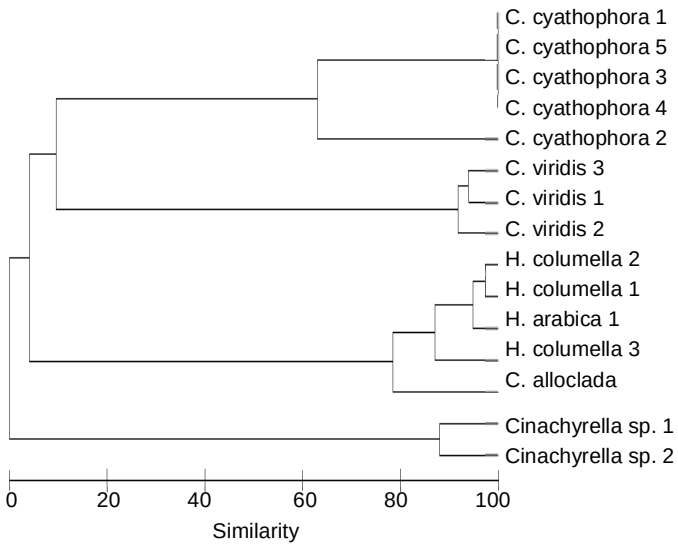

Fig. 4.4. Sponge species clustering based on the Bray-Curtis Index, which represents similarity (%) among those species in which calcibacteria OTUs were detected. *Cinachyrella sp.* was used as outgroup.

Garate et al. (2017, Chapter 3) reported the existence of at least seven calcibacteria species in sponges, corals and seawater samples. In the present study, tag-pyrosequencing analyses showed that all the studied species that harboured calcareous spherules but one, contained calcibacteria. *H. mediterranea* did not harbour calcibacteria OTUs although it contained 1 µm in diameter, calcareous spherules, similar to those of *H. columella*. Thus, other symbiotic bacteria, belonging to different classes within the phylum Proteobacteria, are also able to calcify in the sponge tissues. Different symbiotic microbes with similar metabolic processes (e.g Fan et al. 2012; Ribes et al. 2012; Weigel & Erwin 2017) may have been selected to ensure the holobiont success (Ribes et al. 2012). This could be the case of symbiotic calcifying bacteria, which may enhance the sponge fitness (Garate et al. 2015, Chapter 5). Different microorganisms might have acquired the capacity of precipitate calcium carbonate under similar intra-cellular conditions by convergent evolution or by horizontal gene transfer (HGT).

The sponge symbioses with intracellular calcifying bacteria take relevance in the face of ocean changes, which predict pH decreases in 0.2-0.3 units due to the atmospheric CO_2 dissolution in seawater (Feely et al. 2009). It has been reported that decreases in seawater pH will likely lead to reduction of calcification rates in shell-forming marine organisms (Doney et al. 2009) and calcified seaweeds (e.g. Clements & Chopin 2016). Calcibacteria reach huge densities in the sponge tissues (Uriz et al. 2012; Garate et al. 2017, Chapter 3) so that the symbiosis balance only is possible thanks to a control of these bacteria by the sponge. Sponge cells engulf the calcibacteria into vacuoles where calcification proceeds and most of the calcibacteria die. Changes in pH may prevent bacteria calcification and broke the symbiotic equilibrium with unpredictable but purportedly negative consequences for the sponge.

ACKNOWLEDGEMENTS

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Chapter 5

Calcareous spherules produced by intracellular symbiotic bacteria protect the sponge *Hemimycale columella* from predation better than secondary metabolites

ABSTRACT

Benthic sessile organisms in general, and sponges in particular, have developed an array of defense mechanisms to survive in crowded, resource and/or space-limited environments. Indeed, various defense mechanisms may converge in sponges to accomplish a defensive function in an additive or synergetic way, or to operate at different times during the sponge's life cycle. Moreover, sponges harbor highly diverse microbial communities that contribute in several ways tothe host's success. Although some symbiotic bacteria produce chemical compounds that protect the sponge from predation, the possible deterrent function exerted by the calcareous coat of a sponge's endosymbiotic bacterium has not, to date, been explored. *Hemimycale columella* is an Atlanto-Mediterranean sponge, which produces bioactive metabolites and has been reported to host an intracellular bacterium with a calcite envelope. Calcibacteria accumulate in high densities at the sponge periphery, forming a kind of sub-ectosomal cortex. They have been suggested to provide the sponge with several benefits, one of which is protection from predators. In this study, we assess the relative contribution of the endosymbiotic calcibacteria and bioactive compounds produced by *H. columella* to defend the sponge against sympatric predators. Deterrence experiments have revealed that the sponge combines >1 defense mechanism to dissuade a large array of potential predators; this represents an example of the evolutionary fixation of redundant mechanisms of defense. The chemicals deterred *Paracentrotus lividus*, *Chromis chromis*, *Oblada melanura*, and *Diplodus vulgaris*, but not *Parablennius incognitus* and *Coris julis*, while the spherules of the symbiotic calcibacteria significantly deterred all predators assayed.

INTRODUCTION

Species coexistence, which determines the biodiversity of a given ecosystem, is the result of several long discussed, biological, and ecological mechanisms such as environmental variation (Chesson & Warner 1981), resource and/or niche partitioning (Chesson 2000), and species-specific interactions, which involve species-specific mechanisms of defense (Buss 1976).

Benthic sessile organisms in general, and sponges in particular, have developed an array of defense mechanisms to survive in crowded, resource and/or space-limited environments. Structural materials, such as external and internal skeletons, dermal spines, or protruding spicules serve as defense for benthic invertebrates and fish by protecting their soft tissues and, thus, dissuading most potential benthic predators. Conversely, bioactive chemicals usually act in a less generalist way. Some chemicals may deter one or more species from predation on the producer organism while they may not deter others (Becerro et al. 2003). On the whole, chemical defenses have been reported to significantly contribute to the structure of sponge assemblages on coral reefs (Loh & Pawlik 2014) and have been proposed to favor complex interaction networks, which are responsible for increasing species coexistence and thus biodiversity (Buss 1976; Loh & Pawlik 2014). On the other hand, mineral skeletons would only improve species persistence by offering general protection to the organisms (Uriz et al. 2003).

Various defense mechanisms converge in most sponges to accomplish a defensive function in an additive or synergetic way, or to operate at different times during the sponge's life cycle (Uriz et al. 1996c). However, the opposite is also true: multiple functions have also been reported for a sole defense mechanism (Thacker et al. 1998; Becerro et al. 1997a). Furthermore, the efficiency of a deterrent mechanism can vary according to the predator (Becerro et al. 2003), which makes the results of deterrence assays difficult to generalize.

To add to the complexity of defense mechanisms, sponges harbour highly diverse microbial communities (e.g. Blanquer et al. 2013), which form stable symbiotic associations and contribute in several ways to the host's

success (Taylor et al. 2007b; Thacker & Freeman 2012). For instance, some sponge symbiotic bacteria produce chemical compounds that protect the sponge from predation (Thacker et al. 1998; Haber et al. 2011; Esteves et al. 2013). However, the possible deterrent function of an endosymbiotic bacterium, other than that mediated by bioactive chemicals, has not been explored to date.

Hemimycale columella (Bowerbank, 1874) is a common encrusting demosponge (Order Poecilosclerida) widespread in the Mediterranean and North Atlantic sublittorals. The species, which has a reduced ectosomal skeleton (Van Soest 2002), produces chemical compounds with cytotoxic and antimitotic activities (Uriz et al. 1992a; Becerro et al. 1997a) that might deter its potential predators. However, *H. columella* has also been reported to host an intracellular bacterium with calcifying abilities (Uriz et al. 2012). Bacteria that are surrounded by a 100 nm thick calcite envelope have been detected by catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) and transmission electron microscopy (TEM) in high numbers within a particular sponge cell type called calcibacteriocyte (Uriz et al. 2012; Chapter 3). Thousands of bacterium-produced calcite spherules are accumulated at the sponge periphery, forming a kind of sub-ectosomal cortex that mimics a rudimentary exoskeleton (Uriz et al. 2012). It has been proposed that this unusual, intimate symbiosis, which is vertically transmitted to progeny and constantly present in all populations of *H. columella* examined along the western Mediterranean sublittoral zone, purportedly provides the sponge with several benefits, among which protection from predators has been highlighted (Uriz et al. 2012).

In this study, we aimed to assess the relative contributions of the calcite spherules of endosymbiotic calcibacteria and the bioactive compounds produced by *H. columella* to sponge defense, and whether the combination of secondary metabolites and the calcibacterial calcareous envelope exerted a synergetic effect in deterring potential predators from feeding on the sponge. With this aim, we conducted several deterrence experiments, both in the laboratory and in the sponge's habitat, with an array of sympatric potential predators (echinoderms and fishes).

MATERIALS & METHODS

5.1 Sampling, sponge identification, and location of bacteria

Between 8 and 12 individuals of *Hemimycale columella* were randomly collected from the Blanes littoral zone, NW Mediterranean (41°40.12'N, 2°47.10'E), in each of 3 sampling dives, to prepare the artificial food used in the experiments. The species was taxonomically identified by phenotypic characters (Part 3.4). The sponge samples were taken to the laboratory in hermetic, seawater-filled bowls, blended, and weighed after removing foreign material under a stereomicroscope. Half of the sponge mix was frozen for obtaining the crude chemical extract (potential chemical defenses), while the other half was used to isolate the calcite spherules, which represented the purported physical defenses.

Light-microscope pictures were obtained by using forceps to break up recently collected sponges and by direct observation of the resulting disaggregated cells though a Zeiss (Axioplan) microscope connected to a Jenoptik/Jena (ProgRes C10 plus) digital camera.

For TEM, samples of ca. 2 mm^3 in size were fixed in 1 % OsO4 and 2% glutaraldehyde (1:3) in 0.45 M sodium acetate buffer (pH 6.4) with 10% sucrose (Leys & Reiswig 1998) for 12 h at 4°C. After rinsing in the same buffer, dehydration, and inclusion in Spurr's resin, ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and observed with a TEM (JEOL 1010), implemented with a Bioscan system (Gatan) for image digitalization (Microscopy Unit of the Scientific and Technical Services of the University of Barcelona).

For scanning electron microscopy (SEM), samples were fixed in a cocktail (6:1) of a saturated solution of $HgCl_2$ and 2% aqueous solution of OsO_4 (Johnston & Hildeman 1982), cryofractured in liquid N_2, dehydrated, gold palladium metalized, and observed through a Hitachi S-3520N SEM (Microscopy Service ICM-CSIC, Barcelona).

For experiments in the laboratory, the target predators were the sea urchin *Paracentrotus lividus* and the fish *Parablennius incognitus*, which share habitat with *H. columella*. These 2 predators were collected in sufficient

numbers from the sponge habitat (Blanes littoral, NW Mediterranean; 41°40.12'N, 2°47.10'E), transported to the laboratory in seawater containers, and placed in an open-system aquarium at a similar temperature to that in their habitat (22°C). All individuals were from the same size-class (adults), and no male livery was shown by any of them. They were starved for 7 d before experiments were initiated. Once the experiments were completed all individuals were taken back to their natural habitat.

5.2. Chemical extraction

Ca. 25 g of fresh sponges, corresponding to 40 ml in volume (according to the water volume displaced when submerged in a measuring cylinder), were freeze-dried for 72 h and pounded. Acetone was used for chemical extraction because it has been reported to extract a wide range of secondary metabolites (Cimino et al. 1993). The extraction was done in an ultrasound bath for cell breaking and was performed in 2 steps. First, we added 20 ml of acetone per gram of sponge powder, and the extraction lasted for 25 min. Once the supernatant was removed, we added another 20 ml of acetone per gram sponge and extracted it for 10 min. The supernatants from the 2 extractions were pooled together in a previously weighed tube, and the solvent was totally evaporated in a hood. The tubes were weighed again after drying to determine the amount of crude extract obtained. The procedure was repeated 4 times (25 g of fresh sponge each time) and ended with a total crude extract of 72 mg (0.45 mg ml^{-1} sponge), which was preserved frozen in the darkness until the artificial food was prepared.

5.3. Isolation of calcibacteria spherules

The presence and abundance of calcibacteria in the sampled sponges (i.e. bacteria surrounded by a calcareous coat) were confirmed through optic and electron microscopes. The calcibacteria coats, which are calcium carbonate made according to X-diffraction analysis (Uriz et al. 2012), were obtained directly from fresh sponge samples. Ca. 25 g fresh sponge, 40 ml in volume, were disaggregated and homogenized in sterile seawater to avoid dissolution of the calcite-made, calcibacteria

spherules. The whole process of spherule isolation consisted of a series of centrifugations and re-suspensions in an attempt to be as exhaustive as possible. Siliceous spicules precipitated first, forming part of the pellet after centrifugation, and were discarded.

The spicule-free homogenates were initially centrifuged at 200 rpm for 1 min (Step 1), and the supernatants with the calcite spherules were removed and kept apart. The pellets, which still contained spherules, according to light microscope observation, were re-suspended in 7 ml of sterile seawater and centrifuged again at 500 rpm for 2 min (Step 2). The resultant supernatants were removed and set apart. The upper layer of the pellet, which contained entire calcibacteriocytes, was also removed with a pipette, and re-suspended with RIPA buffer (Sigma) and sterile seawater (1:1) to lyse the calcibacteriocytes; this was centrifuged at 500 rpm for 2 min (Step 3). The various supernatants containing spherules were pooled together and centrifuged at 2000 rpm for 4 min in order to precipitate the calcibacteria spherules (Step 4). The spherule-free supernatant (verified through light microscopy) was discarded, and finally the pellet was re-suspended in 1 ml of sterile seawater. The whole process was repeated 3 times totaling 48 ml of calcite spherules, which represented a concentration of 0.4 ml spherules ml^{-1} sponge.

5.4. Artificial food preparation

Two types of artificial food were prepared according to the feeding behavior of the target predators: 4% carragenate plates for sea urchins and bread pellets for fishes.

5.4.1. *Paracentrotus lividus*

The food controls were prepared by adding 120 g of the fresh alga *Cystoseira mediterranea*, which is part of the diet of *P. lividus* (Verlaque & Nédelec 1983, Verlaque 1984), to 120 ml of 4% carragenate. To detect any deterrent effect of the solvent used in the chemical treatment, acetone controls were also prepared by adding 12 ml of acetone to 120 ml of a 4% carragenate–alga mixture (i.e. 2.25 ml of acetone per carragenate plate). Either the sponge crude extract

(chemical treatment) or the calcibacterial treatments were added to 120 ml of slightly warm 4% carragenate seawater–alga mix.

For the chemical treatment, we re-dissolved ca. 5.3 mg of crude extract in 12 ml of acetone and added this solution to the 120 ml of 4% carragenate–alga mix, which approximately mimicked the crude volumetric concentration in the sponge (ca. 0.45 ml of crude extract per milliliter of carragenate).

For the calcibacterial treatment, we added 48 ml of the concentrated spherule suspension to 120 ml of a 4 % carragenate–alga mix, which approached the calcibacterial density estimated in fresh sponges (0.4 ml of spherules per milliliter of carragenate). A total of 15 ml of the carragenate–alga mix containing either the crude extract or the calcibacterial blend was poured into 8 Petri dishes (6 plates for experi- mental trials and 2 as hydration controls). The treatment combining the crude extract and the calcibacteria was prepared by adding both 5.3 mg of sponge crude extract and 48 ml of concentrated calcibacterial suspension to the carragenate–alga mix. After cooling, the plates were removed from the Petri dishes and weighed immediately before being offered to the sea urchins. Two plates per treatment were kept in aquaria free of sea urchins to estimate the possible weight gains because of carragenate hydration.

5.4.2. *Parablennius incognitus*

Hundreds of ca. 3 mm long, 1 mm thick pellets — an appropriate size considering the mouth size of the target fish— were hand made from smashed bread. Either the sponge crude extract solution or the spherule suspension was added in appropriated volumes to bread pellets to obtain ecologically relevant concentrations (i.e. similar to those present in the sponge tissues). The treatments considered for *P. incognitus* were: sponge crude extract (chemical treatment), calcibacteria spherules, and acetone control. The chemical treatment was prepared by adding ca. 4 mg of crude extract, dissolved in 12 ml acetone, to 10 g (ca. 40 ml of

bread pellets, measured in a measuring cylinder) to obtain a concentration of ca. 0.45 mg of crude extract per milliliter of bread pellets.

The spherule treatment was prepared as described above by adding 16 ml of spherules, suspended in 1 ml of seawater, to 10 g, ca. 40 ml, of bread pellets (resulting in a concentration of ca. 0.4 ml of spherules per milliliter of pellets). The acetone control was prepared by adding 12 ml of acetone to 10 g (40 ml) of bread pellets. The pellets containing the treatments were then air dried to facilitate manipulation. In the previous experiment on sea urchins no differences were found between the carragenate and acetone controls, so we only considered the acetone control in subsequent experiments.

5.4.3. In situ sympatric fish assemblage

The artificial food for the in situ experiment with the sympatric fishes at the sponge habitat was similar to that prepared for the fish experiment in the laboratory (see above) but the pellet size was larger (4 mm long and 1 to 2 mm thick) in order to adapt the food to the mouth size of the fishes targeted. We per formed the same 3 treatments (crude extract, calcibacteria spherules, and acetone control) as in the *P. incognitus* experiment. Treatments were offered to the fish assemblage at random. Fishes at sea were adapted to feed on artificial food offered by divers for 7 d prior to the experiment.

5.5. *Paracentrotus lividus* experiment

The sea urchins were starved for 1 wk and then placed in individual 5 l aquaria, with continuous aeration at 22°C. Three treatments consisting of (1) the sponge crude extract, (2) the calcibacteria spherules, and (3) both components combined were offered. We used a total of 30 individuals, 6 per treatment (including controls). Two other aquaria were disposed under the above conditions to sink 2 plates of each treatment to assess their increase in weight due to hydration during the experiment. The plates were randomly distributed between individuals,

and the experiment lasted for 48 h. The plates were then recovered, slightly toweled, and weighed to calculate weight losses that were due to sea urchin grazing, after discounting the mean increase in weight of plates used to control hydration.

5.6. *Parablennius incognitus* experiment

After 1 week of adaptation to aquarium conditions, each *P. incognitus* individual was placed in a 5 l aquarium (N = 14) with continuous water flow at a constant temperature (22°C). The experiment lasted for 8 d. Every 2 d we offered 10 pellets of each treatment (crude extract, spherules, and acetone control) in random order to 4 randomly selected fishes, and recorded the number of pellets eaten or rejected per treatment. No pellet was ignored when offered to fish in this experiment. At the end, we had a total of 16 replicates per treatment. Those fishes that were not involved in a given trial were fed daily *ad libitum* with Sera® marine granulate.

5.7. Sympatric fish experiment

The field experiment was carried out in the Blanes sublittoral zone (NW Mediterranean; 41°40.12'N, 2°47.10'E), in summer 2013, on a rocky, 10 to 15 m deep, bottom. The most frequent fish species co-occurring in the sponge habitat were *Chromis chromis*, *Diplodus vulgaris*, *Oblada melanura*, and *Coris julis*. Thus, we recorded the behavior of these 4 fishes with respect to the food offered. The number of fish participating in the experiment, as estimated from the number of pellets that they ate or rejected, was >20 per species (see Table 3), although we were unable to ensure that a given individual participated only once in the experiment.

The artificial food was taken to sea in large plastic syringes (1 treatment^{-1}) as described by Becerro et al. (2003). Treatments and controls (5 pellets treatment^{-1}) were randomly offered to fishes by slowly releasing the pellets into the water. Two independent SCUBA divers recorded the number of eaten or rejected pellets. A pellet was considered rejected by a fish if tried and spat out 3 or more times. When a pellet was ignored, or tried by a fish just once or twice and spat out and ignored, the outcome was annulled and a new pellet of the

same treatment was offered later.

5.8. Comparative deterrence quantification

A deterrence index (Becerro et al. 2003) was used for comparing sea urchin and fish deterrence in the 3 experiments. The index (DET) was defined as:

$$DET = \frac{\frac{EC}{OC} - \frac{ET}{OT}}{\frac{EC}{OC}}$$

Where *EC* is either the number of control pellets eaten by fishes or the weight losses in the control agar plates offered to sea urchins and *OC* is the number of control pellets offered or the initial weight of the control plate; *ET* is the number of treatment pellets eaten or the decrease in weight of a treatment plate and *OT* is the number of treatment pellets offered or the initial weight of a give treatment plate. DET varies from 0 (no deterrence) to 1 (total deterrence).

5.9. Statistical analyses

Data from the experiments in the laboratory on the sea urchin *P. lividus* and the fish *P. incognitus* were analyzed by one-way ANOVA after rank transformation, since they did not meet the assumptions for parametric analyses. The significance values of the post-hoc pairwise comparisons (Newman-Keuls test) were adjusted by the false discovery rate (FDR) correction for multiple comparisons (Yekutieli & Benjamini 1999).

Data from the sea experiment were analyzed using log-linear models for contingency tables. We tabulated our data with treatment (control and treated food), fish species tested, and consumption (eaten or rejected) as factors, and the number of occurrences (pellets) in each category as observed cell frequencies (Sokal & Rohlf 1995). The statistical significance of the deviations of the observed frequencies from the expected frequencies was evaluated by Pearson's chi-squared.

All the statistics analyses were performed with STATISTICA 6.0.

RESULTS

Calcibacterial presence/abundance

Calcibacteria were present in the *Hemimycale columella* sponges used for the experiments, as proved by light and electron microscope observations. Calcibacteria spherules were extraordinarily abundant (Fig. 5.1 A–D) in the sponge homogenates either free in suspension (due to their small size [<1 μm in diameter] and low weight) as a result of calcibacteriocyte damage or on the upper layer of the pellets within denser entire calcibacteriocytes. SEM pictures of cryofractured sponge tissue showed calcibacteria with a 100 nm coat of nanospherules arranged in a layer and with inner material corresponding to the bacteria (Fig. 5.1 C). Images of intracellular bacteria deprived of the calcareous coat (likely due to calcium carbonate dissolution during the pH-lowering fixation process) were obtained by TEM. The intracellular vacuoles maintained the size and shape of the calcibacterial coat (Fig. 5.1 D).

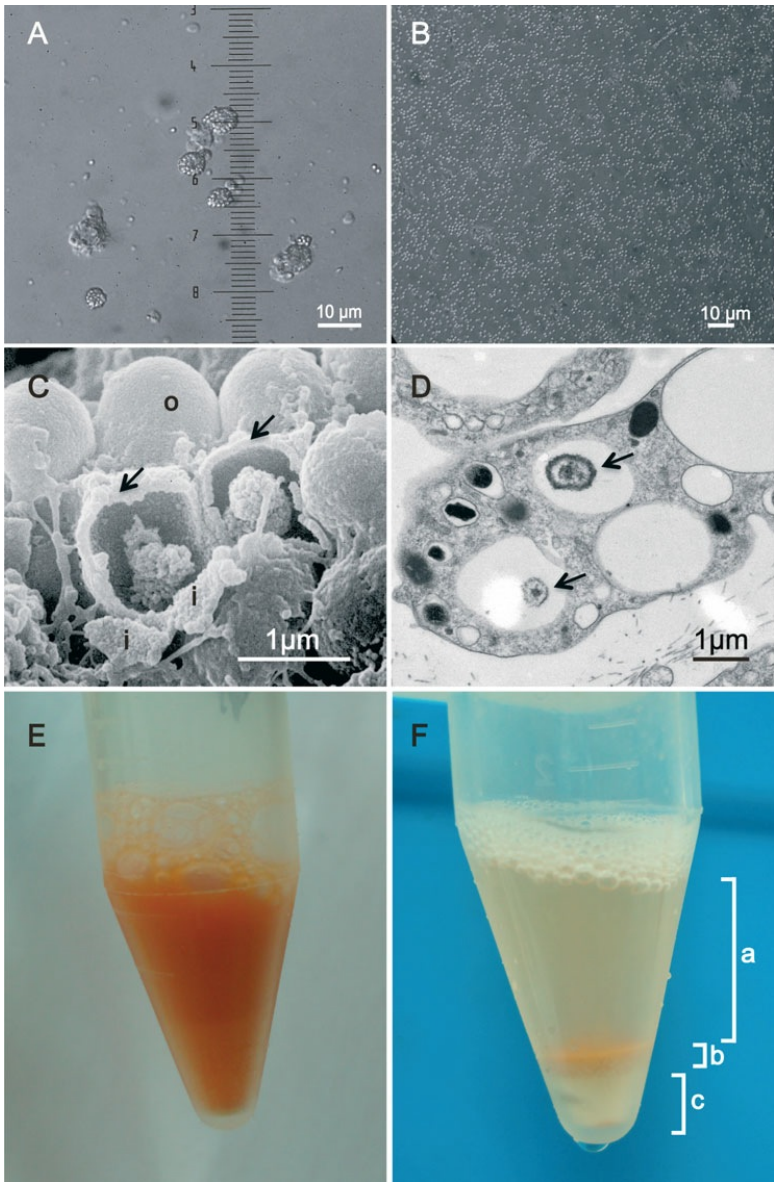

Figure 5.1. Calcibacteria in *Hemmycale columella* (A–D), and the process of calcibacterial isolation (E, F). A) Sponge cells (calcibacteriocytes) containing the calcified bacteria (which appear refringent through the light microscope). B) Huge amounts of calcibacteria after cell dissociation of fresh sponges (light microscope). C) Scanning electron microscope picture of a cryofractured sponge showing entire and broken calcibacteria—i: internal side showing the nanospherules that form the calcite coat; o: outside of the calcite coat (arrows point to the zones where nanospherule arrangement in a layer is more conspicuous). D) Transmission electron

microscope picture of a calcibacteriocyte containing 2 calcibacteria within their respective vacuoles (arrows); the calcareous envelope was dissolved during the fixation process by acidic fixators, i.e. glutaraldehyde). E) Sponge homogenate after spicule removal: Step 1 of the cell dissociation and centrifugation process. F) Step 2 of the process in which the debris of most sponge cells has already been removed. a: supernatant containing isolated calcibacteria in suspension; b: layer of calcibacteriocytes; c: settled calcibacteria.

P. lividus experiment

ANOVA results on ranks showed a significant effect ($p < 0.001$) of treatments on ingested food (Table 1). Post-hoc comparisons (Newman-Keuls test) after FDR correction proved significant ($p < 0.001$) differences between the 3 treatments and the 2 controls (carragenate control and acetone control), which were eaten similarly ($p = 0.22$). There were no significant differences in feeding between the calcibacteria spherules and the chemical treatment ($p = 0.29$), or between the chemical treatment and the chemical+calcibacteria spherule treatment ($p = 0.29$; Fig. 5.2). Thus, the crude extract, the calcibacteria spherules, and the calcibacteria+crude extract similarly deterred sea urchins from feeding on *H. columella*, but the latter combination did not deter the sea urchin in an additive or synergetic way.

Effect	SS	DF	MS	*F*	*p*
Treatment	1653.000	4	413.250	17.3780	0.000001
Error	594.500	25	23.780		

Table 1. One-way ANOVA on the deterrent effect of several treatments (carragenate control, acetone control, crude extract, calcibacteria spherules, and chemical and bacterial components combined) on the sea urchin *Paracentrotus lividus*.

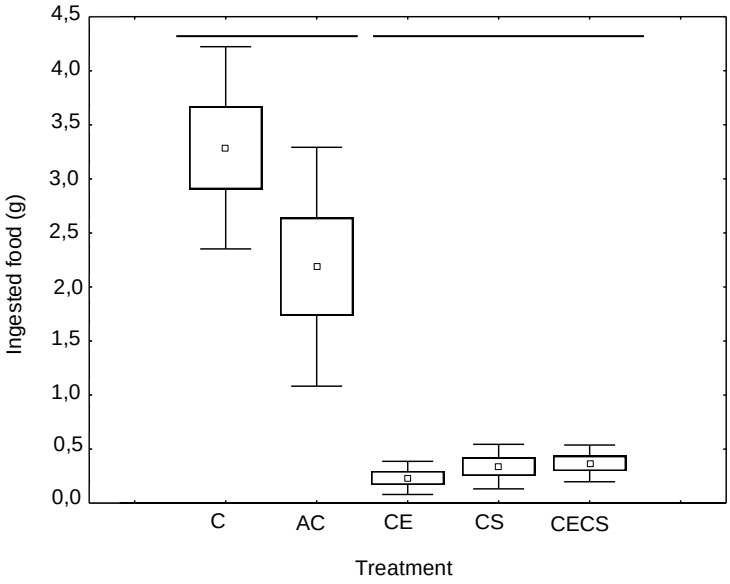

Figure 5.2. Deterrent effect of treatments assayed on the sea urchin *Paracentrotus lividus* (N = 6; C: carragenate control; AC: acetone control; CE: sponge crude extract; CS: calcibacteria spherules; CECS: crude extract+calcibacteria). Horizontal bars at the top of the panel indicate no significant differences between treatments after false discovery rate correction ($p < 0.021$).

P. incognitus experiment

ANOVA on the number of pellets ingested by *P. incognitus* showed significant differences among treatments and the control (Newman-Keuls test, $p < 0.001$; Table 2). Post-hoc multiple comparisons after FDR correction showed significant differences ($p < 0.001$) between the calcibacterial treatment and the control but not ($p = 0.13$) between the chemical treatment and the control (Fig. 5.3). Thus, only the spherules of the symbiotic calcibacteria defended *H. columella* against predation by the small sympatric fish *P. incognitus*.

Effect	SS	DF	MS	*F*	*p*
Treatment	3548.49	2	177.25	25.49	<0.001
Error	2853.01	41	69.59		

Table 2. One-way ANOVA on the deterrent effect of several treatments (control, crude extract, and calcibacteria spherules) on the fish *Parablenius incognitus*.

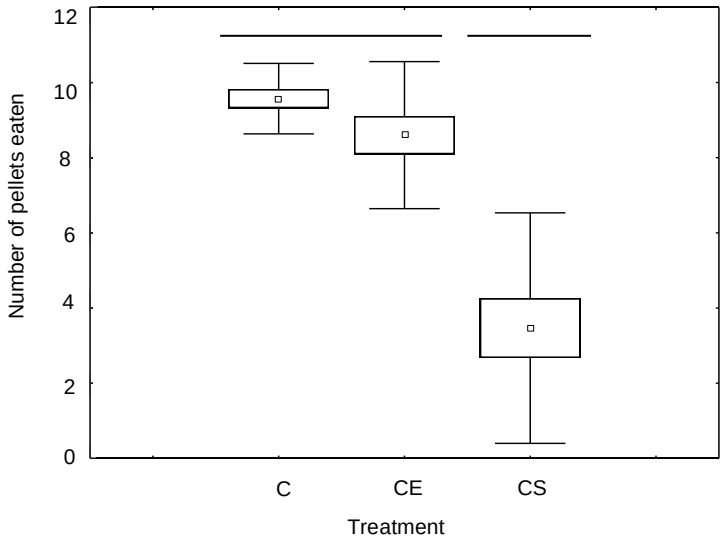

Figure 5.3. Deterrent effects of treatments assayed on the fish *Parablennius incognitus* (N = 16; C: control; CE: sponge crude extract; CS: calcibacteria spherules). Horizontal bars indicate no significant differences between treatments after false discovery rate correction ($p < 0.023$). Boxes represent ±SE, bars ±SD.

Sympatric fish experiment

The 3-way log-linear model for the contingency table with treatment, fish species, and ingested food as factors indicated that the assayed fish species, which shared habitat with the target sponge, were differently deterred from feeding by the 2 treatments assayed (Table 3; $\chi 2$, $p < 0.001$). There were high significant differences ($p < 0.001$) between the calcibacterial treatment and the control for the 4 sympatric fishes. Conversely, the chemical (crude extract) treatment was eaten significantly less often than the control for two out of the four assayed fishes (Table 3; $\chi 2$, $p < 0.001$).

Ingested pellets	*Chromis chromis*	*Oblada melanuta*	*Diplodus vulgaris*	*Coris julis*	Total
Acetone control					
Yes	31	33	27	40	131
No	4	6	1	0	11
Crude extract					
Yes	23	0	17	43	83
No	22^{ns}	24^{**}	14^{**}	0^{ns}	60
Calcibacteria spherules					
Yes	0	0	4	35	39
No	38^{**}	42^{**}	18^{**}	7^{*}	105
Total	118	105	81	125	429

Table 3. Frequency table from the in situ sympatric fish assemblage experiment that was used for con tingency table analysis (143 pellets were offered per treatment). Asterisks indicate deterrent effect on ingestion ($\chi 2$, $^{*}p < 0.05$; $^{**}p < 0.01$; ns: non-significant).

Comparative deterrence quantification

The deterrence index (DET), which represents the relation between the consumed food and the food offered in treatments and controls, varied between treatments and among species (Fig. 5.4). It approached 1 for both the chemical and the calcibacterial treatments in *Paracentrotus lividus*, while it significantly varied between the calcibacterial (DET = 0.64) and chemical (DET = 0.1) treatments for *P. incognitus*.

The fish deterred most by the two treatments (DET = 1, crude extract and calcibacteria spherules) was the sparid *Oblada melanura*. Conversely, the labrid *Coris julis*, the pomacentrid *Chromis chromis*, and the sparid *Diplodus vulgaris* showed significantly lower deterrence indices for the chemical treatment than for the calcibacterial treatment (DET = 0, DET = 0.4, and DET = 0.4, respectively) (Fig. 5.4).

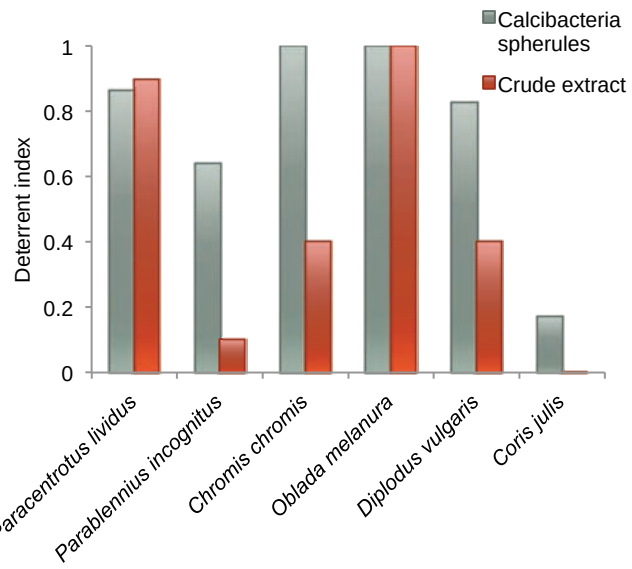

Figure 5.4. Deterrence index of sponge crude extract and calci- bacteria spherules for several species assayed.

DISCUSSION

The various deterrence experiments performed revealed that the sponge *Hemimycale columella* combines >1 defense mechanism to dissuade potential predators. Some predators are deterred by both secondary metabolites and calcibacteria, a case example of the evolutionary fixation of redundant mechanisms of defense in a species to widen the spectrum of predators deterred. On the other hand, sponges are not the only organisms to present two different types of defenses; crude extracts and sclerites have also been reported to exert anti-predatory functions in gorgonians (van Alstyne & Paul 1992).

The spherules produced by the symbiotic calcibacteria significantly deterred all species assayed; thus they appear to represent a generalist defense mechanism. Conversely, the chemical extract of *H. columella* deterred some of the species assayed, but not others; thus it seems to represent a more species-specific defense mechanism.

The sea urchin *Paracentrotus lividus* was deterred from feeding on *H. columella* by both the sponge's chemical extracts and the calcibacteria spherules, as well as by a combination of both components; this finding agrees well with the observed lack of predation of *P. lividus* on *H. columella* in the field (authors' pers. obs.). Several studies reported that some sponge components deterred this sea urchin, but the outcome of the assays performed here varied as a function of the sponge species, the types of defense analyzed, and the sea urchin species used. Sponge spicules, spongin, collagen, or calcium carbonate may deter some sea urchins from predation (Pennings & Svedberg 1993; Uriz et al. 1996c). Conversely, other sea urchin species feed on sponges habitually, despite the presence of siliceous spicules (de Ridder & Lawrence 1982; Santos et al. 2002). These contrasting results illustrate predator-dependent outcomes to the same type of defense.

Paracentrotus lividus has been reported to feed on sponge species devoid of spicules when food resources are limited (Boudouresque & Verlaque 2007). Since *H. columella* shows a relatively poor spicule complement, predation by *P. lividus* on this sponge species would be

expected, but has not been observed. Our results showed that the calcium carbonate spherules of the symbiotic calcibacteria at natural concentrations deter this sea urchin. The spherules may be unpalatable to sea urchins, but not strictly toxic (Birenheide et al. 1993), and, likely, their high concentration in sponge tissues may decrease sponge nutritional quality; thus, field sea urchins may select other more attractive food sources for optimal growth. Moreover, it has been reported that both calcite and aragonite deter some herbivore fishes from feeding (Pennings & Svedberg 1993), which has been related to a decrease in fish gut pH impairing food digestion (Schupp & Paul 1994).

Besides the symbiotic calcibacteria, *H. columella* produces secondary metabolites with demonstrated antimitotic, cytotoxic, and antibacterial activity (Amade et al. 1987, Uriz et al. 1992a; Becerro et al. 1997a). Here we report on another defensive function of these secondary metabolites since they discourage the sea urchin *P. lividus* from grazing. It has also been reported that *P. lividus* is deterred by the crude extract of the sponge *Crambe crambe* (Uriz et al. 1996c; Becerro et al. 1997b) and the seagrass *Posidonia oceanica* (Vergés et al. 2007), while it appears to consume the alga *Caulerpa taxifolia* during the months when it presents the lowest amount of secondary metabolites (Lemee et al. 1996). Protection from the devastating grazing by sea urchins (Guidetti & Dulc˘ic´ 2007) seems to be widespread among many benthic organisms, which have developed deterrent toxicants.

The outcomes of the fish experiments differed according to the fish species assayed. In general, calcareous spherules deterred fishes more efficiently than sponge crude extract did, but, for some species, both components were similarly deterrent. Previous studies reported that sponge skeletal structures are deterrents for fishes (Burns & Ilan 2003; Jones et al. 2005; but see Chanas & Pawlik 1995, 1996). *H. columella* is a spicule-poor species, and the high concentration of calcibacteria spherules at the sponge periphery may replace spicules as deterrent elements for fishes.

The indexes formulated to compare the deterrence intensity among the potential predators assayed (DET) varied across species depending on the treatment. The calcibacteria DET index showed the highest value for *C.*

chromis and *O. melanura*, followed by the sea urchin *P. lividus* (DET = 1), and exerted the lowest effect (DET = 0.19) on *C. julis*. Such differences may be due to differences in the habitual prey preferentially targeted in the field by each predator. Thus, fishes such as *C. julis*, which usually feed on invertebrates provided with an external skeleton, such as mollusks, gastropods, bivalves, and crustaceans (Kabasakal 2001; Stergiou & Karpouzi 2002), may be more adapted to encountering calcareous structures in their diet.

The DET index for the chemical treatment varied drastically with the predator species: while it reached its highest value for the sea urchin *P. lividus* and the fish *Oblada melanura*, and a medium value for *D. vulgaris* and *C. chromis*, it was close to zero for *P. incognitus* and *C. julis*.

The contrasting deterrent effects found for the crude extract may also be related to differences in the natural feeding habits of the species assayed. *O. melanura* and *D. vulgaris* are considered opportunistic predators that feed on an array of both benthic and pelagic organisms (Pallaoro et al. 2003, 2006).

Thus, they may select other, non-toxic food sources in the field. The small bleniid fish (*P. incognitus*) captures small benthic animals in the field, while grazing the surface of rocky substrata and encrusting invertebrates (Goldschmid & Kotrschal 1981); thus, it might be adapted to ingest small amounts of potentially toxic species such as *H. columella* and *C. crambe* (Becerro et al. 1997b) while capturing small invertebrates dwelling on sponges. The labrid *C. julis* is a voracious species that has been reported to predate on crustaceans and gastropod mollusks (Fasola et al. 1997; Kabasakal 2001); apparently it also tolerates, to some extent, the bioactive compounds produced by the alga *Caulerpa prolifera* (Sureda et al. 2006). Thus, the two latter fishes seem to show some resistance to the secondary metabolites of benthic invertebrates. On the other hand, pomacentrid fishes such as *C. chromis* are also opportunistic, omnivorous species that include sponges in their diet (Emery 1973; Emery & Thresher 1980; Horn 1989). *C. chromis*, however, has been reported to avoid artificial food containing the crude

extract of the nudibranch *Discodoris indecora*, which obtains its metabolites from *Ircinia spp*. sponges (Marin et al. 1997).

Reinforcing the invertebrate periphery by mineral materials in order to make it less attractive to potential predators is the main function of mineral exoskeletons (Uriz 2006). Sponges concentrate microscleres at the periphery to form a mineral cortex (Uriz et al. 2003). Rohde & Schupp (2011) reported a higher deterrent effect of artificial food containing siliceous spicules from the sponge cortex than from the choanosome. This is likely related to the spicule sizes and may depend on the mouth size of the predator considered. Small spicules (microscleres) are densely packed in the sponge cortex (Boury-Esnault & Rützler 1997), and thus more likely to deter small predators, while protruding choanosomal megascleres (from hundreds of micrometers to millimeters) likely deter larger mouthed predators (Uriz et al. 2003). The calcified calcibacteria of *H. columella* are spherules of ca. 1 µm size that are transported by amoeboid cells (calcibacteriocytes) to the sponge sub-ectosomal zone (Uriz et al. 2012) where they form a kind of calcareous cortex. Since these calcareous spherules appear to be so efficient in deterring the potential predators assayed, their high concentration at the sponge periphery may make them very efficient in deterring an array of small-mouthed predators.

Although chemical extracts and calcibacteria deterred some potential predators individually, the deterrent effect did not increase in additive or synergistic ways when they were combined in a treatment. The few studies in which the possible synergism between structural defenses and crude extracts from sponges has been considered showed disparate results (e.g. Hay et al. 1994; Burns & Ilan 2003; Hill et al. 2005; Jones et al. 2005; Ribeiro et al. 2012).

The *Hemimycale*-calcibacteria symbiosis is not the only case in which a bacterium protects a sponge from predation. Recently, a symbiotic Beta-proteobacteria of the sponge *C. crambe* has been reported to participate in the metabolic pathways (Croué et al. 2013) of two highly deterrent metabolites of *C. crambe* (Uriz et al. 1996c). On the other hand, polyketide synthethases

(PKS) of bacterial origin, with bioactive functions, have been found in many sponge-bacteria symbioses (Piel et al. 2004; Haber et al. 2011; Esteves et al. 2013). In all cases, the resulting substances produced by symbiotic microorganisms, either secondary metabolites or carbonate spherules, may be used by sponge species to their own benefit (deterrent, antibacterial, or antifouling roles; Uriz et al. 1996c), thus promoting the persistence of sponge-bacteria associations.

Since the concentrations (at a volumetric proportion) of chemicals and calcibacteria used in the experiments were roughly similar to those found in natural sponges, we are confident that protection from predation is one of the benefits that *H. columella* receives from its symbiosis with calcibacteria. Its con- tribution to sponge survival may have helped establish this unique symbiosis between marine sponges and calcifying bacteria. However, the sponge's secondary metabolites also exert a deterrent effect against some potential sponge predators. The calcibacteria and crude extracts together do not seem to have an additive or synergetic effect on potential sponge predators, but rather may be engaged in enlarging the array of potential predators deterred. The chemical defenses of *H. columella* contribute to the complexity of Mediterranean species interactions, which supports the theory of Buss (1976), which was recently substantiated by Loh & Pawlik (2014) for sponge communities of coral reefs. In contrast, symbiotic calcibacteria unambiguously contribute to protect the sponge from generalist predators and thus favor the species' success. This is the first time that a physical defense produced by symbiotic bacteria has been documented.

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General Discussion

Sponge biodiversity is though to be by far larger than that known up to know (van Soest et al. 2012). Traditionally, sponge species have been identified on the basis observation of external and skeletal characters. However, in the last years molecular markers have been incorporated to the sponge taxonomy revealing the existence of many morphologically cryptic species (e.g. Blanquer et al. 2008, Reveilleud et al. 2010; Knapp et al. 2015), which, in some cases have been then confirmed by distinctive biological features (Blanquer et al. 2008b). However, some molecularly discovered species have never been formally described due to the difficulty of finding diagnostic phenotypic characters, and still less their particular life histories have been studied. In the case of the cryptic Mediterranean *Hemimycale* (*H. columella* and *H. mediterranea*), both species have similar spicules, but the three gene partitions used (18S rRNA, 28S rRNA and COI mtDNA), as well as their contrasting ecological distribution, strongly support they are good species. However, the most evident differences between these two cryptic species are their respective life cycles and growth dynamics.

Moreover a more detailed study on their microbiomes revealed that both species have completely different bacteria assemblages, confirming the reported species-specificity of sponge-associated bacteria (e.g. Schmitt et al. 2012). However, our results also point to the existence of exceptions to the generally agreed low, intra-species variability of sponge microbiomes. *H. mediterranea* showed relevant differences in its bacteria assemblages among individuals from different locations, which suggests that a part of its associated bacterial are acquired from the surrounding water as reported for other sponge species (e.g. Alex & Antunes 2015; Sipkema et al. 2015).

No meaningful correlation was found between the growth rates and survival of *H. columella* and *H. mediterranea* with any of the environmental parameters measured at their respective habitats, except temperature. Temperature was negatively correlated with growth rates in *H. columella*, suggesting a North-Atlantic origin for this species with a current Atlanto-Mediterranean distribution. Conversely, although monthly growth rates were very variable in *H. mediterranea*, preventing correlation with temperature, its maximum growth rates occurred in the warmest month (August).

Mediterranean sponge endemisms use to grow faster in summer (e. g Blanquer et al. 2008b), what suggests a Mediterranean origin for this second species, which has been only found up to now in the Mediterranean Sea.

But the most remarkable feature of these *Hemimycale* species is their particular symbiosis with calcifying bacteria. This symbiosis had been recently reported for marine invertebrates (Uriz et al. 2012) and we have proved that it is shared with other sponge species taxonomically and geographically apart. We have retrieved at least seven calcibacteria OTUs, belonging to the SAR116 group (Alpha-proteobacteria class), which form a monophyletic clade in phylogenetic reconstructions. However, calcifying bacteria may also belong to other bacteria class, as no Alpha-proteobacteria were found in individuals of *H. mediterranea* from the Adriatic Sea, although they contained huge amounts of calcareous spherules. In these individuals, Beta-proteobacteria might be responsible for spherule formation as ca. 95% of its microbiome OTUs belonged to this class. Moreover, calcibacteria sequences are not exclusive of sponges, but according to our phylogenetic reconstructions, they have been also found in corals and in seawater. The presence of calcibacteria in several taxonomically distant hosts and in seawater, as well as their taxonomical diversity, suggests that the observed calcification could be the results of the interaction between the intracellular bacteria an their hosts rather than due to specific bacteria exclusively. Calcification occurred in our target symbioses within sponge cell vacuoles. The vacuole microenvironment is expected to change over the course of bacterial growth, because nutrients are removed from the medium and bacteria expel waste products into the medium. We propose that these changes may increase the pH of the vacuole, and calcium carbonate nucleation and precipitation on the bacteria membrane is biologically induced (De Nooijer et al. 2009; Weiner & Dove 2003), as observed previously in experimental studies with *Chromohalobacter marismortui* (Rivadeneyra et al. 2010).

We have shown that calcibateria accumulated at the sponge periphery, forming a kind of exoskeleton, analogous to the cortex of

siliceous microscleres of many sponges (Uriz 2006), which confer them protection against predators.

The calcibacterium of *H. columella* (Uriz et al. 2012) is transported to the sponge periphery by particular, mobile sponge-cells (calcibacteriocytes) providing a whitish colour to the sponge surface. Calcibacteriocytes do not differ significantly from archaeocytes, moving cells that are genetically programmed to remove debris and undesired substances from the sponge mesohy (Simpson 1984). Moreover, archaeocyte-like cells pack bioactive metabolites in the form of spherules to prevent sponge self-toxicity (Uriz et al. 1996a, b). Thus, the sponge mechanism of particle capture and transport may have also evolved for harbouring symbiotic bacteria. This bacteria-mediated calcification process does not seem to be restricted to *H. columella*. Likely, it also occurs in the other calcibacteria-bearing sponges, which show a whitish ectosome.

This type of symbiosis has been speculated to play a role on skeletonization or early animals (Uriz et al. 2012). Protection against an increase in predators has been proposed as an evolutionary driver of exoskeletons in ancient animals during the Cambrian explosion (Bengtson & Zhao 1992). The experiments carried out to test the role of calcibacteria accumulation in *H. columella* against predators (sea urchins and fishes), demonstrated that calcibacteria represent a defence mechanism, actually more effective than the chemical compounds isolated from the species (Becerro el al. 1997a). The bacteria calcareous coats deterred all the potential predators assayed so that it seems to be a generalist defence mechanism, while chemical compounds deterred some species assayed but not others, acting as a species-specific defence mechanism.

If the symbiosis with calcifying bacteria has become evolutionarily fixed, benefits at the species level should compensate for the costs to the associated partners (Roughgarden 1975). The most obvious benefit to sponges from their association with calcifying bacteria is the 'low cost' construction of an exoskeleton, which may serve as structural purpose and has been proved to deter potential sponge predators (Garate et al. 2015; Chapter 5). By assuming that calcium precipitation around the bacteria is

spontaneously triggered by increases in pH within the vacuole, the only cost of formation of the sponge exoskeleton would be the transport of calcified calcibacteria to the sponge peripheral zone.

In contrast, the benefits for calcibacteria are more difficult to ascertain. Sponge tissues might offer protection from pathogens and predators, which are abundant in non-host environments (García & Gerardo 2014), and buffer nutrient ocean fluctuations that prevent the steady growth of bacteria over long periods (Navarro-Llorens et al. 2010). However, considering the detrimental consequence of calcification for the bacteria, calcibacteria may also be more akin to 'prisoners' or 'farmed crops' than equal partners as in other bacteria-invertebrate symbioses (García & Gerardo 2014). Benefits might therefore be related to the propagation of the species. Symbiosis ensures that calcibacteria persist across sponge generations via the vertical transmission to sponge progeny.

Moreover, the presence of free calcibacteria in seawater also suggests that viable calcibacteria are released back into the environment, which would allow the bacteria to form a species reservoir to facilitate dispersal and colonization of new invertebrate hosts. Calcification appears to be the cost to bacteria for living in a more stable, predator-free, nutrient-rich environment.

The current-day animal-bacteria symbioses, which likely existed when animals first appeared (McFall-Ngai et al. 2013), can provide key insights into Metazoa evolution. The reporting of bacteria-mediated calcification mechanisms in phylogenetically apart sponges suggests the implication of bacteria in the early evolution of the skeleton in the pre-Cambrian metazoans (Ayala & Andrey 1998). Although several molecular mechanisms are responsible for calcium precipitation and skeleton formation in animals (Taylor et al. 2007b), those involving bacteria might be evolutionarily older and, thus, acquire new relevance in the light of these results.

Conclusions

❖ Cryptic species

A new *Hemimycale* species: *Hemimycale mediterranea* sp. nov.*,* which is morphologically cryptic with *H. columella,* has been described. The new species is widely distributed across the western, central, and eastern Mediterranean at shallow depths.

Hemimycale columella, the type species of the genus, likely with an Atlantic origin, inhabits deeper rocky/coralligenous assemblages of the North-western Mediterranean, although its presence in eastern Mediterranean cannot be discarded.

Cryptic species represent something more that wrong taxonomic identifications or biodiversity underestimates. They may feature contrasting biological cycles and life spans, and puzzle biological studies, what may invalidate conservation policies based on wrong biological species data.

❖ Phylogeny of *Hemimycale* and *Crella*

Hemimycale is a polyphyletic genus and the species *H. arabica* from the Red Sea belongs in a different genus.

The genus *Crella* is polyphyletic and the species *Crella cyatophora* from the Red Sea belongs to a different genus, closer to *H. arabica* than to the Atlanto-Mediterranean *Crella spp.*

Hemimycale and *Crella* form a monophyletic group, which suggests the former genus should be better placed in Crellidae than in Hymedesmiidae. However, more Hymedesmiidae and Crellidae representative should be included in a phylogenetic analysis before such a decision is proposed.

❖ Ecological and biological traits of Mediterranean *Hemimycale* species

The cryptic sponge species *H. columella* and *H. mediterranea* present contrasting life spans being *H. columella* multiannual and the *H. mediterranea* annual. To our knowledge, this is the first report of an annual life span in a Demospongiae.

H. columella growth in the western Mediterranean was correlated negatively with water temperature, so that the species presented the highest growth rates in cold months. Conversely, its cryptic species *H. mediterranea* grew more in summer, but without any correlation with the environmental parameters monitored.

The environmental factors measured at the two species habitats were significantly different and may have contributed to their contrasting ecological distributions, despite sharing geographical locations.

Food depletion after the reproduction period at the *H. mediterranea* habitat might contribute to the sponge decay. A higher availability of particulate matter at the *H. columella* habitat after the larval release period might have help this species to persist for years. Thus, we cannot totally discard that *H. mediterranea* populations persist longer in places where food conditions are more appropriated for the species to cope with the delicate post-reproduction period.

The strong biological differences showed by these sponge species contrast with their slight differences in phenotypic characters and highlight the need of untangling the cryptic diversity of ecosystems to guarantee the reliability of ecological studies.

❖ Localization and quantification of calcibacteria

CARD-FISH experiments with a calcibacteria specific probe confirmed the presence of this bacterium within the tissues of the sponges *H. columella, Cliona viridis, Cinachyrella alloclada* and *Prosuberites sp.*, as it was suggested by the calcareous spherules observed though light microscope.

Calcibacteria are actively accumulated at the periphery of whitish individuals of *H. columella*, which harbour higher number of calcibacteria than pinkish individuals. In contrast, the choanosome of both colour morphs revealed similar calcibacteria concentrations.

Calcibacteriocytes full of calcibacteria are present in tissues of both non-reproductive and reproductive stages of the sponge *H. columella, as* observed

through CARD-FISH and confocal and TEM studies, which support a vertical transmission of the calcibacteria.

❖ Calcibacteria taxonomy and phylogeny

There are at least seven OTUs of calcibacteria present in taxonomical apart sponges (all of them harbouring calcareous spherules), corals and seawater of different oceans. Thus no phylogenetic signal has been found in the calcibacteria hosts, what points towards the absence of sponge-bacteria coevolution.

These calcibacteria belong to the SAR116, within the Ricketssiales, as a sister group of the Rhodospirillales, within the Alpha-Proteobacteria class of phylum Proteobacteria.

However, other taxonomically distant bacteria are also be able to produce calcareous spherules similar to those mediated by the calcibacteria (Alpha-proteobacteria), as calcareous spherules are also abundant in *H. mediterranea*, which lacks the Alpha-proteobacteria OTUs responsible for calcification in the other studied species.

❖ Microbial composition of sponges harbouring calcibacteria

The sponge species studied presented in general species-specific microbial communities, with low variation among replicates, except *H. mediterranea*, which showed high microbiome differences among replicates from the north-western Mediterranean and the Adriatic Sea, suggesting the possible incorporation of bacteria from the water.

The Phylum Proteobacteria was relatively more abundant in those species previously classified as "low microbial abundance" (LMA) sponges than in the species considered as "high microbial abundance" (HMA) sponges, with the exception of *H. mediterranea* from north-western Mediterranean, where bacteria belonging to phylum Tenericutes were similarly abundant than Proteobacteria.

❖ Anti-predatory defences of *H. columella*

This is the first time that a physical defence produced by symbiotic bacteria has been documented in sponges.

Calcibacteria protect *H. columella* from predation by several fishes and sea urchins thanks to the production of calcareous spherules. Thus calcibacteria rather represent a generalist mechanism of defence, while the sponge chemical defences work as a specific mechanism of defence as they deter some potential predators but not others.

Protection from predation is one of the benefits that *H. columella* receives from its symbiosis with calcibacteria, which may have contributed to the symbiosis persistence.

The calcibacteria and the bioactive compounds (crude extracts) produced by H. columella altogether do not seem to have an additive or synergetic effect on deterring its potential predators, but rather the two mechanisms of defence may enlarge the array of potential predators deterred.

❖ Evolutionary implications of the sponge-calcibacteria symbioses

The widespread distribution of calcibacteria across Early Metazoa (Porifera and Cnidarian), and several oceans, their accumulation at the sponge periphery mimicking an exoskeleton, with a defensive function against potential predators proved for the sponge *H. columella,* add support to previous hypotheses on the possible role of endosymbiotic bacteria in the skeletonization of Early Metazoan.

Research in progress

My knowledge about calcibacteria has increased noticeably along this thesis. The taxonomical study placed them within the SAR116 group, CARD-FISH assays with a specific probe confirmed their accumulation at the peripheral zone of *Hemimycale columella* individuals, their vertical transmission to the sponge larva, and their deterrent role against sea urchins and fishes. Moreover, it has been proposed a "calcibacteria cycle" through which bacteria acquisition, calcification within sponge cell vacuoles, and transmission might take place. These calcibacteria undergo calcification within the sponge calcibacteriocytes, purportedly as a consequence of an increase of pH within vacuoles where they are include. However, the purported metabolic processes ending in the formation of calcium carbonate spherules still remains elusive. Calcification may be biologically induced by the bacteria metabolism, by the sponge cell metabolism, or by the interaction of the metabolism of both partners.

In the last years many studies have focused on unravelling the function that bacterial symbionts play within the sponge. The "omics" techniques can help in assessing the genes expressed by both the prokaryotes and eukaryotes involved in the symbioses and thus, elucidation the role of each partner in the whole metabolism of the holobiont. For example, some symbiotic microorganisms have been reported to be the producers of chemical compounds with pharmacological interest, are involved in the transference of nutrients to the hosts, and participate in complex metabolic cycles (Hoffman et al. 2009; Freeman et al. 2013; Kamke et al. 2013).

Thus, we have sequenced the genome of the calcibacteria by "single cell genomics" to ascertain the genomic repertoire of these bacteria and to unveil the molecular mechanisms involved in the establishment of the symbiosis and in the calcification process.

An enriched population of calcibacteria was obtained by scraping the inner part of the ectosome of fresh *Hemimycale columella* individuals, and subjected to several steps of centrifugation (200 rpm and 800 rpm) and filtration (2 μm and 0.8 μm). The calcibacteria-enriched solution was cryoconserved in Gly-TE. Single cell genomics (SCG) was carried out in the Single Cell Genomic Centre of Bigelow (MA, USA). The calcibacteria-

enriched solution was subjected to fluorescence-activated cell sorting (FACS) and once identified the calcibacteria sequence (alignment with already known sequence of its 16S rRNA gene), the genome of a single cell was amplified through multiple displacement amplification (MDA) with Phi29 polymerase, and then sequenced through Sanger/Illumina 1.9.

Preliminary results showed a genome fragment of almost 600 kbp with 617 predicted genes. Only 19% of bacterial genes were present, which may indicate that the calcibacterium genome has only been partially sequenced, although some essential genes may be absent due to the endosymbiont condition of calcibacteria (e.g. Tian et al. 2017).

We retrieved the gene categories available of the genomes of the closest bacteria similar to our calcibacterium, which corresponded to three bacteria belonging to the SAR116 group, from the IMG/M database (Integrated Microbial Genomes & Microbial Samples). Comparisons of the metabolic repertoire (based on KEGG orthologs) of these bacteria with the calcibacterium showed a high metabolic difference of the calcibacterium (Fig. 1). This bacterium presents an important proportion of calcium-related proteins, although their related metabolic pathways still remain elusive. Thus, in the near future we will continue exploring the genomic repertoire related with the calcification mechanisms.

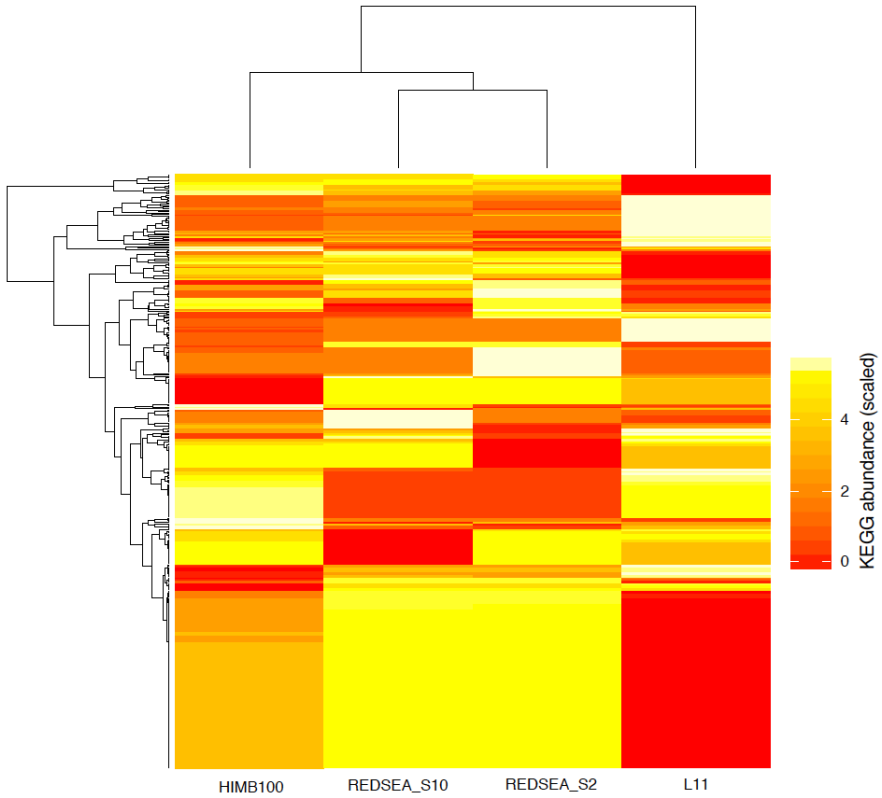

Fig. 1. Heatmap of the abundance of genes (scaled) involved in metabolic pathways from the KEGG database (Kyoto Encyclopedia of Genes and Genomes). The calcibacterium sequenced correspond to L11, and three representatives of SAR116 obtained from IMG/M.

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Appendix

Molecular phylogenies confirm the presence of two cryptic *Hemimycale* species in the Mediterranean and reveal the polyphyly of the genera *Crella* and *Hemimycale* (Demospongiae: Poecilosclerida)

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ABSTRACT

Background: Sponges are particularly prone to hiding cryptic species as their paradigmatic plasticity often favors species phenotypic convergence as a result of adaptation to similar habitat conditions. *Hemimycale* is a sponge genus (Family Hymedesmiidae, Order Poecilosclerida) with four formally described species, from which only *Hemimycale columella* has been recorded in the Atlanto-Mediterranean basin, on shallow to 80 m deep bottoms. Contrasting biological features between shallow and deep individuals of *Hemimycale columella* suggested larger genetic differences than those expected between sponge populations. To assess whether shallow and deep populations indeed belong to different species, we performed a phylogenetic study of *Hemimycale columella* across the Mediterranean. We also included other *Hemimycale* and *Crella* species from the Red Sea, with the additional aim of clarifying the relationships of the genus *Hemimycale*.

Methods: *Hemimycale columella* was sampled across the Mediterranean, and Adriatic Seas. *Hemimycale arabica* and *Crella cyathophora* were collected from the Red Sea and Pacific. From two to three specimens per species and locality were extracted, amplified for Cytochrome C Oxidase I (COI) (M1–M6 partition), 18S rRNA, and 28S (D3–D5 partition) and sequenced. Sequences were aligned using Clustal W v.1.81. Phylogenetic trees were constructed under neighbor joining (NJ), Bayesian inference (BI), and maximum likelihood (ML) criteria as implemented in Geneious software 9.01. Moreover, spicules of the target species were observed through a Scanning Electron microscope.

Results: The several phylogenetic reconstructions retrieved both *Crella* and *Hemimycale* polyphyletic. Strong differences in COI sequences indicated that *C. cyathophora* from the Red Sea might belong in a different genus, closer to *Hemimycale arabica* than to the Atlanto-Mediterranean *Crella* spp. Molecular and external morphological differences between *Hemimycale arabica* and the Atlanto-Mediterranean *Hemimycale* also suggest that *Hemimycale arabica* fit in a separate genus. On the other hand, the Atlanto-Mediterranean Crellidae appeared in 18S and 28S phylogenies as a sister group of the Atlanto-Mediterranean *Hemimycale*. Moreover, what was known up to now as *Hemimycale columella,* is formed by

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two cryptic species with contrasting bathymetric distributions. Some small but consistent morphological differences allow species distinction.
Conclusions: A new family (Hemimycalidae) including the genus *Hemimycale* and the two purported new genera receiving *C. cyathophora* and *Hemimycale arabica* might be proposed according to our phylogenetic results. However, the inclusion of additional Operational Taxonomic Unit (OTUs) appears convenient before taking definite taxonomical decisions. A new cryptic species (*Hemimycale mediterranea* sp. nov.) is described. Morphologically undifferentiated species with contrasting biological traits, as those here reported, confirm that unidentified cryptic species may confound ecological studies.

Subjects Aquaculture, Fisheries and Fish Science, Biodiversity, Marine Biology, Taxonomy, Zoology
Keywords Biodiversity, Molecular taxonomy, Morphological taxonomy, Cryptic species, Marine sponges, Phylogeny, *Hemimycale mediterranea*, *Hemimycale columella*, *Hemimycale arabica*, *Crella cyathophora*

INTRODUCTION

The discovery of cryptic species is continuously improving our knowledge on real ecosystem biodiversity and functioning, which are intimately related (*Frainer, McKie & Malmqvist, 2014*). Unrecognized cryptic diversity may mask biological features such as divergent reproduction patterns, growth dynamics, and inter-species interactions, among others (*Knowlton, 1993*; *Prada et al., 2014*; *de Meester et al., 2016*; *Loreau, 2004*), which may confound conservation studies (*Forsman et al., 2010*) and obscure the introduction pathway of invasive species (*Knapp et al., 2015*).

Molecular tools help to confirm suspected hidden species. However, molecular based identifications alone do not solve the problem of species misidentification, in particular when the cryptic species have overlapping distributions (e.g., *Knowlton & Jackson, 1994*; *Tarjuelo et al., 2001*; *De Caralt et al., 2002*; *Blanquer & Uriz, 2007*, *2008*; *Pérez-Portela et al., 2007*). In these cases, deep studies on their morphology, biology (e.g., life-history traits), and ecology (e.g., growth dynamics) become crucial to understand the mechanisms underlying their coexistence (*López-Legentil et al., 2005*; *Pérez-Portela et al., 2007*; *Blanquer, Uriz & Agell, 2008*; *Payo et al., 2013*).

Sponges are sessile, aquatic filter-feeders that are widespread across oceans, depths, and ecosystems (*Van Soest et al., 2012*), with so far 8,789 accepted species inventoried in 2016 (*Van Soest et al., 2016*) and ca. 29,000 predicted to be discovered in the forthcoming years (*Hooper & Lévi, 1994*; *Appeltans et al., 2012*), many of which remain currently hidden among supposed widespread morpho-species (*Uriz & Turon, 2012*).

The poor dispersal capacities of sponges prevent in most cases gene flow among populations even at short geographical distances (*Boury-Esnault, Pansini & Uriz, 1993*; *Uriz et al., 1998*; *Nichols & Barnes, 2005*; *Mariani et al., 2006*; *Uriz, Turon & Mariani, 2008*). Consequently, sponge populations become genetically structured (*Boury-Esnault, Pansini & Uriz, 1993*; *Duran, Pascual & Turon, 2004*; *Blanquer, Uriz & Caujapé-Castells, 2009*; *Guardiola, Frotscher & Uriz, 2012*, *2016*), which favors speciation, while the

sponge plasticity fosters phenotypic (morphological) convergence to similar habitats (*Blanquer & Uriz, 2008*).

Many new cryptic sponge species have been discovered in the last decades thanks to the use of molecular markers (see *Uriz & Turon, 2012* for a review until 2012, *Knapp et al., 2011*; *de Paula et al., 2012*). However, less often, molecularly discovered new species have also been described morphologically (but see *Blanquer & Uriz, 2008*; *Cárdenas & Rapp, 2012*; *Reveillaud et al., 2011*, *2012*), which is necessary if phylogeny is aimed to translate into taxonomy, and the new species are wanted to be considered in ecological studies.

Sponge species can be both morphologically (e.g., *Uriz & Turon, 2012*) and, more rarely, molecularly (with the markers used) cryptic (*Carella et al., 2016*; *Vargas et al., 2016*) but show contrasting biological features. For instance, *Scopalina blanensis* (*Blanquer & Uriz, 2008*), which is sympatric with *Scopalina lophyropoda*, mainly grows in winter. Conversely, *Scopalina lophyropoda* regresses in winter and grows principally in summer–autumn (*Blanquer, Uriz & Agell, 2008*), thus indicating temporal niche partition.

The Order Poecilosclerida (Porifera: Demospongiae) harbors the highest number of species within the Class Demospongiae (Systema Porifera) and it is far from being resolved from a phylogenetic point of view (*Morrow et al., 2012*; *Thacker et al., 2013*). Within Poecilosclerida, the Family Hymedesmiidae represents a hotchpotch where genera of dubious adscription have been placed (*Van Soest, 2002*). As expected, this family appeared clearly polyphyletic in a molecular phylogeny of the so-called G4 clade based on 28S rRNA gene (*Morrow et al., 2012*).

Hymedesmiidae currently contains 10 accepted genera among which, *Hemimycale* Burton, 1934 (*Van Soest et al., 2016*). The position of genus *Hemimycale,* which shares with *Hymedesmia*, and *Phorbas* (Hymedesmiidae) and with *Crella* (Crellidae), the so-called aerolate areas with an inhaling function, has changed from Hymeniacidonidae in Halichondrida (*Lévi, 1973*) to Hymedesmiidae in Poecilosclerida (*Van Soest, 2002*). More recently, in 18S phylogenies of Poecilosclerida, *Hemimycale columella* was retrieved within the Crellidae clade, although with low support (*Redmond et al., 2013*).

Hemimycale harbors only four formally described species (*Van Soest et al., 2016*)*:* the type species *Hemimycale columella* (Bowerbank, 1874), from Northwestern Atlantic and Mediterranean, *Hemimycale rhodus* (Hentchel, 1929) from the North Sea, *Hemimycale arabica* Illan et al., 2004 from the Red Sea and *Hemimycale insularis* Moraes, 2011 from Brazil. However, the simple spicule complement of the genus, which only consists of strongyles with some occasional styles, may propitiate the existence of morphologically (based on the spicules) cryptic species.

Hemimycale columella, the type species of *Hemimycale,* is widely distributed across the Atlanto-Mediterranean basin, from shallow (ca. 10 m) to deep (ca. 60 m) waters (*Uriz, Rossell & Martín, 1992*). Assays performed with eight microsatellite loci developed from deep specimens of *Hemimycale columella* (*González-Ramos, Agell & Uriz, 2015*) failed to amplify a high percentage of the assayed individuals from a shallow population, which suggested larger genetic differences than those expected between intra-species sponge populations.

Furthermore, the life cycle of species has been monitored in a shallow Northwestern Mediterranean population of what was thought to be *Hemimycale columella* (*Pérez-Porro, González & Uriz, 2012*), where all individuals disappeared after larval release in early November and new individuals arose the forthcoming year but on different rocky sites, which pointed to annual mortality and subsequent recruitment from sexually produced propagula (settling larvae). Conversely, during a study of deeper populations of *Hemimycale columella* (*González-Ramos, Agell & Uriz, 2015*), we recorded their survival for more than three years. Thus, shallow and deep populations of *Hemimycale columella* seemed to show contrasting life spans, which were thought to be a result of contrasting habitat characteristics. However, a 2-year monitoring of two, some km apart, populations (one deep and one shallow) and the main environmental factors at both locations, confirmed their contrasting life span and growth traits, as well as proved no correlation between biological features and environmental factors (M. J. Uriz, L. Garate & G. Agell, 2013–2014, unpublished data), which rather pointed to population intrinsic (genetic) differences.

To assess whether these two population types with contrasting biological traits but without clearly distinct morphological characters belonged or not to different species, we performed a phylogenetic study of individuals considered as *Hemimycale columella* across the Mediterranean, using three molecular (nuclear and mitochondrial) gene partitions. We incorporated additional species to the analyses to gain knowledge on the relationships between *Hemimycale* species and other genera of families Hymedesmiidae and Crellidae.

MATERIALS AND METHODS

Sampling

Fragments of what a priori was thought to be *Hemimycale columella* were collected by SCUBA diving across the Northwestern, central and eastern Mediterranean, and Adriatic Sea, between 12 and 45 m of depth during several campaigns (Coconet, Benthomics, and MarSymbiOmics projects) (Table 1). Moreover, fragments of *Hemimycale arabica* and *Crella cyathophora* from the Red Sea (Dedalos and Ephistone) and Pacific (Bempton Islands) between 5 and 20 m depth were also collected (Table 1). Individuals were photographed underwater before sampling. Collected fragments were divided into two pieces, one of them was preserved in 100% ethanol, and after three alcohol changes, kept at −20 °C until DNA extraction; the other fragment was fixed in 5% formalin in seawater and preserved in 70% ethanol as a voucher for morphological and spicule studies. All vouchers have been deposited at the Sponge collection of the Centre d'Estudis Avançats de Blanes (numbers CEAB.POR.GEN.001 to CEAB.POR.GEN.029).

DNA extraction, amplification, and sequencing

DNA extractions were performed on two to three specimens per species and locality (totaling 18 individuals). *Hemimycale* spp. were extracted with QIAmp DNA stool kit (Qiagen), while *Crella* spp. were extracted with DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's protocol. Standard primers were used for COI partitions

Table 1 Geographical origin and ecological distribution of the individuals used in the phylogenetic study, with accession numbers.

Species	Sea/Ocean	Locality	Voucher numbers	Accession numbers
***Hemimycale arabica* ind. 1**	Red Sea	Dedalos, Brother Islands	CEAB.POR.GEN.001	COI: KY002124 18S: KY002171 28S: KY002181
***Hemimycale arabica* ind. 2**	Red Sea	Elphinstone, Brother Islands	CEAB.POR.GEN.002	COI: KY002125 18S: KY002172 28S: KY002182
Hemimycale columella	Northeastern Atlantic	Plymouth, Wales, UK		28S: HQ379300.1 18S: KC902127.1
***Hemimycale columella* ind. 1**	Northwestern Mediterranean	Arenys de Mar, Spain	CEAB.POR.GEN.003	28S: KY002183
***Hemimycale columella* ind. 2**	Northwestern Mediterranean	Arenys de Mar, Spain	CEAB.POR.GEN.004	28S: KY002184
***Hemimycale columella* ind. 3**	Northwestern Mediterranean	Arenys de Mar, Spain	CEAB.POR.GEN.005	COI: KY002126
***Hemimycale columella* ind.1**	Northwestern Mediterranean	Tossa de Mar, Spain	CEAB.POR.GEN.006	COI: KY002127 18S: KY002160 28S: KY002185
***Hemimycale columella* ind. 2**	Northwestern Mediterranean	Tossa de Mar, Spain	CEAB.POR.GEN.007	COI: KY002128 18S: KY002161 28S: KY002186
***Hemimycale columella* ind. 3**	Northwestern Mediterranean	Tossa de Mar, Spain	CEAB.POR.GEN.008	COI: KY002129 28S: KY002187
***Hemimycale columella* ind. 4**	Northwestern Mediterranean	Tossa de Mar, Spain	CEAB.POR.GEN.009	28S: KY002188
***Hemimycale mediterranea* sp. nov. ind. 1**	Northwestern Mediterranean	Tossa de Mar, Spain	CEAB.POR.GEN.010	COI: KY002130 18S: KY002162 28S: KY002189
***Hemimycale mediterranea* sp. nov. ind. 2**	Northwestern Mediterranean	Tossa de Mar, Spain	CEAB.POR.GEN.011	18S: KY002163 28S: KY002190
***Hemimycale mediterranea* sp. nov. ind. 4**	Northwestern Mediterranean	Tossa de Mar, Spain	CEAB.POR.GEN.012	COI: KY002131
***Hemimycale mediterranea* sp. nov. ind. 5**	Northwestern Mediterranean	Tossa de Mar, Spain	CEAB.POR.GEN.013	COI: KY002132
***H. mediterránea* sp. nov. ind. 3**	Adriatic Sea	Koznati, Croatia	CEAB.POR.GEN.014	COI: KY002134
***H. mediterránea* sp. nov. ind. 7**	Adriatic Sea	Koznati, Croatia	CEAB.POR.GEN.015	18S: KY002170 28S: KY002193
***H. mediterránea* sp. nov. ind. 8**	Adriatic Sea	Koznati, Croatia	CEAB.POR.GEN.016	28S: KY002194
***H. mediterránea* sp. nov. ind. 2**	Adriatic Sea	Tremity, Italy	CEAB.POR.GEN.017	COI: KY002133
***H. mediterránea* sp. nov. ind. 11**	Adriatic Sea	Tremity, Italy	CEAB.POR.GEN.018	28S: KY002199
***H. mediterránea* sp. nov. ind. 8**	Central Mediterranean	Porto Cesareo, Italy	CEAB.POR.GEN.019	18S: KY002164
***H. mediterránea* sp. nov. ind. 9**	Central Mediterranean	Porto Cesareo, Italy	CEAB.POR.GEN.020	18S: KY002165 28S: KY002197
***H. mediterránea* sp. nov. ind. 10**	Central Mediterranean	Porto Cesareo, Italy	CEAB.POR.GEN.021	28S: KY002198
***H. mediterránea* nov. sp. ind. 5**	Adriatic Sea	Karaburum, Albania	CEAB.POR.GEN.022	18S: KY002166 28S: KY002191
***H. mediterránea* nov. sp. ind. 6**	Adriatic Sea	Karaburum, Albania	CEAB.POR.GEN.023	18S: KY002167 28S: KY002192

(Continued)

Table 1 (continued).

Species	Sea/Ocean	Locality	Voucher numbers	Accession numbers
***H. mediterránea* sp. nov. ind. 3**	Eastern Mediterranean	Othonoi, Greece	CEAB.POR.GEN.024	18S: KY002168 28S: KY002195
***H. mediterránea* sp. nov. ind. 4**	Eastern Mediterranean	Othonoi, Greece	CEAB.POR.GEN.025	18S: KY002169 28S: KY002196
***Crella cyatophora* ind.1**	Red Sea	Dedalos, Brother Islands	CEAB.POR.GEN.026	COI: KY002120 18S: KY002173 28S: KY002177
***Crella cyatophora* ind. 2**	Red Sea	Elphinstone, Brother Islands	CEAB.POR.GEN.027	COI: KY002121 18S: KY002174 28S: KY002178
***Crella cyatophora* ind. 3**	Pacific	Bempton Patch Reef (beween New Caledonian and Australia)	CEAB.POR.GEN.028	COI: KY002122 18S: KY002175 28S: KY002179
***Crella cyatophora* ind. 4**	Pacific	Bempton Patch Reef (between New Caledonian and Australia)	CEAB.POR.GEN.029	COI: KY002123 18S: KY002176 28S: KY002180
Crella elegans	Mediterranean	France		18S: KC902282
Crella elegans	Mediterranean	France		18S: AY348882
Crella elegans	Mediterranean	France		28S: HQ393898
Crella plana	Northeastern Atlantic	Northern Ireland		18S: KC9023009
Crella rosea	Northeastern Atlantic	Northern Ireland		28S: HQ379299
Crella rosea	Northeastern Atlantic	Northern Ireland		18S: KC902282
Phorbas bihamiger	Northeastern Atlantic	English Channel		18S: KC901921.1 28S: KC869431
Phorbas punctatus	Northeastern Atlantic	Wales		18S: KC869439.1 28S: KC869439.1
Phorbas dives	Northeastern Atlantic	English Channel		28S: HQ379303
Phorbas fictitioides	North Pacific	–		COI: HE611617.1
Phorbas tenacior	Northeastern Atlantic	–		18S: AY348881
Phorbas glaberrimus	Antarctic	Ross Sea		COI: LN850216.1
Hymedesmia paupertas	Northeastern Atlantic			18S: KC902073.1 28S: KF018118.1
Hymedesmia pansa				18S: KC902027.1
Hymedesmia paupertas	Northeastern Atlantic			28S: KF018118.1
Kirkpatrickia variolosa	Antarctic	Ross Sea		COI: LN850202.1

Note:
Individuals sequenced de novo are in bold.

M1–M6 (*Folmer et al., 1994*) and 18S rRNA (1F and 1795R, from *Medlin et al., 1988*), and Porifera primers for the D3–D5 partition of 28S rRNA (Por28S–830F and Por28S–1520R, from *Morrow et al., 2012*). Different amplification protocols were performed for each gene (Table 2). COI (M1–M6 partition) amplifications were performed in a 50 μL volume reaction, containing 37.6 μL H_2O, 5 μL buffer KCl (BIORON; F Holzinger Sales & Support, Germany), 2 μL BSA, 2 μL dNTP (Sigma; Sigma_Aldrich, Germany), 1 μL of primers, 0.4 μL Taq (BIORON; F Holzinger Sales & Support, Germany), and 1 μL of genomic DNA. 18S rRNA amplifications were performed in a 50 μL volume reaction,

Table 2 PCR conditions for the three partitions used (COI, 28S and 18S).

PCR Stage	COI (M1–M6)	28S (D3–D5)	18S
Denaturalization	94 °C 2 min	94 °C 5 min	94 °C 5 min
	35 cycles	35–40 cycles	30 cycles
Denaturalization	94 °C 1 min	94 °C 1 min	94 °C 30 s
Annealing	43 °C 1 min	50–55 °C 1 min	53 °C 30 s
Elongation	72 °C 1 min	72 °C 1 min	72 °C 30 s
Final elongation	72 °C 5 min	72 °C 5 min	72 °C 5 min

containing 36.85 μL H_2O, 5 μL buffer (Invitrogen, Carlsbad, CA, USA), 0.75 μL MgCl (Invitrogen, Carlsbad, CA, USA), 1.2 μL DMSO (dimethyl sulfoxide), 1 μL BSA, 1.5 μL dNTP (Sigma; Sigma_Aldrich, Germany), 1 μL of primers, 0.7 μL Taq (Invitrogen, Carlsbad, CA, USA), and 1 μL of genomic DNA. Finally, partition D3–D5 of 28S rRNA amplifications were performed in a 50 μL volume reaction, containing 36.85 μL H_2O, 5 μL buffer (Invitrogen, Carlsbad, CA, USA), 0.75 μL MgCl (Invitrogen, Carlsbad, CA, USA), 2 μL BSA, 2 μL dNTP (Sigma; Sigma_Aldrich, Germany), 1 μL of primers, 0.4 μL Taq (Invitrogen, Carlsbad, CA, USA), and 1 μL of genomic DNA. Polymerase chain reaction products were purified and sequenced in both directions using Applied Biosystems 3730xl DNA analyzers in Macrogen, Korea.

Sequence alignment and phylogenetic reconstructions

Sequences of COI, 28S, and 18S were aligned using Clustal W v.1.81, once their poriferan origin was verified using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), as implemented in Genieous 9.01 (*Kearse et al., 2012*). When sequences were identical, only one sequence per locality and species was included in the phylogenetic trees. After alignment, ambiguous regions were determined with Gblocks v.091 b software (*Castresana, 2000*), which removes from 1 to 4% of poorly aligned positions and divergent regions of an alignment of DNA. Representatives of family Hymedesmiidae (i.e., genera *Phorbas* and *Hymedesmia*) and Crambeidae (i.e., genera *Crambe* and *Monanchora*) were selected as outgroups. The inclusion of Crambeidae as an outgroup was decided because the species *Hemimycale arabica* had been reported to contain similar secondary metabolites (polycyclic guanidine alkaloids) to those of *Crambe* and *Monanchora* (Ilan et al. 2004).

JModelTest 0.1.1 (*Posada, 2008*) was used to determine the best-fitting evolutionary model for each dataset. The model GTR + I + G was used for both mitochondrial and nuclear genes. Phylogenetic trees were constructed under neighbor joining (NJ) (default parameters), Bayesian inference (BI), and maximum likelihood (ML) using Geneious software 9.01 (*Kearse et al., 2012*). NJ generates unrooted minimum evolution trees (*Gascuel & Steel, 2006*). BI analyses were performed with MrBayes 3.2.1 (*Ronquist & Huelsenbeck, 2003*). Four Markov Chains were run with one million generations sampled every 1,000 generations. The chains converged significantly and the average standard deviation of split frequencies was less than 0.01 at the end of the run. Early tree generations were discarded by default (25%) until the probabilities reached a stable

plateau (burn-in) and the remaining trees were used to generate a 50% majority-rule consensus tree. ML analyses were executed with PhyML v3.0 program (*Guindon & Gascuel, 2003*; *Guindon et al., 2005*). The robustness of the tree clades was determined by a nonparametric bootstrap resampling with 1,000 replicates in PhyML. MrBayes and PhyML were downloaded by Genieous.

Incongruence length difference (ILD) test (PAUP 4.0b10) was run (*Swofford, 2002*) to verify sequence homogeneity or incongruence between the 18S rRNA and COI markers and the 18S and 28S rRNA markers. The ILD test indicated no significant conflict ($p = 0.93$ and $p = 0.91$, respectively) between the marker pairs to be concatenated. Thus, concatenated 18S COI and 18S–28S rRNA datasets were constructed for the species with sequences available for both markers. The phylogeny on the three genes concatenated was not performed due to the few species/individuals for which the three genes were available.

Phenotypic characters

To assess whether molecular differences among the target populations and species (*Hemimycale columella, Senso latus*, *Hemimycale arabica,* and *C. cyathophora*) were supported by morphological and spicule traits, the target species were observed both in situ and on recently collected samples. Moreover, spicules of all the species were observed through light and scanning electron microscopes (SEM) after removing the sponge organic matter from small (3 mm^3) pieces of each individual by boiling them in 85% Nitric acid in a Pyrex tube and then washed three times with distilled water and dehydrated with ethanol 96% (three changes). A drop of a spicule suspension in ethanol per individual was placed on 5 mm diameter stuffs, air dry, and gold–palladium metalized (*Uriz, Turon & Mariani, 2008*) in a Sputtering Quorum Q150RS. Observation was performed through a Hitachi M-3000 Scanning Electron Microscope at the Centre d'Estudis Avançats de Blanes.

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can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix "http://www.mycobank.org/mb/283905". The online version of this work is archived and available from the following digital repositories: PeerJ, PubMed Central, and CLOCKSS.

RESULTS

18S rRNA phylogeny

The resulting phylogeny using the 18S rRNA partition on 25 sequences (17 new) of 695 nt. (46 variable positions, from which 38 were parsimony informative) was congruent under BI, and ML and just differed in the position of *Hemimycale arabica* which appeared as a sister group of the remaining *Crella* spp. and *Hemimycale* spp. under NJ (Fig. S1). The representatives of the family Crambeidae (*Monanchora*) appeared as outgroups and the genus *Phorbas* was a sister group of the remaining species. In the BI, NJ, and ML trees, the genera *Hemimycale* and *Crella* appeared polyphyletic, with the Red Sea species *Hemimycale arabica* and *C. cyathophora*, far away from the Atlanto-Mediterranean *Hemimycale* and *Crella* species. The Atlanto-Mediterranean *Crella* formed a well-supported clade (1/81/98, posterior probability/bootstrapping values), which was the sister group of the Atlanto-Mediterranean *Hemimycale* (1/97/98). Moreover, the deep *Hemimycale columella* clustered with an Atlantic sequence downloaded from the GenBank (0.89/89/88) forming a separate clade from the also well-supported (1/97/98) group containing the shallow Mediterranean *Hemimycale*. No genetic differences for this partition were found among shallow individuals. In the BI and ML trees, the two individuals of *Hemimycale arabica* appeared in unresolved positions while they formed a poorly supported (75%) clade in the tree under the NJ criterion (not shown).

28S rRNA (D3–D5) phylogeny

The 28S rRNA (D3–D5) dataset comprised 31 sequences (24 new) of 623 nt. (84 variable positions from which, 60 parsimony informative).

The resulting phylogenies were congruent with the three clustering criteria and matched in most cases the phylogeny based on the 18S rRNA partition, although the supporting values of some clades were in some cases slightly lower (Fig. S2).

The three phylogenies retrieved the representatives of Family Crambeidae (*Monanchora* and *Crambe*) as an outgroup. The monophyly of the in-group containing *Crella* spp. and *Hemimycale* spp. was strongly supported under the BI, NJ, and ML criteria (1/100/100). The genus *Phorbas* was a sister group of the remaining species considered. *Crella* was polyphyletic, with *C. cyathophora* separated from the well-supported clade (1/100/100) encompassing the Atlanto-Mediterranean *Crella*. The latter appeared as a sister clade of a poorly supported group (0.7/77/70) harboring *C. cyathophora and Hemimycale spp.* The *Hemimycale* spp. group, although monophyletic, was poorly supported under the NJ and ML criteria (77/70) while the Atlanto-Mediterranean *Hemimycale* clade was well supported under the three clustering criteria (1/92/95).

The deep and shallow Mediterranean populations of *Hemimycale* formed two well-supported monophyletic groups (0.96/87/83 and 0.96/ 100/98, for deep and shallow

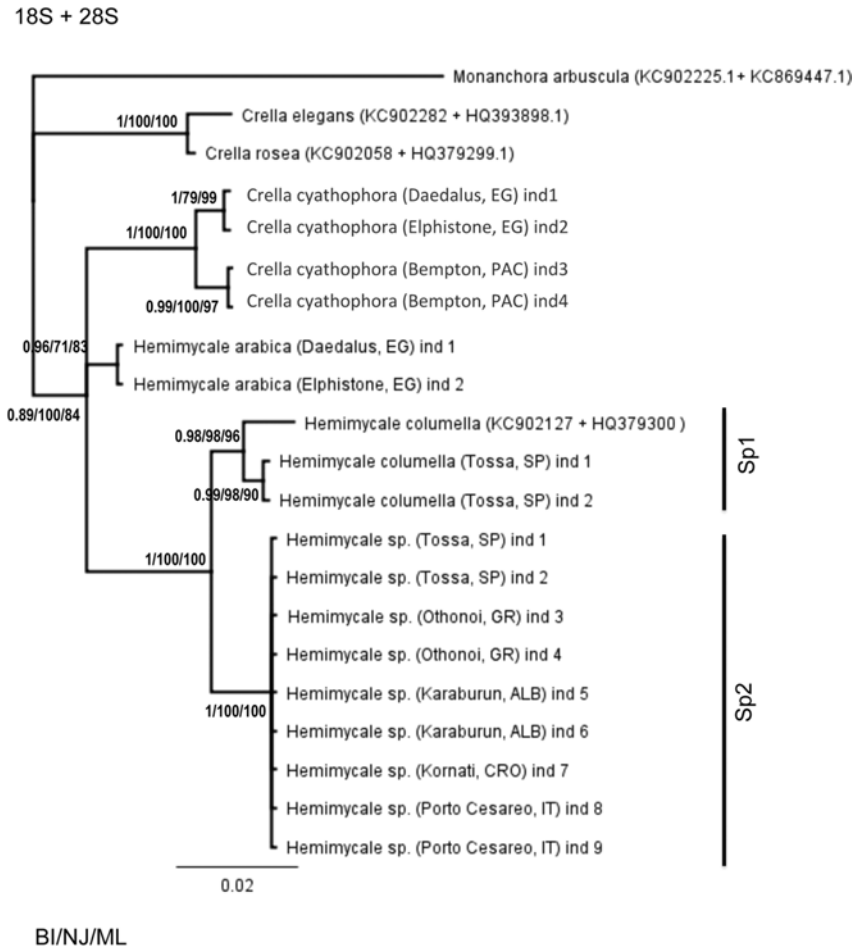

Figure 1 Phylogenetic tree using concatenated (18S rRNA + COI) partitions. BI, NJ and ML gave the same topologies. Posterior probability, neighbor joining, and maximum likelihood supporting values are at the base of clades.

individuals, respectively), the former containing the Atlantic sequence of *Hemimycale columella*. No genetic differences for this partition were retrieved for shallow individuals despite their spread distribution across the Mediterranean. The individuals of *C. cyathophora* from the Red Sea clustered with those from the Pacific collected between Australia and Nouvelle Caledonie (1/89/76).

COI phylogeny

The COI dataset included 21 sequences (15 new) of 535 nt. (169 variable positions, from which 149 parsimony informative).

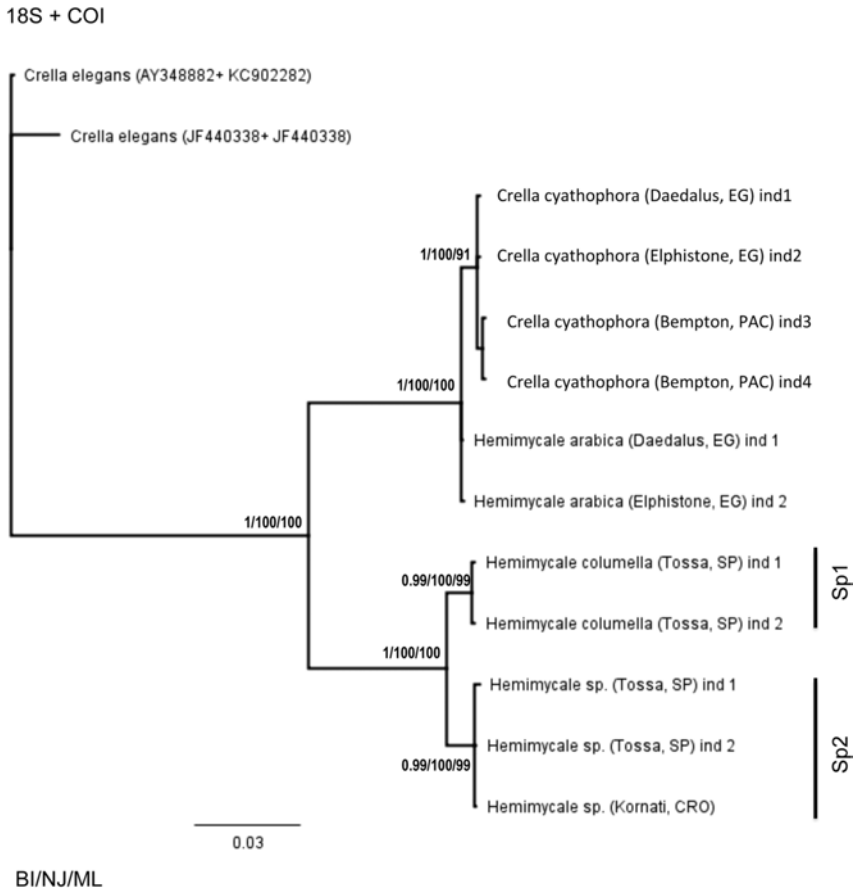

Figure 2 Phylogenetic tree using concatenated (18S + 28S rRNA) partitions. BI, NJ and ML gave the same topologies. Posterior probability, neighbor joining, and maximum likelihood supporting values are at the base of clades.

The COI phylogeny, which was congruent under BI, NJ, and ML, also retrieved the representatives of Crambeidae as outgroups of the group formed by *Crella, Phorbas,* and *Hemimycale*. The genus *Phorbas* clustered with the Atlanto-Mediterranean *Crella* spp. (0.98/100/86) likely because we only included one individual/species of *Phorbas* (Fig. S3).

A clade containing *Hemimycale* spp. and *C. cyathophora* was well supported (0.94/94/80). The *Hemimycale* clade was divided into two subclades corresponding to deep and shallow individuals. No genetic differences among shallow individuals were found. A sister, well supported group (1/100/94) contained *C. cyathophora* and *Hemimycale arabica* representatives with almost no genetic differences between them (Fig. S3).

Concatenated trees

The concatenated 18S + 28S rRNA (Fig. 1) confirmed the outgroup position for the Crambeidae representative (*Monanchora*), the polyphyly of *Crella* with the Red Sea and Pacific species forming a separate clade (1/100/100) from the Atlanto-Mediterran *Crella*, which appeared in a non-resolved position. *Hemimycale* also appeared polyphyletic, but the position of *Hemimycale arabica* was unresolved. The Atlanto-Mediterranean *Hemimycale* clade was confirmed as well as its division into two subclades: one containing the deep Mediterranean individuals together with two Atlantic sequences of the species and the other one harboring the shallow Mediterranean individuals, which did not show any genetic difference across the Mediterranean and Adriatic Sea.

The concatenated 18S rRNA + COI (Fig. 2) tree contained only 13 sequences and no representative of Crambeidae could be included. The representatives of the Atlanto-Mediterranean *Crella* appeared as outgroups of the remaining target species, which formed two well-supported clades: one containing *C. cyathophora* and *Hemimycale arabica* representatives (1/100/100) and the other with the Atlanto-Mediterranean *Hemimycale* (1/100/100) divided into two monophyletic well-supported groups (deep and shallow individuals).

DISCUSSION

The phylogenetic reconstructions performed with 18S, 28S rRNA and COI, as well as with concatenated genes (18S rRNA + COI and 18S + 28S rRNA) support the polyphyly of *Crella* and *Hemimycale*, under the three clustering criteria used. As although *Hemimycale* was monophyletic with the 28S rRNA (D3–D5) marker, the clade was not statistically supported.

Crella cyathophora sequences differ from those of the Atlanto-Mediterranean *Crella* spp. in 2% (18S rRNA), 2.19% (28S rRNA), and 10.24% (COI). These genetic distances suggest that, despite some spicule similitude (presence of acanthoxeas and smooth diactines with Atlanto-Mediterranean *Crella* spp.), the former species belongs in a different genus, closer to *Hemimycale arabica* (0.71% with 18S rRNA, 1.37% with 28S rRNA, and none with COI) than to the Atlanto-Mediterranean *Crella* spp.

Hemimycale arabica differs from the Atlanto-Mediterranean *Hemimycale* spp. in 1.43–1.86% with 18S rRNA, 1.78–2.19 with 28S rRNA, and in 8.38–8.64% with COI. These strong COI differences and the contrasting morphological traits (blue external color, irregular, rim-free, aerolate areas and abundance of true styles in *Hemimycale arabica* vs. orange–pinkish color, circular, rimmed aerolate areas, and slightly asymmetrical any strongyles almost exclusively in *Hemimycale* spp.) also indicate that *Hemimycale arabica* would belong in a different genus, which might also include *C. cyathophora*, as there are not COI differences between these two species.

Moreover, the Atlanto-Mediterranean Crellidae appeared in 18S and 28S rRNA phylogenies as a sister group of the Atlanto-Mediterranean *Hemimycale*, which suggests higher affinities of this genus with Crellidae than with Hymedesmiidae (its current family). However, more complete analyses including additional Crellidae and Hymedesmiidae OUT's are needed to move *Hemimycale* from Hymedesmiidae to Crellidae.

The phylogenetic trees with any of the three gene partitions either separately or concatenated confirm the presence of two cryptic *Hemimycale* species in the Mediterranean within what was considered until now *Hemimycale columella*. The new species that we name *Hemimycale mediterranean* sp. nov. (see description below) has a shallower distribution across the whole Mediterranean than *Hemimycale columella*, which has Atlantic affinities. *Hemimycale columella* differs from *Hemimycale mediterranea* in 0.85% (18S rRNA), 1.23% (28S rRNA), and in 1–1.2% (COI).

The lack of genetic diversity among the distant populations of *Hemimycale mediterranea* analyzed points to its recent presence in the Mediterranean, which is compatible with a recent introduction. However, the new species has not been recorded out of the Mediterranean, and thus, its origin cannot be established at the present time.

Many cryptic species that were revealed by molecular markers have never been formally described owing to the difficulty of finding diagnostic phenotypic characters. Although after exhaustive observation, only slight, morphological differences have been found to differentiate *Hemimycale mediterranea* sp. nov. from *Hemimycale columella* (see species description below), these phenotypic differences are consistent across individuals and thus, add to molecular differences and biological traits (L. Garate et al., 2013–2014, unpublished data) to consistently differentiate these two species.

Species description

Genus *Hemimycale* Burton, 1934

Sequence accession Numbers GenBank (Table 1)

Type species *Hemimycale columella* (Bowerbank, 1874)

Hemimycale is the only genus of Hymedesmiidae that has smooth diactines and monactines exclusively (*Van Soest, 2002*). The genus was described by Burton (1934) as "reduced Mycaleae with skeleton of loose fibers of styli, sometimes modified into anisostrongyles, running vertically to the surface; fibers tending to branch and anastomose; no special dermal skeleton, no microscleres."

The spicule complement described by Burton; however, seems different from that reported in the several modern redescriptions of *Hemimycale columella* (*Vacelet, Donadey & Froget, 1987*), which report predominant straight anisostrongyles with rare or absent styles. Indeed, Burton stated that the Bowerbank representation of *Hemimycale columella* spicules was wrong because it figured anisostrongyles instead of styles, and was precisely the dominance of styles what induced Burton to place the species among the *Mycaleae*. The termination of the diactines either round or pointed ends may be the result of different silica concentration in the water masses, as reported for other siliceous sponge skeletons (*Uriz, 2006*), but it cannot be totally discarded that the Burton *Hemimycale columella* belonged in another *Hemimycale* species.

Species: *Hemimycale columella* (Bowerbank, 1874)

Sequence accession numbers GenBank (Table 1)

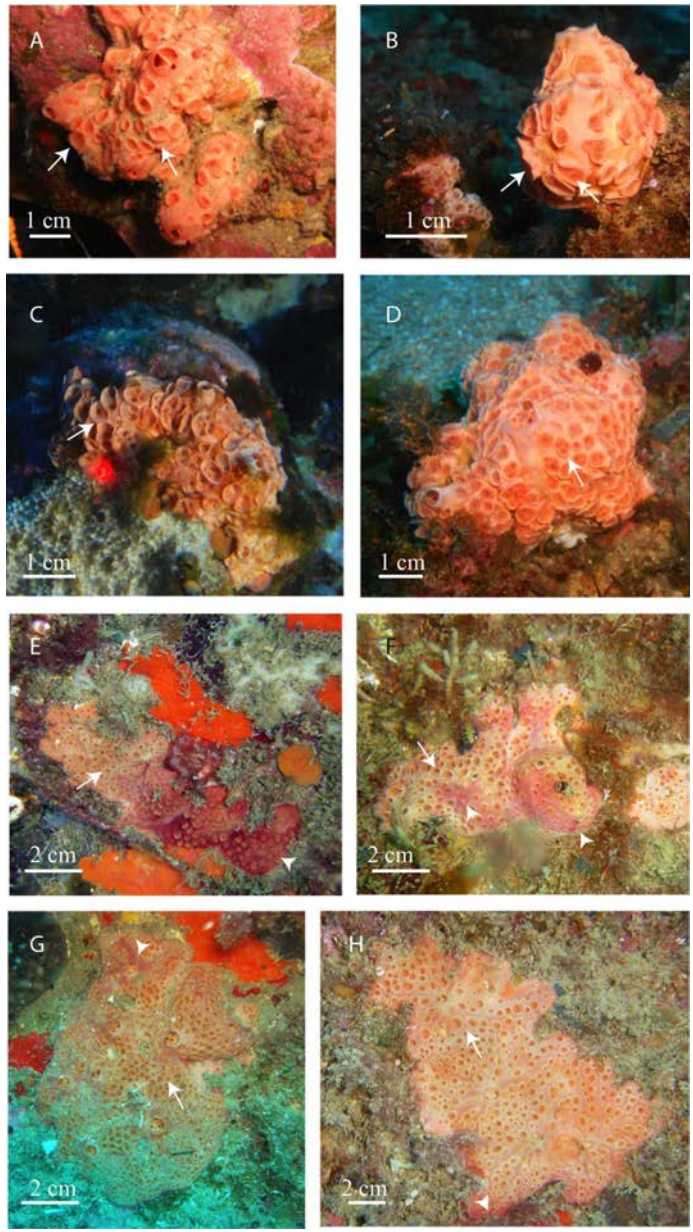

Figure 3 In situ pictures of Atlanto-Mediterranean *Hemimycale* spp. (A, B, C, D) *Hemimycale columella* from 35 to 40 m of depth. (E, F, G, H) *Hemimycale mediterranea* sp. nov. from 12 to 17 m of depth. Whitish tinge is due to calcibacteria accumulation. Red tinges are due to several species of epibiotic cyanobacteria. Arrows point to aerolate inhaling areas; arrowheads indicate the epibiont cyanophycea on *Hemimycale mediterranea* specimens.

Table 3 Locality and spicule sizes of the studied individuals, and comparison with descriptions by other authors.

Species	Author	Locality	Depth (m)/ Assemblage	Styles	Strongyles (range/mean)	Acanthoxeas
Hemimycale arabica ind. 1	This study	Red Sea (Egypt)	14/coral reef	160–189 (179.6) × 7–8 (7.5)	210–290 (273) × 2.8–4.1 (3.6)	–
Hemimycale arabica	Illan et al. 2004	Red Sea (Egypt)		190–250 (218) × 3.5–5 (4.7)	200–290 (266) × 2.5–4 (3.5)	–
H. mediterránea ind. 7	This study	Adriatic (Croatia)	10–15/rocky sub-horizontal	–	233–330 (274.8) × 3–4.6 (4.0)	–
H. mediterránea ind. 11	This study	Adriatic (Italy)	10–15/rocky sub-horizontal	–	251–300 (276.6) × 2.1–4 (3.0)	–
H. mediterránea ind. 5	This study	Adriatic (Albania)	10–15/rocky sub-horizontal	–	274–317 (296.4) × 2.9–4.5 (4.0)–	–
H. mediterránea ind. 10	This study	Central Med. (Italy)	10–15/rocky sub-horizontal	–	229–328 (291.3) × 2.4–5.2 (3.5)	–
H. mediterránea ind. 3	This study	Eastern Med. (Greece)	10–15/rocky sub-horizontal	–	242–340 (272.7) × 2.6–4 (3.2)	–
H. mediterránea ind. 1	This study	NW Med. (Spain)	12–16/rocky wall	–	261–320 (296.3) × 3.1–3.8 (3.5)	–
Hemimycale columella ind. 1	This study	NW Med. (Spain)	27–29/ coralligenous	–	302–435 (370) × 3–4 (3.7)	–
"*Hemimycale columella*"	Vacelet 1987	NW Med. (France)	–	–	225–310 (285) × 2–4 (3)	–
Hemimycale columella	Vacelet 1987	NW Med. (France)	–	–	320–410 (369) × 2.5–3.8 (3.1)	–
"*Hemimycale columella*"	Vacelet 1987	NW Med. (France)	–	–	220–320 (273) × 2–4 (2,7)	–
Hemimycale columella	Vacelet 1987	North Atlantic (France)	–	–	290–465 (394) × 4–7 (5.1)	–
Hemimycale columella	Topsent 1925	North Atlantic (France)	–	–	400 × 6	–
"*Hemimycale columella*"	Foster 1995	North Atlantic (UK)	–	–	330–420 (373) × 5–6 (5.85)	–
Hemimycale columella	Bowerbank 1874	North Atlantic (UK)	–	–	376 × 7	–
Crella cyatophora ind. 3	This study	Indo-Pacific (Bemptom)	18m/coral reef	–	205–308 (263.9) × 2.2–4.3 (3.4)	92–115 (105.4) × 2–2.3 (2)
C. cyatophora ind. 1	This study	Red Sea (Egypt)	12/coral reef	–	227–293 (267.8) × 2.5–3.9 (3.4)	89–120 (109.4) × 1.8–2.5(2.47)

Description (Figs. 3A–3D)**:** Encrusting to massive sponges. Surface smooth, covered with circular inhaling, areas up to 6 mm in diameter with an up to 3 mm high rim. Morbid and fleshy consistence. Translucent to whitish ectosome, difficult to separate from the choanosome. Thousands of calcareous spherules, 1 μm in diameter formed by intracellular calcifying bacteria (*Uriz et al., 2012*) are spread through the sponge mesohyl and specially accumulated at the sponge periphery of whitish individuals (L. Garate et al., 2013–2014, unpublished data).

Color from pinkish-orange to whitish outside, dark orange inside.

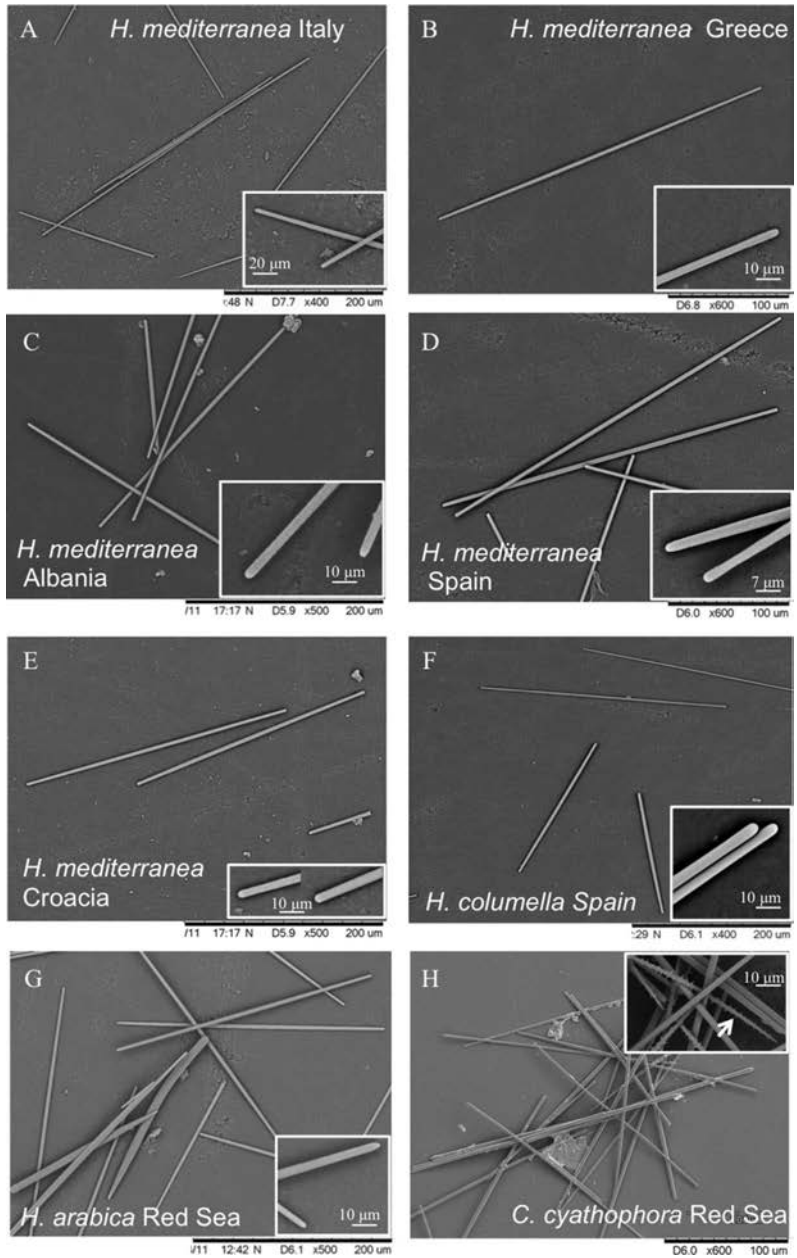

Figure 4 Spicules of *Hemimycale* spp. and *Crella cyathophora* though SEM. (A, B, C, D, E) Anysostrongyles (*Hemimycale mediterranea*). (F) Anisostrongyles (Hemimycale columella). (G) Anisostrongyles and one style (*Hemimycale arabica*). (H) Anysotrongyles and acantoxeas (*Crella cyatophora*). Inserts on each picture correspond to magnifications of the spicule ends.

Spicules (Table 3; Fig. 4F): Asymmetric strongyles (anysotrongyles), straight, 302–435 × 3–4 μm in size. Styles rare or completely absent from the Mediterranean specimens (this study) and Canary Islands (*Cruz, 2002*).

Skeletal arrangement: Plumose branching bundles of anysostrongyles together with spread spicules. A palisade of vertical anysotrongyles forms the rim around the inhaling areas.

Distribution: Northeastern Atlantic (United Kingdom and Ireland coasts) Canarias Islands (*Cruz, 2002*), western Mediterranean: Tossa de Mar, Arenys de Mar, from 28 to 60 m depth (this study). It is not possible to confirm whether previous Mediterranean records of the species (see *Vacelet & Donadey, 1977*) belonged to *Hemimycale columella* or to *Hemimycale mediterranea.*

Biology: Multiannual life span, ca. 60% survival after two monitoring years; maximum growth in autumn–winter (L. Garate et al., 2013–2014, unpublished data). Larval release occurs at the beginning of November in Mediterranean populations (M. J. Uriz, L. Garate & G. Agell, 2013–2014, unpublished data).

Species: *Hemimycale mediterranea* sp. nov. (Figs. 3E–3H)

Sequence accession numbers GenBank (Table 1)

Description: Thick encrusting sponges with aerolate inhaling areas up to 3 mm in diameter, surrounded by an up to 1.5–2 mm high rim, which in some cases barely surpasses the sponge surface. Thousands of calcareous spherules, 1 μm in diameter formed by intracellular calcifying bacteria are spread through the sponge mesohyl and specially accumulated at the sponge periphery (*Garate et al., in press*).

Ectosome: Firmly attached to the choanosome.

Color: Flesh to clear brownish externally, more or less whitish depending on calcibacteria accumulation at the surface, sometimes partially covered by an epibiotic (reddish or pinkish) cyanobacteria.

Spicules (Table 3; Figs. 4A–4E): Smooth, uniform in size, straight, anysostrongyles, 200–296 × 3–4 μm in size. Styles completely absent.

Skeletal arrangement: Plumose undulating bundles of anysostrongyles together with spread spicules. A palisade of vertical anysotrongyles forms the rim around the inhaling areas.

Known distribution: Northwestern Mediterranean, central Mediterranean, Adriatic, eastern Mediterranean (Spain: Cap De Creus, Tossa, Blanes, Arenys, South Italy: Croatia, Tremiti, Turkey, Greece) between 3 and 17 m deep.

Biology: Annual life span, maximum growth rates in summer (M. J. Uriz, L. Garate & G. Agell, 2013–2014, unpublished data). Larval release at the end of September beginning of October (M. J. Uriz, L. Garate & G. Agell, 2013–2014, unpublished data).

In most cases, it is difficult to ascertain whether individuals of *Hemimycale columella* recorded by other authors belong to *Hemimycale columella* or *Hemimycale mediterranea.* The redescription of *Hemimycale columella* by *Van Soest (2002)* based on the holotype

(from the Atlantic) reported large aerolate porefields with elevated rims, which are shared with the deep Mediterranean specimens of *Hemimycale columella* (Figs. 3A–3D) in contrast to the small, short-rimmed porefields showed by *Hemimycale mediterranea* sp. nov. Both species have mainly straight slightly asymmetric strongyles but the spicule sizes are systematically larger in *Hemimycale columella* (Table 3). However, while styles were rarely present in *Hemimycale columella* individuals, they have not been found in specimens of *Hemimycale mediterranea* sp. nov. The external color also differs between the two species, being orange to pinkish in *Hemimycale columella* and flesh color to brownish *Hemimycale mediterranea* sp. nov. (Figs. 3E–3H). *Vacelet & Donadey (1977)* reported two different color forms occurring side by side on the littoral of Provence (France), one pink cream and the other one brownish. Likely the second color morph, which besides had smaller strongyles, corresponded to the *Hemimycale mediterranea* sp. nov.

Color has not received much attention as a diagnostic character in sponges because it has been generally considered to be a response to higher or lower light irradiance at the sponge habitat, or to the presence of epibiotic or symbiotic cyanobacteria. However, color has proven to be taxonomically relevant to distinguish other invertebrates such as shrimp species (*Knowlton & Mills, 1992*) and also sponge species of the genus *Scopalina* (*Blanquer & Uriz, 2008*), and thus it seems worthy to be taken into account in sponge taxonomy.

The slight phenotypic differences found between the two species appear; however, consistent across individuals and localities within the Atlanto-Mediterranean basin. Moreover, their ecological distribution and bacterial symbionts, strongly differentiate these two cryptic species. For instance, although calcareous spherules produced by intracellular bacteria are present in the two species, the producer bacteria belong in different species (*Garate et al., in press*), and the respective microbial communities totally differ (*Garate et al., in press*). Symbionts, as predators do (e.g., *Wulff, 2006*), often distinguish their target sponge preys or hosts while the species remain morphologically cryptic to taxonomists. Moreover, *Hemimycale mediterranea* sp. nov. shows an annual life span, with individuals disappearing after larval release, while *Hemimycale columella* has a multiannual life span (M. J. Uriz, L. Garate & G. Agell, 2013–2014, unpublished data) and growth dynamics also differs between the two species, as *Hemimycale mediterranea* sp. nov. grows more in summer, while *Hemimycale columella* grows preferentially in autumn–winter (M. J. Uriz, L. Garate & G. Agell, 2013–2014, unpublished data).

The contrasting ecological distribution of these two cryptic species in the Mediterranean helps to predict their identity in the field. *Hemimycale mediterranean* sp. nov. inhabits shallower zones than *Hemimycale columella*. However, it is likely that both species may share occasionally habitats, as the record of the two color morphs side by side (*Vacelet & Donadey, 1977*) indicate. *Hemimycale mediterranea* sp. nov. seems to be more abundant and widespread in the Mediterranean than *Hemimycale columella*. Molecular differences between groups of individuals of *Hemimycale columella* suggest the possible presence of additional cryptic species among the deep Mediterranean *Hemimycale*.

The presence of two morphologically cryptic *Hemimycale* species in the Mediterranean, which show contrasting biological traits, reinforces the idea that cryptic species represent something more than wrong taxonomic identifications or biodiversity underestimates. They may feature contrasting biological cycles and life spans, and puzzle biological studies, which may invalidate conservation policies based on those studies.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Maria J. Uriz conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, and reviewed drafts of the paper.
- Leire Garate conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, and reviewed drafts of the paper.
- Gemma Agell performed the experiments, analyzed the data.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The 18 S, 28 S and COI sequences described here are accessible at GenBank https://www.ncbi.nlm.nih.gov/genbank/.

Accession numbers: KY002124, KY002171, KY002181, KY002125, KY002172, KY002182, KY002183, KY002184, KY002126.

COI: KY002127.

18S: KY002160, KY002185, KY002128, KY002161, KY002186, KY002129, KY002187, KY002188, KY002130, KY002162, KY002189, KY002163, KY002190, KY002131, KY002132, KY002134, KY002170, KY002193, KY002194, KY002133, KY002199, KY002164, KY002165, KY002197, KY002198, KY002166, KY002191, KY002167, KY002192, KY002168, KY002195, KY002169, KY002196, KY002120, KY002173, KY002177, KY002121, KY002174, KY002178, KY002122, KY002175, KY002179, KY002123, KY002176, KY002180.

Data Deposition

The following information was supplied regarding data availability:

CEAB Porifera Collection.

Vouchers: CEAB.POR.GEN.001, CEAB.POR.GEN.002, CEAB.POR.GEN.003, CEAB.POR.GEN.004, CEAB.POR.GEN.005, CEAB.POR.GEN.006, CEAB.POR.GEN.007, CEAB.POR.GEN.008, CEAB.POR.GEN.009, CEAB.POR.GEN.010, CEAB.POR.GEN.011, CEAB.POR.GEN.012, CEAB.POR.GEN.013, CEAB.POR.GEN.014, CEAB.POR.GEN.015, CEAB.POR.GEN.016, CEAB.POR.GEN.017, CEAB.POR.GEN.018, CEAB.POR.GEN.019, CEAB.POR.GEN.020, CEAB.POR.GEN.021, CEAB.POR.GEN.022, CEAB.POR.GEN.023, CEAB.POR.GEN.024, CEAB.POR.GEN.025, CEAB.POR.GEN.026, CEAB.POR.GEN.027, CEAB.POR.GEN.028, CEAB.POR.GEN.029.

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Endosymbiotic calcifying bacteria across sponge species and oceans

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From an evolutionary point of view, sponges are ideal targets to study marine symbioses as they are the most ancient living metazoans and harbour highly diverse microbial communities. A recently discovered association between the sponge *Hemimycale columella* and an intracellular bacterium that generates large amounts of calcite spherules has prompted speculation on the possible role of intracellular bacteria in the evolution of the skeleton in early animals. To gain insight into this purportedly ancestral symbiosis, we investigated the presence of symbiotic bacteria in Mediterranean and Caribbean sponges. We found four new calcibacteria OTUs belonging to the SAR116 in two orders (Poecilosclerida and Clionaida) and three families of Demospongiae, two additional OTUs in cnidarians and one more in seawater (at 98.5% similarity). Using a calcibacteria targeted probe and CARD-FISH, we also found calcibacteria in Spirophorida and Suberitida and proved that the calcifying bacteria accumulated at the sponge periphery, forming a skeletal cortex, analogous to that of siliceous microscleres in other demosponges. Bacteria-mediated skeletonization is spread in a range of phylogenetically distant species and thus the purported implication of bacteria in skeleton formation and evolution of early animals gains relevance.

Symbiosis, whereby different biological species live together in intimate, long-term interactions, is regarded as a major source of evolutionary innovation[1]. Symbiotic associations of animals and microbes are widespread in marine ecosystems and play key ecological roles by contributing in an important, and often cryptic, way to the ecosystem biodiversity and stability. For example, shifts in symbiotic bacterial communities have been attributed to recurrent mass mortalities of corals and sponges[2–5].

From an evolutionary point of view, sponges are ideal targets to study marine microbial symbioses, as they are the most ancient living metazoans on Earth and harbour highly diverse microbial communities[6]. The establishment of symbiotic relationships between sponges and prokaryotes has been traced back to the pre-Cambrian period[7]. However, despite the abundance of available sequences from sponge-associated bacteria and their long history, the evolutionary origins of these associations and the adaptive traits of the species involved are only beginning to be understood[8–11].

Recently, a symbiotic association between the Atlanto-Mediterranean sponge *Hemimycale columella* (Bowerbank, 1874) and an unidentified calcifying bacterium, has been shown to produce thousands of calcite spherules[12]. The calcifying bacteria are contained within vacuoles in amoeboid, archaeocyte-like cells[13], or 'calcibacteriocytes', where they divide by bipartition before becoming enclosed within a 100-nm thick calcite envelope. The calcifying bacterium is vertically transmitted to the sponge progeny[12] via phagocytosis of maternal calcibacteriocytes as embryos grow[14]. Uriz *et al.*[12] speculated that this type of eukaryote-prokaryote symbiosis might represent a relict mechanism involved in the evolution of skeletons in Lower Metazoa. Later, Blanquer *et al.*[15] used pyrosequencing to retrieve a dominant alpha-proteobacterium in *H. columella* that represented up to 67% of the total sequences in the sponge and was similar to that obtained from the sponge *Cliona viridis* (Schmidt, 1862).

To gain insight into this newly identified symbiosis and its possible role in the evolution of skeletons in Lower Metazoa, we investigated whether the dominant alpha-proteobacteria reported by Blanquer *et al.*[15] corresponded to the extremely abundant calcifying bacteria that form calcareous spherules in the sponge tissues[12]. We also explored the molecular diversity of the symbiotic calcibacteria, and their presence in other sponge species, to assess the occurrence of this symbiosis across sponges and oceans. Moreover, we explored whether the calcibacterium of *H. columella* is evenly distributed within the sponge tissues or accumulate in particular sponge zones, thus, fulfilling a potential skeletal function, and analyse the purported costs and benefits for the symbiotic partners.

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To achieve these objectives, we performed ultrastructure studies using transmission electron microscopy (TEM) and scanning electron microscopy (SEM), and designed a molecular probe based on the dominant bacterial species of *H. columella*. We also conducted CARD-FISH experiments and cloned the 16 S rRNA gene of the calcibacterium from *H. columella* and *C. viridis* to examine their phylogenetic relationships.

Results

Light microscope observations. Morphologically similar calcareous spherules, 1 μm in size, were observed in the sponges *Hemimycale columella, Cliona viridis*, *Prosuberites* sp., *Crella cyathophora* (Carter, 1869) (Fig. 1), and *Cinachyrella alloclada* (Uliczka, 1929) (not shown). Profuse numbers of spherules were released from the squeezed tissues of all the sponge species examined, except in *C. viridis* and *C. alloclada*, where they were less abundant.

The calcareous spherules were concentrated at the periphery of whitish individuals of *H. columella* (Fig. 2a,b). Moving sponge cells (calcibacteriocytes) full of calcified calcibacteria were recorded immediately after sponge disaggregation (Fig. 2c), which confirmed the amoeboid-like properties of these cells and their capacity to transport calcibacteria across the sponge tissues.

Ultrastructure. SEM images of *H. columella* (whitish morph) showed huge numbers of calcareous spherules that had been released into the sponge mesohyl (Fig. 2b), as well as abundant, 10–15 μm calcibacteria-full calcibacteriocytes (Fig. 2d). The calcareous spherules were accumulated at the sponge periphery (Fig. 2b) forming a 2–3 mm thick layer, which concurred with light microscope observations (Fig. 2a). Some calcibacteriocytes showed hemispherical, ca. 1 μm in diameter holes, which corresponded to the space previously occupied by released calcibacteria (Fig. 2d). There were frequent images of calcibacteria enclosed within the calcareous coat as they divided (Fig. 3c).

TEM pictures of calcibacteriocytes showed abundant cellular vacuoles containing single or dividing calcibacteria (Fig. 3a). Most vacuoles contained a single bacterium, but several bacteria were also observed enclosed within a common calcareous coat after successive incomplete divisions (Fig. 3c). Calcified calcibacteria degraded in most cases, as indicated by the scarce or absent organic content within the calcareous crusts, but also formed condensed bodies, ca. 200 nm in size, that might correspond to starved forms (Fig. 3b). Uncalcified calcibacteria were spherical and relatively small (ca. 0.2–0.8 μm in cross-section), showed a thin bacterial wall and were found abundantly in the mesohyl of sponge larvae, after being released from engulfed maternal cells (Fig. 3f). There, they divided profusely by bipartition (Fig. 3g). Archaeocyte-like embryo cells (newly differentiated calcibacteriocytes) contained calcified calcibacteria (Fig. 3e). The calcareous crust that surrounded calcibacteria was clearly visible in SEM images of samples that were fixed for just 2 h (see methods) (Fig. 3h).

Calcibacteria-specific probe. Table 1 shows the best 18 nt. long probe that targeted the prevalent alpha-proteobacteria sequence in *H. columella* (CAL32), as well as the 5′–3′ helpers, and competitors.

The non-sense probe, which was used as a negative control, did not hybridize in the samples (images not shown), and no hybridization occurred when the specific probe was assayed in the species *Crambe crambe* (Schmidt, 1862) (negative sponge control, images not shown), which confirmed that the hybridization signal observed was not an artefact and corresponded to the target calcibacteria.

Catalysed reporter deposition fluorescence *in-situ* hybridization (CARD-FISH). CARD-FISH using the designed probe (CAL32L), helpers, and competitors allowed us to detect the presence of the target *Ca.* Calcibacterium at high densities within the tissues of *H. columella,* (adults and larvae) and *Prosuberites* sp. and at lower densities in *C. alloclada* and *C. viridis* (Fig. 4). There were numerous hybridization points in the sponge mesohyl and within sponge cells (calcibacteriocytes) (Fig. 4g,h,i). Similarly, isolated calcibacteria from *H. columella* tissue on filters also hybridized in high numbers (Fig. 4a,b). This confirmed that the bacteria, which form the calcareous spherules within calcibacteriocytes, corresponded to the dominant alpha-proteobacteria sequence of *H. columella*.

The DAPI staining showed two discrete cell sizes, corresponding to DNA material from sponge cells and calcibacteria. Strong hybridization signals that overlapped with the majority of the DAPI-stained small points were observed with the CAL32L specific probe (Fig. 4a,b) in both tissue sections and extracted calcified calcibacteria, which proved the correspondence of the target bacteria with the calcareous spherules and the abundance of calcibacteria in the sponge tissues and cells. Negative controls, including the non-sense probe, a confirmed calcibacteria-free sponge, and a non-probe control yielded no hybridization signals confirming the precision of the hybridization and a lack of endogenous peroxidases in the sponge tissue. However, a few other non-hybridized bacteria were also DAPI stained in the sponge tissues. Moreover, although targeting cyanobacteria CARD-FISH was not performed, sporadic red/orange fluorescence was observed under green excitation (Fig. 4), indicating the occasional presence of photosynthetic bacteria in the sponge tissues.

Two-way ANOVA with colour morph and sponge zone as orthogonal factors (5 replicates per zone and colour morph) showed significant differences ($N = 5$, $F = 12.14$, $p < 0.05$) in the number of calcibacteria extracted from *H. columella* tissues and recorded through epifluorescence microscopy (EM) (Fig. 5a). However, the interaction between colour morph and sponge zone was also significant ($p < 0.05$), so that the abundance of calcibacteria in the two colour morphs depended on the sponge zone considered. T-tests on colour morph and sponge zone separately showed that whitish morphs had significantly higher numbers of calcibacteria (i.e. hybridized points) in the ectosomal region than pinkish morphs (t-value $= -3.98$, $p < 0.001$), whereas there were no differences in calcibacteria density between the choanosomes of both colour-morphs (t-value $= 0.52$, $p = 0.67$). Confocal microscopy of sponge sections confirmed the significant differences in calcibacteria density in the extracted spherules observed using EM ($N = 3$ each colour, $Z = -1.96$, $p < 0.05$, Mann-Whitney test) (Fig. 5b). Together, these results

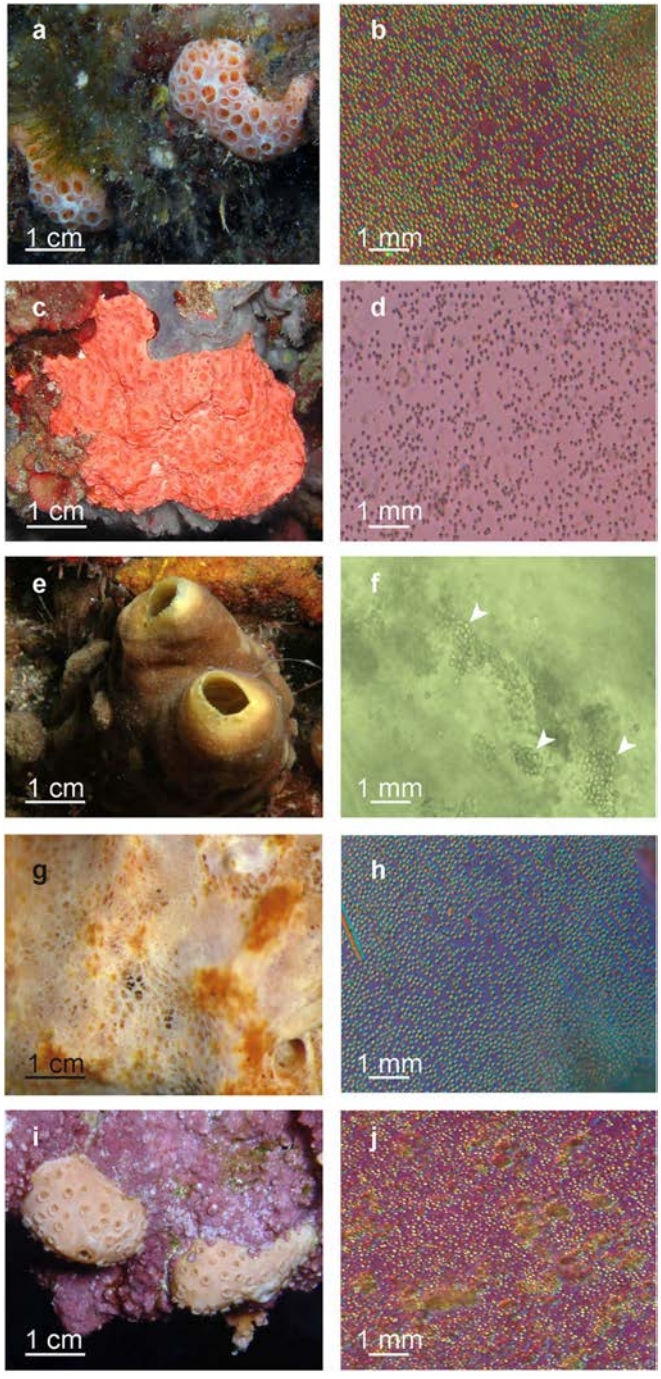

Figure 1. Living target sponge species and their calcareous spherules after fresh tissue squeeze. (**a**,**b**) Whitish morph of *Hemimycale columella*. (**c**,**d**) Pinkish morph of *H. columella*. (**e**,**f**) *Cliona viridis*. (**g**,**h**) *Prosuberites* sp. (**i**,**j**) *Crella cyathophora*.

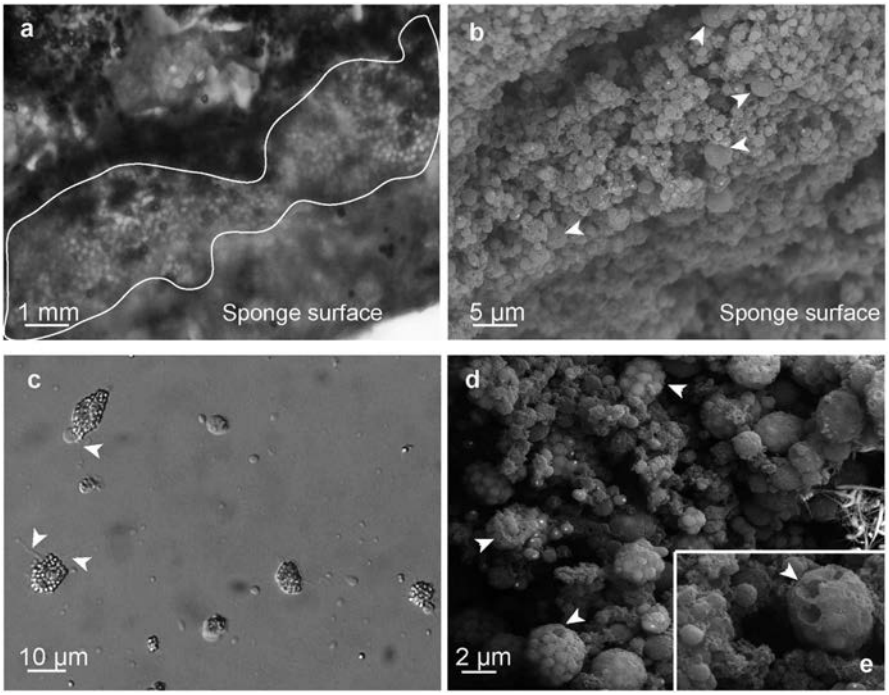

Figure 2. Calcibacteria and calcibacteriocytes from *Hemimycale columella* observed using light and SE microscopes. (**a**) Light microscope and (**b**) SEM pictures of calcareous spherules accumulated at the sponge periphery forming a kind of cortex. A few sponge non-granulose cells (arrowheads) are shown (**b**) among the dense layer of calcareous spherules. (**c**) Calcibacteriocytes showing pseudopodia and phyllopodia (arrowheads) while creeping across a solid surface (light microscopy). (**d**) Calcibacteriocytes full of calcified calcibacteria. (**e**) Close view of calcibacteriocytes (SEM). Hemispherical holes (arrowheads) correspond to the space previously occupied by calcibacteria.

confirmed that calcified calcibacteria accumulated in large numbers at the sponge ectosome, conferring a whitish tinge to some individuals of *H. columella*, and that bacterial DNA remained within the calcareous spherules for an undetermined time after bacteria became calcified.

Larvae contained lower numbers of calcibacteria than adult tissue as non-reproductive individuals showed higher fluorescence values per tissue unit ($Z = 7.32$, $p < 0.001$, Mann-Whitney test) than sponges harbouring abundant larvae (Fig. 5c). The presence of larvae in reproductive individuals decreased the total integrated fluorescence of the sponge sections.

Calcibacteria phylogeny. Cloning 16 S rRNA gene of the bacterial symbionts present in *H. columella* and *C. viridis* allowed us to recover two sequences, ca. 1,400 nt. long, that contained the 273 nt. calcibacterium fragment.

Bayesian phylogenetic reconstruction using the cloned sequences, plus their closest neighbouring sequences in the 16 S SILVA database and several outgroup sequences, retrieved a well-supported calcibacteria clade (1, posterior probability) that clustered bacteria from sponges harbouring calcareous spherules similar to those of *H. columella* along with sequences from two corals and two tropical seawater bacteria (Fig. 6). The calcibacteria clade contained 17 sequences, belonging to seven distinct calcibacteria OTUs with >98.5% intra-OTU similarity, which would correspond to seven calcibacteria species according to Kim *et al.*[16]. The calcifying bacterium of *C. cyathophora* differed from our cloned sequences of *C. viridis* and *H. columella*, in >5% and likely belonged to a different genus[17].

According to the Bayesian phylogeny, the calcibacteria clade belonged to SAR116 (Alpha-proteobacteria), which appeared as a sister clade of some Rhodospirillales. It was split in two well supported subclades (1, posterior probability): subclade A contained the clone of *H. columella* (GenBank ID:KU985279), and that of the Alcyonacea coral *Erythropodium caribaeorum* (Duchassaing & Michelotti, 1860), and another clade with two bacterial sequences from tropical marine waters; subclade B clustered the *C. viridis* clone (GenBank ID: KU985280) with a clone from the Alcyonacea coral *Scleronephthya gracillimum* (Kükenthal, 1906) (previously *Alcyonium gracillimum*) from the North Pacific. This subclade was a sister clade of the *C. cyathophora* clone from the Indo-Pacific.

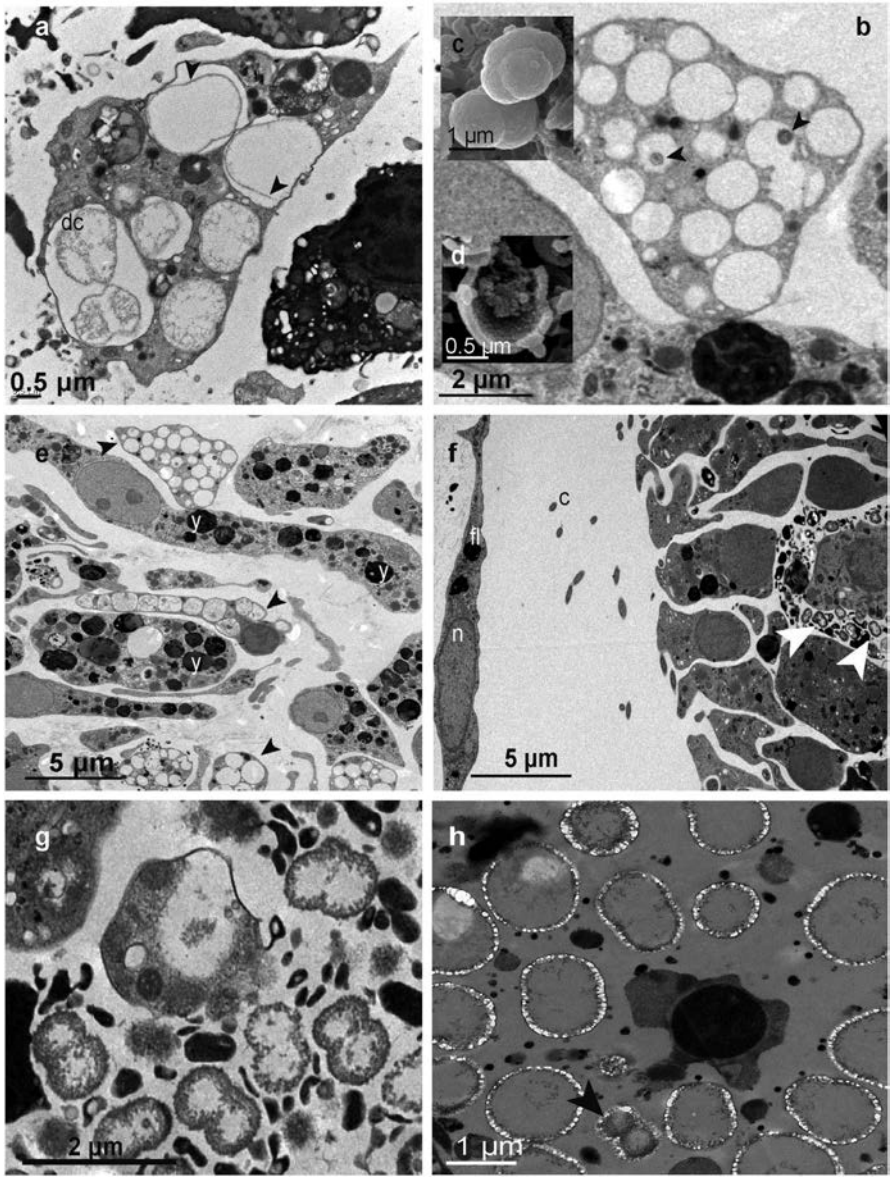

Figure 3. Ultrastructure images of *Hemimycale columella* tissues from adults and larvae. (**a**) Calcibacteria in adult tissue while dividing (dc) within a calcibacteriocyte (TEM). (**b**) Calcibacteriocyte showing the empty vacuoles previously occupied by calcified calcibacteria: arrowheads point to condensed bacterial remains (TEM). (**c**) SEM images of calcified calcibacteria undergoing division. (**d**) SEM image of a broken calcified calcibacterium showing a 100 nm thick calcareous crust and reduced organic matter inside. (**e**) Larval inner cells with yolk reserves (y) and larval calcibacteriocytes (arrowheads) full of remains of calcified calcibacteria (shown in the image as light to electron vacuoles with scarce organic material) (TEM). (**f**) Section of a larva within the follicle showing a calcibacteriocyte of maternal origin surrounded by non-calcified calcibacteria (arrowheads) released into the larval mesohyl: fl, follicular cell; (**c**) cilia of the larval peripheral cells; n, nucleus (TEM). (**g**) Free calcibacteria within the larva mesohyl showing profuse cell division by bipartition (TEM). (**h**) Pictures of free calcibacteria undergoing calcification in the sponge mesohyl (TEM). Calcification in the form of nanospherules can be observed. Arrowhead points to a calcibacterium enclosed within the calcareous crust while dividing.

Step	Description
1. Sample fixation	1. Incubate sponge samples within 4% paraformaldehyde solution for 4 h 2. Incubate samples in ethanol 70% during 18–24 h at 4 °C 3. Keep samples in ethanol 70% at −20 °C
2. Sample dehydration	Sequential incubation of sponge samples in ethanol 96% and 100%, ethanol:toluene (1:1) 30 min each, and absolute toluene 15 min
3. Embedding in paraffin and tissue sectioning	1. Include samples in paraffin at 50 °C for 24–48 h 2. Cut thick (6 μm) sections with an Autocut 2030 (Reichert-Jung) microtome and dry for 3 h at 40 °C
4. Deparaffinization	1. Incubate sections within Xylene for 10 min 2. Rehydratation by sequential incubation in ethanol 100%, 96%, 70%, 10 min each 3. Three baths in MiliQ water 5 min each, air dry
5. Membrane permeabilization	1. Incubate sections in 10 mg/ml Lysozime solution (Sigma USA), 0.05 M EDTA, 0.1 M Tris-HCl for 1 h at 37 °C 2. Wash with MiliQ water for 2 min, air dry
6. Endogenous peroxidases inactivation	1. Incubate sections in 0.1 M HCl solution for 30–60 sec 2. Wash with 1X PBS for 2 min 3. Incubate in 3% H2O2 solution for 10 min 4. Wash with MiliQ water and ethanol 96% 2 min each, air dry
7. Hybridization	1. Cover sections with hybridization buffer (1) solution together with the probe, helpers and competitors in a 3:100 volumetric ratio. Sequences (5′–3′) are:
	CAL32L: CCCCTCTATCTGCGGCGG
	Competitor I: CCCCTCTTTCTCCGGCGG
	Competitor II: CCCCTCATTCTGCGGCGG
	Helper 3′: YACAAGCTAATCGGACGCGGG
	Helper 5′: YACAAGCTAATCGGACGCGGG
	2. Incubate at 46 °C for 5 h with a solution of 45% formamide in humid chambers.
	3. Wash the sections with pre-warmed washing buffer (2) at 48 °C for 10 min
8. CARD	1. Wash sections in 1X PBS (pH = 8) for 15 min
	2. Cover sections with primary CARD substrate mix (3)
	3. Incubate at 46 °C for 20 min
	4. Wash twice in 1X PBS for 6 min at 46 °C and RT
	5. Wash twice in MiliQ water for 2 min each and air dry.

Table 1. Different steps of the followed CARD-FISH protocol. (1) The hybridization buffer was made by mixing 5 M NaCl, 1 M Tris-HCl (pH7.5), 20% sodium dodecyl sulfate (SDS), 10% (w/v) dextran sulfate, 10% Blocking Reagent and 45% formamide (Sigma). (2) Fresh whashing buffer was prepared by mixing 0.5 M EDTA, 1 M Tris-HCl, NaCl and 20% SDS in steryle miliQ water and warmed at 48 °C previously to the wash step. (3) The primary CARD substrate was made by mixing amplification buffer (10% (w/v) dextran sulfate, 2 M NaCl, 0.1% (w/v) blocking reagent, in 1X PBS (pH = 8)) with a freshly prepared H2O2 solution (0.15% in 1X PBS) at a ratio of 100:1. The needed volume of that primary CARD mix solution was mixed with 1 mg dye ml-1 tiramide-Alexa488 solution (Molecular Probes, Inc., Eugene, OR, USA) at a ratio of 500:5.

Discussion

Ultrastructure images combined with molecular analyses have significantly improved our understanding of the symbiosis first discovered between *Hemimycale columella* sponges and calcibacteria. The specific probe designed enabled us to confirm the identity of the endosymbiont calcifying bacteria, which was surrounded by a calcareous crust, to quantify their abundance within the sponge tissues of *H. columella*, and to prove their accumulation at the sponge periphery, which suggest an exoskeleton function. A thin bacterial wall was made evident through TEM in healthy bacteria, which contradicts the lack of bacterial wall suggested by Uriz *et al.*[12]. The phylogenetic reconstruction of near-complete 16 S sequences from cloned *H. columella* and *Cliona viridis* classified the calcibacteria within the SAR116, close to one of the three Rhodospirillales clades[18].

Altogether, the results from CARD-FISH and bacterial phylogeny indicate a temperate-warm geographical distribution of this symbiosis, which comprises at least seven bacterial OTUs and two potential genera, and appears to include demosponges and cnidarian hosts. The inclusion of two bacteria sequences from tropical waters within the calcibacteria clade suggests the presence of calcibacteria stocks in the water from which sponges and cnidarians might acquire them. Indeed, the symbiosis may be propagated by two mechanisms: vertical transmission from maternal tissues to the progeny, as evident in our study, and horizontal transmission from the environment, as suggested by the presence of free calcibacteria in seawater. Redundant mechanisms for assuring a relevant biological function are frequent in nature[19] and two acquisition modes of symbiotic microbes have also been reported for other sponges[20].

In marine environments, calcium carbonates and calcium phosphates are the most commonly precipitated minerals and have formed most invertebrate skeletons since the Cambrian explosion[21]. Particular metabolisms of both autotrophic and heterotrophic bacteria are known to induce mineralisation[22–29]. However, only a few cases of calcium precipitation mediated by endosymbiotic microorganisms have been reported so far in marine

eukaryotes: calcification is facilitated by symbiotic microalgae (*Phaeocystis*) in the radiolarian *Acantharia*[30], and by bacteria in the Foraminifera[31].

Increased pH, which may result from bacterial metabolism, could promote calcium precipitation. Significant increases in pH have been recorded during the growth of *Escherichia coli*[32], and an increased pH of at least one unit above seawater pH fostered calcification in Foraminifera vacuoles, even at high Mg2 + and low Ca2 + concentrations and low temperature[33].

In our target symbiosis, calcification occurred within sponge cell vacuoles. We propose that the vacuole microenvironment changed over the course of bacterial growth, as nutrients are removed from the medium and bacteria expel waste products into the medium (Fig. 7). As a consequence, the pH of the vacuole may increase, and calcium carbonate nucleation and precipitation on the bacteria membrane is biologically induced[34]. A similar process of calcification has been observed in experimental studies of *Chromohalobacter marismortui*[28].

In general, sponge skeletons are either siliceous or calcareous[35]. Only a few relict sponges (the sclerosponges), which formed sponge reefs during the late Palaeozoic and Mesozoic eras[36], have a double mineralisation system. Some parallels can be drawn between sclerosponges and the sponge species in this study. Both have siliceous spicules and a complementary calcareous skeleton layer. In both cases, spherules (spherulites in sclerosponges) are produced within the vacuoles of sponge cells (sclerocytes in sclerosponges or calcibacteriocytes in sponges in this study), which are then excreted and accumulate at the sponge periphery. However, some differences between the calcareous bodies produced by the two sponge types should be noted. In the sclerosponge *Astrosclera willeyana* Lister, 1900, calcareous bodies are solid, and 5 μm in diameter, and become cemented forming a mass skeleton at the sponge base[37], whereas those of the sponges in this study are hollow and 1 μm in size, and remain free forming a cortical layer analogous to the siliceous, microsclere-constructed cortex of some Astrophorida demosponges[35].

Studies on the formation of intracellular spherulites in the sclerosponge *A. willeyana* also report parallel traits with the bacteria-mediated calcareous spherules. Jackson *et al.*[38] proposed that sponge genes of bacterial origin promoted calcification. Later, Jackson and Wörheide[39] suggested that sponge cells use the remains of intracellular bacteria as a framework on which to initiate calcification. In both cases, the bacterial wall may act as a nucleation centre for the precipitation of calcium carbonate. Calcification, resulting in either 5 μm spherulites or 1 μm spherules, appears to be caused by the particular conditions (e. g. host enzymes or increased pH) in the sponge vacuoles, as it occurs within cell vacuoles.

It has been reported that many symbiotic microorganisms do not grow unconstrained in hosts[40]. Conversely, it is generally accepted that for mutualistic symbioses to become evolutionaryly fixed, benefits at the species level should compensate for the costs to the associated partners[41]. The most obvious benefit to sponges from their association with calcifying bacteria is the 'low cost' construction of an exoskeleton, which may serve as structural purpose and deter potential sponge predators better than secondary metabolites[42]. Protection against an increase in predators has been proposed as an evolutionary driver of exoskeletons in ancient animals during the Cambrian explosion[43]. By assuming that calcium precipitation around the bacteria is spontaneously triggered by increases in pH within the vacuole, the only cost of formation of the sponge exoskeleton would be the transport of calcified calcibacteria to the sponge peripheral zone.

However, the sponge mechanism of particle capture and transport may have not evolved primarily for bacteria. Calcibacteriocytes do not differ significantly from archaeocytes, which are moving cells that are genetically programmed to remove debris and undesired substances from the sponge mesohyl[14]. Archaeocyte-like cells pack bioactive metabolites in the form of spherules to prevent sponge self-toxicity[44,45]. These spherule-containing cells, or spherulous cells from their ultrastructural aspects[46], have been observed to migrate to the sponge surface where toxins are released to the sponge boundaries to function in deterrent and/or allelochemical roles[44].

In contrast, the benefits for calcibacteria are more difficult to ascertain. It has been experimentally demonstrated that some symbiotic microorganisms have an increased reproductive capacity and higher fitness within hosts relative to non-host environments[47]. Sponge tissues might offer protection from pathogens and predators, which are abundant in non-host environments[40], and buffer nutrient ocean fluctuations that prevent the steady growth of bacteria over long periods[48]. However, considering the detrimental consequence of calcification for the bacteria, calcibacteria may also be more akin to 'prisoners' or 'farmed crops' than equal partners as in other bacteria-invertebrate symbioses[40]. Benefits might therefore be related to the propagation of the species. Symbiosis ensures that calcibacteria persist across sponge generations via the vertical transmission to sponge progeny. Moreover, the presence of free calcibacteria in seawater also suggests that viable calcibacteria are released back into the environment, which would allow the bacteria to form a species reservoir to facilitate dispersal and colonization of new invertebrate hosts.

Symbiotic relationships have been observed to be mostly stable over the lifetime of an individual host, from generation to generation, and over evolutionary time[49]. Thus, mechanisms must have evolved to correct potential deviations from the necessary holobiont homeostasis. In the calcibacteria-sponge association, the host should predominantly maintain proliferation of the dominant bacteria to a level compatible with host survival. Calcification appears to be the cost to bacteria for living in a more stable, predator-free, nutrient-rich environment. Once calcification prevents metabolic exchange between the bacteria and the vacuole medium, the calcibacteria may degrade or become starved. However, according to our TEM images, calcified calcibacteria recovered a steady growth phase as calcibacteriocytes broke and released purported resistant forms into the nutrient-rich mesohyl of sponge larvae. Then larval archaeocyte-like cells may engulf and transport them into cell vacuoles where calcification occurred (Fig. 7). This interpretation is based on *H. columella* observations but it can be safety extrapolated to other calcibacteria-bearing sponge species, as the cellular types involved in the process are similar in all of them (images not shown).

The current-day animal-bacteria symbioses, which likely existed when animals first appeared[50], can provide key insights into Metazoa evolution. The reporting of bacteria-mediated calcification mechanisms in

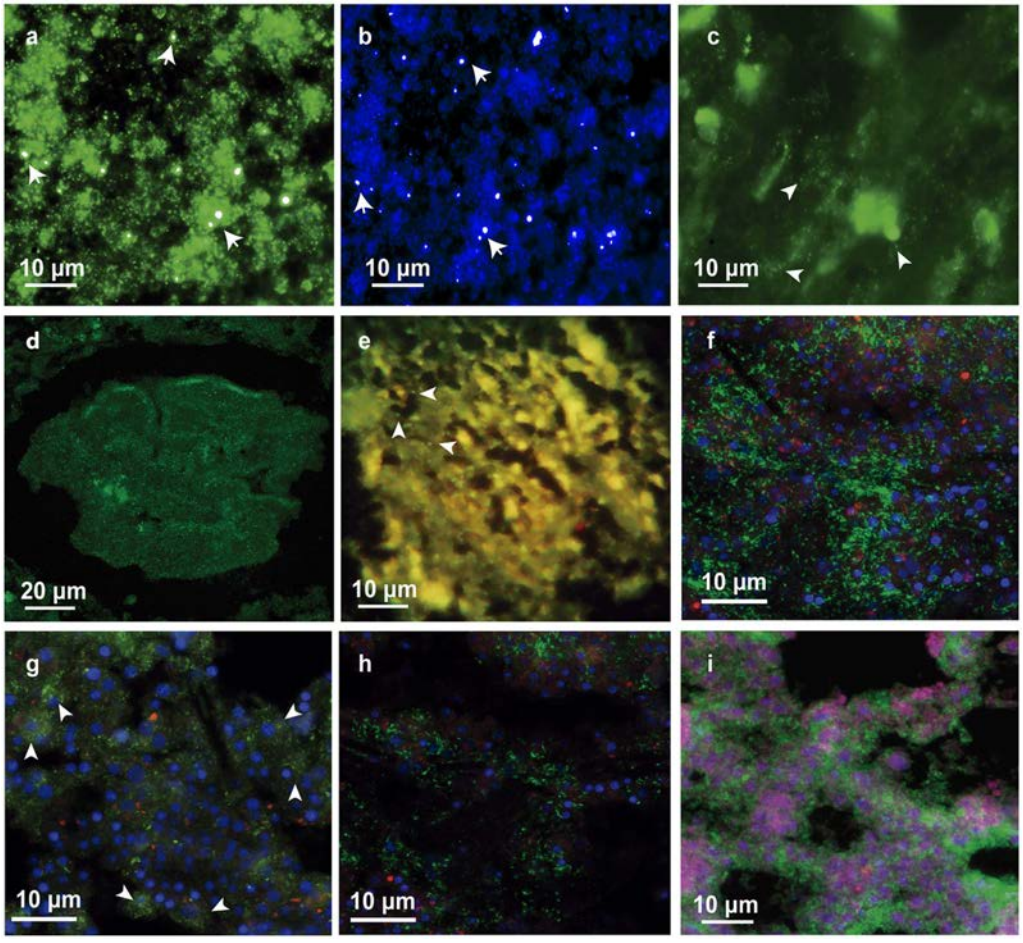

Figure 4. Study species hybridized with the CAL32L probe targeting the calcibacterium. (**a**,**b**) Filters containing isolated calcibacteria from *Hemimycale columella* (EM). (**c**) Filters containing isolated calcibacteria from *Cliona viridis* (EM). (**d**) *H. columella* choanosome harbouring larvae (CM). (**e**) Tissue section of *Cinachyrella alloclada* (EM). (**f**) Ectosome of *H. columella* whitish morph (CM); note that most calcibacteria are released into the sponge mesohyl forming dense aggregates. (**g**) Choanosome of *H. columella* whitish morph (CM); note that most calcibacteria are contained within calcibacteriocytes, surrounding the cell nucleus (arrowheads). (**h**) Ectosome section of *H. columella*, pinkish morph (CM). (**i**) Hybridized ectosome section of *Protosuberites* sp. (CM). Blue colour corresponds to sponge nuclei and bacteria nucleoid; green colour represents hybridization points; reddish colour results from self-fluorescence of cyanobacteria and pinkish colour results from overlapping DAPI blue-stained nuclei and reddish self-fluorescence of cyanobacteria. EM, Epifluorescence Microscopy; CM, Confocal Microscopy.

phylogenetically apart sponges suggests the implication of bacteria in the early evolution of the skeleton in the pre-Cambrian metazoans[51]. Although several molecular mechanisms are responsible for calcium precipitation and skeleton formation in Lower Metazoa[52], those involving bacteria might be evolutionarily older and, thus, acquire new relevance in the light of these results. Paleogenomics approaches may help in the near future to confirm the presence of calcifying bacteria in early animals.

Materials and Methods

Sampling, sample preservation, and treatment. The sponge species studied all harboured 1 μm diameter calcareous spherules, similar to those reported in *Hemimycale columella*[12] (Fig. 1). From one to three samples per species (depending on the species availability) were collected by SCUBA diving between 10 and 30 m depth in several seas: *H. columella* (whitish and pinkish morphs) and *Cliona viridis* from the Northwestern Mediterranean

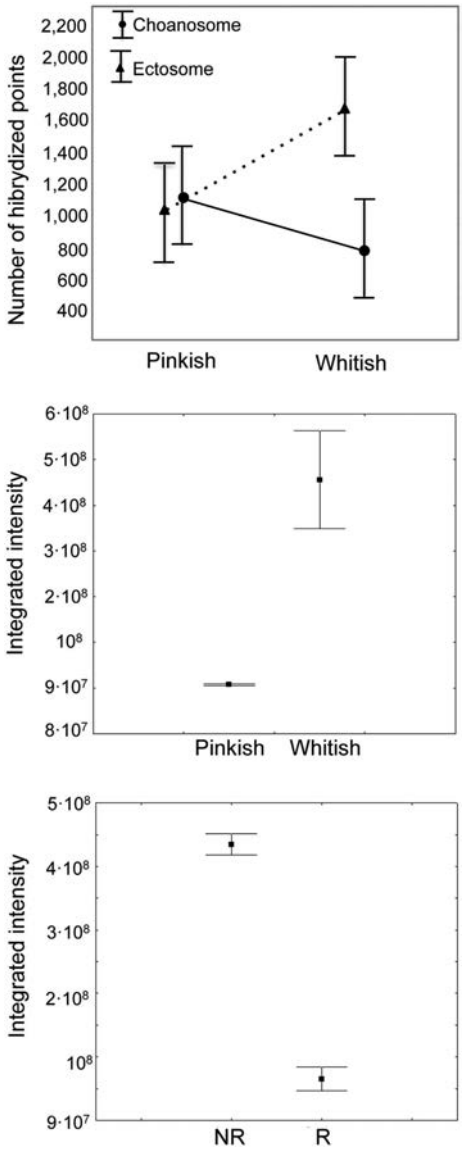

Figure 5. Average abundance of calcibacteria in *Hemimycale columella*. (**a**) Direct hybridization of calcibacteria extracted from the sponge tissue and quantified using epifluorescence microscopy. Vertical bars represented ± 95% confidence intervals. (**b**) Integrated intensity of the hybridization signal in the two colour morphs. Vertical bars represent ± standard errors. (**c**) Integrated intensity of the hybridization signal in two lifecycle stages (NR, non-reproductive; R, reproductive). Vertical bars represent ± standard errors.

(Arenys de Mar, Spain); *Protosuberites* sp., and *Cinachyrella alloclada* from the Caribbean Sea (Florida, USA), and *Crella cyatophora* Carter from the Red Sea (Sharm el-Sheikh, Egypt).

Samples were preserved and treated according to the study purposes:

(i) For light microscopy, 50 mm^3 samples were fixed in 4% paraformaldehyde, embedded in paraffin, and cut with an Autocat 2030 microtome (Reichert-Jung) to obtain 5 μm thick sections.
(ii) For Scanning Electron Microscopy (SEM), ca. 4 mm^3 sponge samples were fixed, critical point dried, and coated with gold-palladium[53]. Samples were observed using a Hitachi SEM at the Institute of Marine Sciences (ICM-CSIC).

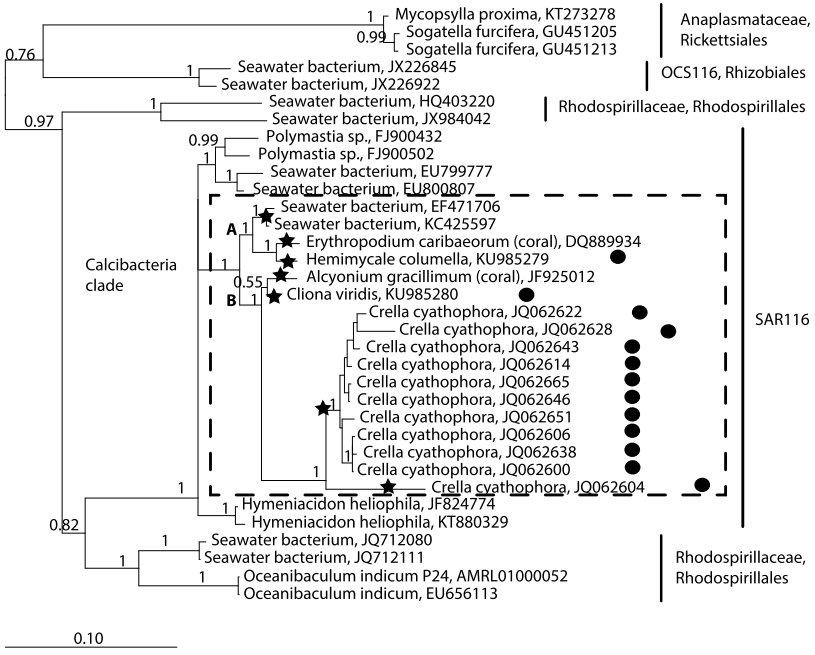

Figure 6. Bayesian phylogeny of the 16S rRNA clones of calcibacteria from *Cliona viridis* and *Hemimycale columella*, and their closest sequences from the SILVA database. Sequence sources are included. The calcibacteria clade is shown within the dashed frame. Posterior probability values are indicated at each node. Bullets on the right indicate presence of ca. 1 μm calcareous spherules (the presence of spherules in cnidarians has not been explored). Seven calcibacteria OTU's at >98.5% similarity (stars), are identified.

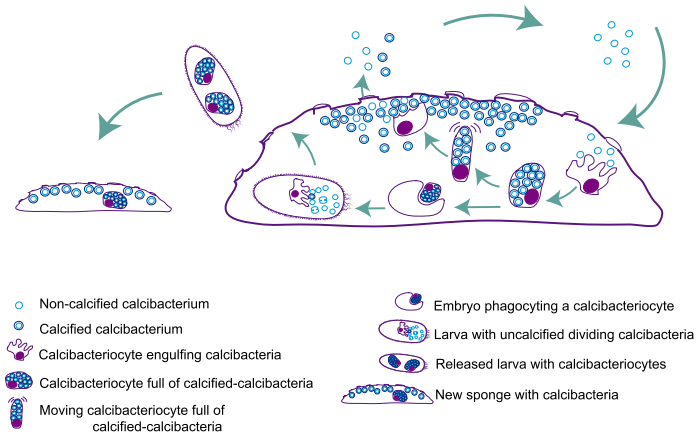

Figure 7. Proposed cycle of calcibacteria acquisition and vertical transmission to sponge settlers. Uncalcified calcibacteria enter the sponge with the inhaled water, are engulfed by the sponge ameboid archeocyte-like cells (calcibacteriocytes), which place them in cell vacuoles where bacteria calcification follows. Calcibacteria-full calcibacteriocytes move to the sponge periphery, where they disintegrate releasing the calcified calcibacteria, which accumulate forming a cortical calcareous layer. Embryos engulf maternal calcibacteriocytes during the maturation process. Maternal calcibacteriocytes disintegrate, releasing the calcibacteria to the larva mesohyl, where the extracellular pH conditions would prevent their calcification. Uncalcified calcibacteria divide in the larval mesohyl until they are captured by larval calcibacteriocytes where they calcified. Free larvae carry out calcibacteriocytes, and the cycle resume after larval settlement.

(iii) For Transmission Electron Microscopy (TEM), ca. 3 mm^3 samples were fixed, rinsed with buffer, dehydrated, and embedded in a plastic resin[54]. Ultrathin sections were cut using an ultramicrotome (Ultracut Leica), and stained with uranyl acetate and lead citrate. Samples were observed using a JEOL 1010 TEM, implemented with Bioscan (Gatan) for image digitalization (Microscopy Unit of the Scientific and Technical Services of the University of Barcelona).
(vi) For CARD-FISH experiments, ca. 50 mm^3 samples were fixed in 4% paraformaldehyde for 4 h, then transferred to 70% ethanol, and embedded in paraffin. Histological sections were prepared according to the study needs (Table 1).
(v) For bacterial cloning, samples of *C. viridis* and *H. columella* were submersed in absolute ethanol immediately after collection and taken to the laboratory in a cooled container.

Different analyses were performed on different sub-sets of sponge species (see Supplementary Table S1).

Calcibacterium location and quantification. We quantified the calcibacteria in the same set of *H. columella* individuals by two procedures to confirm that the calcibacteria were inside the calcareous spherules and to avoid possible biases in bacteria quantification in hybridized sponge sections due to signal overlapping in Epiflourescence Microscopy (EM):

(i) Hybridization of a filtered aliquot of the extracted calcibacteria (see procedure below) and observation using EM.
(ii) Direct tissue hybridization and observation though Confocal Laser-Scanning Microscopy (CLSM).

Calcibacteria extraction. Calcibacteria spherules were exhaustively extracted from fresh *H. columella* pieces. Pieces of ca. 1 g of fresh sponge (three individuals per colour morph and two regions per individual) were disaggregated and homogenized. Siliceous spicules were precipitated and then the pellet was discarded. The spicule-free homogenates were subjected to a series of centrifugations and re-suspensions[42]. One aliquot of the each final spherule suspensions (three per individual) was filtered through 0.2 μm pore filters and filters were CARD-FISH treated (Table 1).

Oligonucleotide probe design. An oligonucleotide probe targeting the prevalent alpha-proteobacteria sequence in the sponge *H. columella* was designed using ARB software (http://www.arb-home.de/). The target sequence matched with two alpha-proteobacteria from the water column (GenBank ID: KC425597.1; GenBank ID: EF471706) and another from the cnidarian *Erytropodium caribaeorum* (GenBank ID:889934.1). The best probe was checked in silico with the online software MathFish (http://mathfish.cee.wisc.edu/) and its efficacy confirmed using the probe match tool in ARB. We also designed two 'competitor sequences' to avoid non-specific hybridizations, and two 'helper sequences' (Table 1) to hybridize the flanking regions of the specific probe. A non-sense probe (Non-EUB 338-I 5′-ACTCCTACGGGAGGCAGC-3′)[55] was used as a negative control (Table 1). All the probes were synthesized using Biomers (http://www.biomers.net/).

Catalyzed reporter deposition fluorescence *in-situ* hybridization (CARD-FISH). We used CARD-FISH with the designed probe to verify whether the dominant sequence in the species *H. columella* corresponded to the abundant intracellular calcifying bacteria, observed using TEM and SEM, and to detect its presence in other sponge species harbouring 1 μm diameter calcareous spherules: *H. columella* (Poecilosclerida, Hymedesmiidae), *C. viridis* (Hadromerida, Clionaidae), *Protosuberites* sp. (Hadromerida, Suberitidae), and *C. alloclada* (Spirophorida, Tetillidae). CARD-FISH was also used to quantify calcibacteria within the main sponge regions (ectosome and choanosome) of whitish and pinkish morphs of *H. columella* (N = 3) and in two stages of the sponge life cycle: reproductive individuals incubating larvae and non-reproductive individuals.

Tissue samples that were fixed in 4% paraformaldehyde were dehydrated, embedded in paraffin, cut, deparaffined, and subjected to membrane permeabilization and inactivation of endogeneous peroxidases following procedures listed in Table 1 (modified from Pernthaler & Pernthaler[56]). Filters containing the extracted calcareous spherules were directly subjected to bacteria membrane permeabilization and inhibition of endogenous peroxidases. The optimum formamide concentration for the specific probe (i.e. 45%) was determined from assays at concentrations of 55%, 45%, and 35%. Following CARD-FISH, the sponge sections and the isolated spherules were DAPI stained to observe DNA. Dehydrated samples were mounted using Citifluor.

To discount self-fluorescence from the hybridized tissue, three sponge sections were treated according to the CARD-FISH protocol without adding the probe. Moreover, hybridization was assayed in tissues of the sponge *Crambe crambe*, a species that does not harbour the target calcibacteria[57], to confirm that the hybridization signal observed was not an artefact.

Quantification using epifluorescence microscopy. Hybridization was conducted on extracted calcibacteria from the sponge ectosome and choanosome of whitish and pinkish morphs. The resulting calcibacteria were re-suspended in sterilized seawater and an aliquot of this suspension was filtered through a 0.2 μm polycarbonate filter. Filters containing the calcibacteria were hybridized using CARD-FISH and observed using an epifluorescence microscope (EM) (Axioimager, Zeiss). Pictures were captured from 10 randomly selected fields at 100 × magnification using an Axiocam MR3 (Zeiss) digital camera (820 ms exposure time) attached to the microscope. We divided each field into 16 quadrats and counted the number of calcibacteria (hybridization points) in four randomly selected quadrats using Adobe Photoshop. The average number of calcibacteria per quadrat was multiplied by a factor related to the spherule concentration in the initial suspension.

Quantification using confocal laser-scanning microscopy. We quantified calcibacteria in whitish and pinkish morphs of *H. columella* (N = 3) using a Leica TCS-SP5 confocal spectral microscope (Leica Microsystems Heidelberg GmbH, Universitat Autònoma of Barcelona) with a Plan-Apochromatic 63 ¥ 1.4 (oil HC ¥ PL APO lambda blue objective). A series of images (three fields per section) were taken every 1 μm (axis-z) across 6 μm thick histological sections to observe the emission signals of Alexa 488 and DAPI. The images were processed using Metamorph Imaging software (Universal Imaging Corporation, West Chester, PA, USA). We measured the integrated fluorescence intensity of the signal emitted by hybridized bacteria after removing the background fluorescence from the control samples.

DNA extraction, amplification, and cloning. Samples of *C. viridis* and *H. columella* were submersed in absolute ethanol immediately after collection and taken to the laboratory in a cooled container. *H. columella* was extracted using Qiamp DNA stool kit (Qiagen) and *C. viridis* with DNeasy blood and tissue kit (Qiagen). One 16S rRNA gene fragment, ca. 1,450 nt. in size, was amplified using universal primers 26F and 1492R[58]. PCR conditions were as described previously[59]. PCR products were purified using QIAquick PCR Purification kit (Qiagen) and cloned using TOPO® TA Cloning® Kit for sequencing using One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen), according to the manufacturer instructions. Following colony growth, correct-size inserts were identified using PCR with T3–T7 primers, and purified and sequenced using the Sanger method (Macrogen Europe). Sequences containing the 273 nt. calcibacteria fragment[15] were selected and aligned with the SILVA database using SINA web aligner. The alignment was merged into ARB software and improved using the Fast Aligner tool according to the secondary structure.

Statistical analysis. Normality and homoscedasticity of data was verified[60]. Differences in calcibacteria abundance between sponge zones in different colour morphs were analysed using two-way ANOVA, with sponge colour (whitish or pinkish) and zone (ectosome or choanosome) as fixed orthogonal factors. Integrated fluorescence intensities obtained using CLSM were analysed using the Mann Whitney U-test, since data did not meet the assumptions of normality and/or homoscedasticity. Cloned sequences containing the 273 nt. calcibacterium fragment[5], and the closest sequences were exported from ARB and used to construct a Bayesian phylogenetic tree using MrBayes 3.2 software. The GTR evolutionary model was used. Four Markov Chains were run with ten million generations sampled every 1000 generations. The chains converged significantly and the average standard deviation of split frequencies was less than 0.01 at the end of the run. Early tree generations were discarded by default (25%) until the probabilities reached a stable plateau (burn-in) and the remaining trees were used to generate a 50% majority-rule consensus tree.

The threshold used for considering a group of sequences belonging to the same "species" (now OTU) was a sequence similarity higher than 98.5% 16 while similarities >95% suggest same genus[17].

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Author Contributions

L.G. performed sampling, microscope observation, CARD-FISH, cloning, phylogenetic reconstructions and wrote the manuscript. J.S. designed the calcibacteria probe and conducted sampling and CARD-FISH experiments, and contributed to the manuscript writing. G.A., helped with probe design and CARD-FISH, participated in microscope observation and analyses, and contributed to the manuscript writing. M.J.U. designed the study, interpreted the results, led manuscript writing and integration.

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Calcareous spherules produced by intracellular symbiotic bacteria protect the sponge *Hemimycale columella* from predation better than secondary metabolites

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ABSTRACT: Benthic sessile organisms in general, and sponges in particular, have developed an array of defense mechanisms to survive in crowded, resource and/or space-limited environments. Indeed, various defense mechanisms may converge in sponges to accomplish a defensive function in an additive or synergetic way, or to operate at different times during the sponge's life cycle. Moreover, sponges harbor highly diverse microbial communities that contribute in several ways to the host's success. Although some symbiotic bacteria produce chemical compounds that protect the sponge from predation, the possible deterrent function exerted by the calcareous coat of a sponge's endosymbiotic bacterium has not, to date, been explored. *Hemimycale columella* is an Atlanto-Mediterranean sponge, which produces bioactive metabolites and has been reported to host an intracellular bacterium with a calcite envelope. Calcibacteria accumulate in high densities at the sponge periphery, forming a kind of sub-ectosomal cortex. They have been suggested to provide the sponge with several benefits, one of which is protection from predators. In this study, we assess the relative contribution of the endosymbiotic calcibacteria and bioactive compounds produced by *H. columella* to defend the sponge against sympatric predators. Deterrence experiments have revealed that the sponge combines >1 defense mechanism to dissuade a large array of potential predators; this represents an example of the evolutionary fixation of redundant mechanisms of defense. The chemicals deterred *Paracentrotus lividus*, *Chromis chromis*, *Oblada melanura*, and *Diplodus vulgaris*, but not *Parablennius incognitus* and *Coris julis*, while the spherules of the symbiotic calcibacteria significantly deterred all predators assayed.

KEY WORDS: Chemical defenses · Calcifying bacteria · Sponge endosymbiosis · Sponge deterrence · Calcite spherules · *Hemimycale columella* · Atlanto-Mediterranean

INTRODUCTION

Species coexistence, which determines the biodiversity of a given ecosystem, is the result of several long discussed, biological, and ecological mechanisms such as environmental variation (Chesson & Warner 1981), resource and/or niche partitioning (Chesson 2000), and species-specific interactions, which involve species-specific mechanisms of defense (Buss 1976).

Benthic sessile organisms in general, and sponges in particular, have developed an array of defense mechanisms to survive in crowded, resource and/or space-limited environments. Structural materials, such as external and internal skeletons, dermal spines, or protruding spicules serve as defense for benthic invertebrates and fish by protecting their soft tissues and, thus, dissuading most potential benthic predators. Conversely, bioactive chemicals usually act in a

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less generalist way. Some chemicals may deter one or more species from predation on the producer organism while they may not deter others (Becerro et al. 2003). On the whole, chemical defenses have been reported to significantly contribute to the structure of sponge assemblages on coral reefs (Loh & Pawlik 2014) and have been proposed to favor complex interaction networks, which are responsible for increasing species coexistence and thus biodiversity (Buss 1976, Loh & Pawlik 2014). On the other hand, mineral skeletons would only improve species persistence by offering general protection to the organisms (Uriz et al. 2003).

Sponges are a notable component of the marine benthos, where they share habitat with an array of potential predators (McClintock et al. 1994, Wulff 2000, Santos et al. 2002, Leon & Bjorndal 2002, Knowlton & Highsmith 2005). Thus, besides competing for growth space with other benthic organisms such as algae, corals, ascidians, and bryozoans, sponges must also handle predation. Indeed, sponge survival has required the development of several defense mechanisms, which comprise the production of chemical compounds and structural elements (Uriz et al. 2003, Jones et al. 2005), along with cryptic growth habits (Bertolino et al. 2013).

Many of the bioactive compounds produced by sponges with antimitotic, cytotoxic, antibacterial, and/or antitumor activities (Amade et al. 1987, Uriz et al. 1992, Becerro et al. 1994, Monks et al. 2002, Sipkema et al. 2005, Blunt et al. 2009, Hardoim & Costa 2014) inhibit the settlement of foreign larvae in the proximity of the sponge (Martin & Uriz 1993, Becerro et al. 1997a, 2003, De Caralt et al. 2013) or deter predation (Uriz et al. 1996, Ribeiro et al. 2010, Arias et al. 2011). Spicules have also been reported to dissuade sponge predators to some extent (Burns & Ilan 2003, Hill et al. 2005, Jones et al. 2005, but see Chanas & Pawlik 1995). Thus, various defense mechanisms converge in most sponges to accomplish a defensive function in an additive or synergetic way, or to operate at different times during the sponge's life cycle (Uriz et al. 1996). However, the opposite is also true: multiple functions have also been reported for a sole defense mechanism (Thacker et al. 1998, Becerro et al. 1997a). Furthermore, the efficiency of a deterrent mechanism can vary according to the predator (Becerro et al. 2003), which makes the results of deterrence assays difficult to generalize.

To add to the complexity of defense mechanisms, sponges harbor highly diverse microbial communities (e.g. Blanquer et al. 2013), which form stable symbiotic associations and contribute in several ways to the host's success (Taylor et al. 2007, Thacker & Freeman 2012). For instance, some sponge symbiotic bacteria produce chemical compounds that protect the sponge from predation (Thacker et al. 1998, Haber et al. 2011, Esteves et al. 2013). However, the possible deterrent function of an endosymbiotic bacterium, other than that mediated by bioactive chemicals, has not been explored to date.

Hemimycale columella (Bowerbank, 1874) is a common encrusting demosponge (Order Poecilosclerida) widespread in the Mediterranean and North Atlantic sublittorals. The species, which has a reduced ectosomal skeleton (Van Soest 2002), produces chemical compounds with cytotoxic and antimitotic activities (Uriz et al. 1992, Becerro et al. 1997a) that might deter its potential predators. However, *H. columella* has also been reported to host an intracellular bacterium with calcifying abilities (Uriz et al. 2012). Bacteria that are surrounded by a 100 nm thick calcite envelope have been detected by catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) and transmission electron microscopy (TEM) in high numbers within a particular sponge cell type called calcibacteriocyte (Uriz et al. 2012). Thousands of bacterium-produced calcite spherules are accumulated at the sponge periphery, forming a kind of sub-ectosomal cortex that mimics a rudimentary exoskeleton (Uriz et al. 2012). It has been proposed that this unusual, intimate symbiosis, which is vertically transmitted to progeny and constantly present in all populations of *H. columella* examined along the western Mediterranean sublittoral zone, purportedly provides the sponge with several benefits, among which protection from predators has been highlighted (Uriz et al. 2012).

In this study, we aimed to assess the relative contributions of the calcite spherules of endosymbiotic calcibacteria and the bioactive compounds produced by *H. columella* to sponge defense, and whether the combination of secondary metabolites and the calcibacterial calcareous envelope exerted a synergetic effect in deterring potential predators from feeding on the sponge. With this aim, we conducted several deterrence experiments, both in the laboratory and in the sponge's habitat, with an array of sympatric potential predators (echinoderms and fishes).

MATERIALS AND METHODS

Sampling, sponge identification, and location of bacteria

Between 8 and 12 individuals of *Hemimycale columella* were randomly collected from the Blanes lit-

toral zone, NW Mediterranean (41°40.12′N, 2°47.10′E), in each of 3 sampling dives, to prepare the artificial food used in the experiments. The species was taxonomically identified by phenotypic characters, such as external morphology (i.e. thick encrusting shape, presence of characteristic rounded pore sieves with elevated rims, pale orange to pink color, and spicule shape [strongyles to styles], size [320 to 461 µm × 2.5 to 7.5 µm), and plumose arrangement [Van Soest 2002]). The sponge samples were taken to the laboratory in hermetic, seawater-filled bowls, blended, and weighed after removing foreign material under a stereo-microscope. Half of the sponge mix was frozen for obtaining the crude chemical extract (potential chemical defenses), while the other half was used to isolate the calcite spherules, which represented the purported physical defenses.

Light-microscope pictures were obtained by using forceps to break up recently collected sponges and by direct observation of the resulting disaggregated cells though a Zeiss (Axioplan) microscope connected to a Jenoptik/Jena (ProgRes C10 plus) digital camera.

For TEM, samples of ca. 2 mm^3 in size were fixed in 1% OsO_4 and 2% glutaraldehyde (1:3) in 0.45 M sodium acetate buffer (pH 6.4) with 10% sucrose (Leys & Reiswig 1998) for 12 h at 4°C. After rinsing in the same buffer, dehydration, and inclusion in Spurr's resin, ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and observed with a TEM (JEOL 1010), implemented with a Bioscan system (Gatan) for image digitalization (Microscopy Unit of the Scientific and Technical Services of the University of Barcelona).

For scanning electron microscopy (SEM), samples were fixed in a cocktail (6:1) of a saturated solution of $HgCl_2$ and 2% aqueous solution of OsO_4 (Johnston & Hildeman 1982), cryofractured in liquid N_2, dehydrated, gold palladium metalized, and observed through a Hitachi S-3520N SEM (Microscopy Service ICM-CSIC, Barcelona).

For experiments in the laboratory, the target predators were the sea urchin *Paracentrotus lividus* and the fish *Parablennius incognitus*, which share habitat with *H. columella*. These 2 predators were collected in sufficient numbers from the sponge habitat (Blanes littoral, NW Mediterranean; 41°40.12′N, 2°47.10′E), transported to the laboratory in seawater containers, and placed in an open-system aquarium at a similar temperature to that in their habitat (22°C). All individuals were from the same size-class (adults), and no male livery was shown by any of them. They were starved for 7 d before experiments were initiated. Once the experiments were completed all individuals were taken back to their natural habitat.

Chemical extraction

Ca. 25 g of fresh sponges, corresponding to 40 ml in volume (according to the water volume displaced when submerged in a measuring cylinder), were freeze-dried for 72 h and pounded. Acetone was used for chemical extraction because it has been reported to extract a wide range of secondary metabolites (Cimino et al. 1993). The extraction was done in an ultrasound bath for cell breaking and was performed in 2 steps. First, we added 20 ml of acetone per gram of sponge powder, and the extraction lasted for 25 min. Once the supernatant was removed, we added another 20 ml of acetone per gram sponge and extracted it for 10 min. The supernatants from the 2 extractions were pooled together in a previously weighed tube, and the solvent was totally evaporated in a hood. The tubes were weighed again after drying to determine the amount of crude extract obtained. The procedure was repeated 4 times (25 g of fresh sponge each time) and ended with a total crude extract of 72 mg (0.45 mg ml^{-1} sponge), which was preserved frozen in the darkness until the artificial food was prepared.

Isolation of calcibacteria spherules

The presence and abundance of calcibacteria in the sampled sponges (i.e. bacteria surrounded by a calcareous coat) were confirmed through optic and electron microscopes. The calcibacteria coats, which are calcium carbonate made according to X-diffraction analysis (Uriz et al. 2012), were obtained directly from fresh sponge samples. Ca. 25 g fresh sponge, 40 ml in volume, were disaggregated and homogenized in sterile seawater to avoid dissolution of the calcite-made, calcibacteria spherules. The whole process of spherule isolation consisted of a series of centrifugations and re-suspensions (Fig. 1E,F) in an attempt to be as exhaustive as possible. Siliceous spicules precipitated first, forming part of the pellet after centrifugation, and were discarded.

The spicule-free homogenates were initially centrifuged at 200 rpm for 1 min (Step 1), and the supernatants with the calcite spherules were removed and kept apart. The pellets, which still contained spherules, according to light microscope observation, were re-

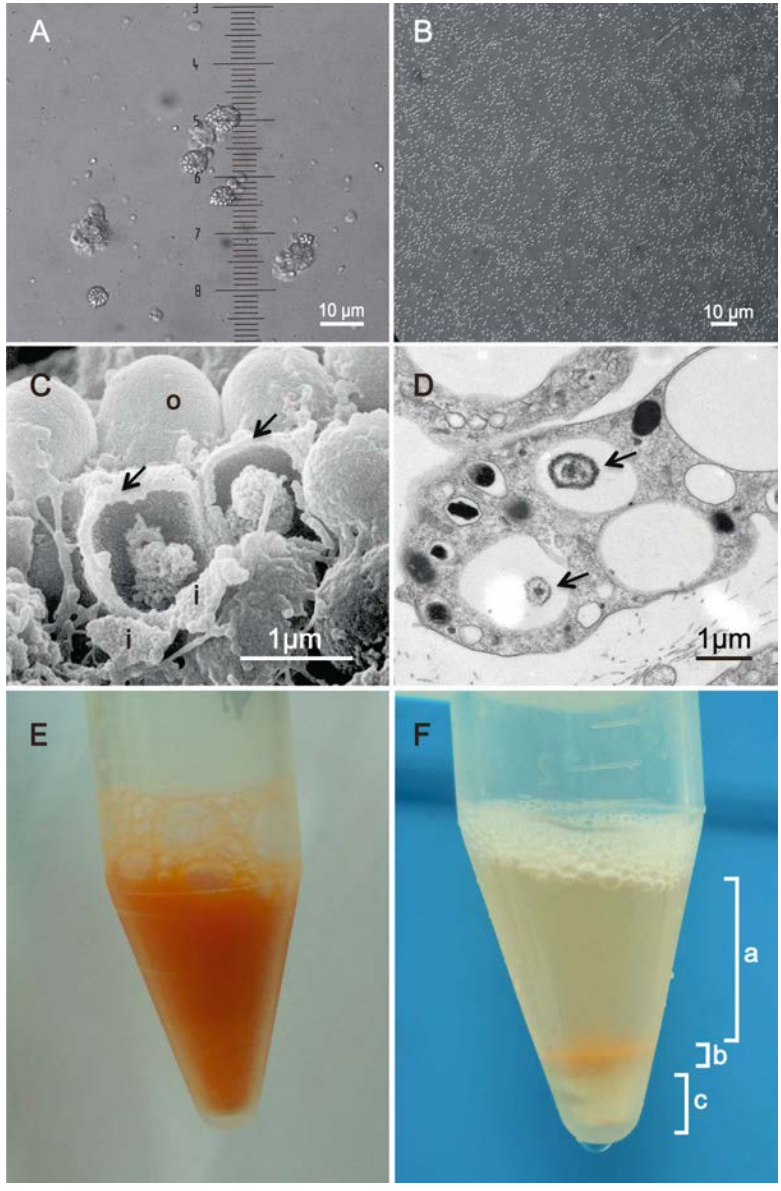

Fig. 1. Calcibacteria in (A–D) *Hemmycale columella*, and (E,F) the process of calcibacterial isolation. (A) Sponge cells (calcibacteriocytes) containing the calcified bacteria (which appear refringent through the light microscope). (B) Huge amounts of calcibacteria after cell dissociation of fresh sponges (light microscope). (C) Scanning electron microscope image of a cryofractured sponge showing entire and broken calcibacteria—i: internal side showing the nanospherules that form the calcite coat; o: outside of the calcite coat (arrows point to the zones where nanospherule arrangement in a layer is more conspicuous). (D) Transmission electron microscope image of a calcibacteriocyte containing 2 calcibacteria within their respective vacuoles (arrows); the calcareous envelope was dissolved during the fixation process by acidic fixators, i.e. glutaraldehyde). (E) Sponge homogenate after spicule removal: Step 1 of the cell dissociation and centrifugation process (see 'Materials and methods: Isolation of calcibacteria spherules'). (F) Step 2 of the process in which the debris of most sponge cells has already been removed—a: supernatant containing isolated calcibacteria in suspension; b: layer of calcibacteriocytes; c: settled calcibacteria

suspended in 7 ml of sterile seawater and centrifuged again at 500 rpm for 2 min (Step 2). The resultant supernatants were removed and set apart. The upper layer of the pellet, which contained entire calcibacteriocytes, was also removed with a pipette, and re-suspended with RIPA buffer (Sigma) and sterile seawater (1:1) to lyse the calcibacteriocytes; this was centrifuged at 500 rpm for 2 min (Step 3). The various supernatants containing spherules were pooled together and centrifuged at 2000 rpm for 4 min in order to precipitate the calcibacteria spherules (Step 4). The spherule-free supernatant (verified through light microscopy) was discarded, and finally the pellet was re-suspended in 1 ml of sterile seawater. The whole process was repeated 3 times totaling 48 ml of calcite spherules, which represented a concentration of 0.4 ml spherules ml^{-1} sponge.

Artificial food preparation

Two types of artificial food were prepared according to the feeding behavior of the target predators: 4% carragenate plates for sea urchins and bread pellets for fishes.

Paracentrotus lividus

The food controls were prepared by adding 120 g of the fresh alga *Cystoseira mediterranea*, which is part of the diet of *P. lividus*' (Verlaque & Nédelec 1983, Verlaque 1984), to 120 ml of 4% carragenate. To detect any deterrent effect of the solvent used in the chemical treatment, acetone controls were also prepared by adding 12 ml of acetone to 120 ml of a 4% carragenate–alga mixture (i.e. 2.25 ml of acetone per carragenate plate). Either the sponge crude extract (chemical treatment) or the calcibacterial treatments were added to 120 ml of slightly warm 4% carragenate seawater–alga mix.

For the chemical treatment, we re-dissolved ca. 5.3 mg of crude extract in 12 ml of acetone and added this solution to the 120 ml of 4% carragenate–alga mix, which approximately mimicked the crude volumetric concentration in the sponge (ca. 0.45 ml of crude extract per milliliter of carragenate).

For the calcibacterial treatment, we added 48 ml of the concentrated spherule suspension to 120 ml of a 4% carragenate–alga mix, which approached the calcibacterial density estimated in fresh sponges (0.4 ml of spherules per milliliter of carragenate). A total of 15 ml of the carragenate–alga mix containing either the crude extract or the calcibacterial blend was poured into 8 Petri dishes (6 plates for experimental trials and 2 as hydration controls). The treatment combining the crude extract and the calcibacteria was prepared by adding both 5.3 mg of sponge crude extract and 48 ml of concentrated calcibacterial suspension to the carragenate–alga mix. After cooling, the plates were removed from the Petri dishes and weighed immediately before being offered to the sea urchins. Two plates per treatment were kept in aquaria free of sea urchins to estimate the possible weight gains because of carragenate hydration.

Parablennius incognitus

Hundreds of ca. 3 mm long, 1 mm thick pellets—an appropriate size considering the mouth size of the target fish—were hand made from smashed bread. Either the sponge crude extract solution or the spherule suspension was added in appropriated volumes to bread pellets to obtain ecologically relevant concentrations (i.e. similar to those present in the sponge tissues). The treatments considered for *P. incognitus* were: sponge crude extract (chemical treatment), calcibacteria spherules, and acetone control. The chemical treatment was prepared by adding ca. 4 mg of crude extract, dissolved in 12 ml acetone, to 10 g (ca. 40 ml of bread pellets, measured in a measuring cylinder) to obtain a concentration of ca. 0.45 mg of crude extract per milliliter of bread pellets.

The spherule treatment was prepared as described above by adding 16 ml of spherules, suspended in 1 ml of seawater, to 10 g, ca. 40 ml, of bread pellets (resulting in a concentration of ca. 0.4 ml of spherules per milliliter of pellets). The acetone control was prepared by adding 12 ml of acetone to 10 g (40 ml) of bread pellets. The pellets containing the treatments were then air dried to facilitate manipulation. In the previous experiment on sea urchins no differences were found between the carragenate and acetone controls, so we only considered the acetone control in subsequent experiments.

In situ sympatric fish assemblage

The artificial food for the *in situ* experiment with the sympatric fishes at the sponge habitat was similar to that prepared for the fish experiment in the laboratory (see above) but the pellet size was larger (4 mm long and 1 to 2 mm thick) in order to adapt the food to the mouth size of the fishes targeted. We per-

formed the same 3 treatments (crude extract, calcibacteria spherules, and acetone control) as in the *P. incognitus* experiment. Treatments were offered to the fish assemblage at random. Fishes at sea were adapted to feed on artificial food offered by divers for 7 d prior to the experiment.

P. lividus experiment

The sea urchins were starved for 1 wk and then placed in individual 5 l aquaria, with continuous aeration at 22°C. Three treatments consisting of (1) the sponge crude extract, (2) the calcibacteria spherules, and (3) both components combined were offered. We used a total of 30 individuals, 6 per treatment (including controls). Two other aquaria were disposed under the above conditions to sink 2 plates of each treatment to assess their increase in weight due to hydration during the experiment. The plates were randomly distributed between individuals, and the experiment lasted for 48 h. The plates were then recovered, slightly toweled, and weighed to calculate weight losses that were due to sea urchin grazing, after discounting the mean increase in weight of plates used to control hydration.

P. incognitus experiment

After 1 wk of adaptation to aquarium conditions, each *P. incognitus* individual was placed in a 5 l aquarium (N = 14) with continuous water flow at a constant temperature (22°C). The experiment lasted for 8 d. Every 2 d we offered 10 pellets of each treatment (crude extract, spherules, and acetone control) in random order to 4 randomly selected fishes, and recorded the number of pellets eaten or rejected per treatment. No pellet was ignored when offered to fish in this experiment. At the end, we had a total of 16 replicates per treatment. Those fishes that were not involved in a given trial were fed daily ad libitum with Sera® marine granulate.

Sympatric fish experiment

The field experiment was carried out in the Blanes sublittoral zone (NW Mediterranean; 41°40.12′N, 2°47.10′E), in summer 2013, on a rocky, 10 to 15 m deep, bottom. The most frequent fish species co-occurring in the sponge habitat were *Chromis chromis*, *Diplodus vulgaris*, *Oblada melanura*, and *Coris julis.* Thus, we recorded the behavior of these 4 fishes with respect to the food offered. The number of fish participating in the experiment, as estimated from the number of pellets that they ate or rejected, was >20 per species (see Table 3), although we were unable to ensure that a given individual participated only once in the experiment.

The artificial food was taken to sea in large plastic syringes (1 treatment^{-1}) as described by Becerro et al. (2003). Treatments and controls (5 pellets treatment^{-1}) were randomly offered to fishes by slowly releasing the pellets into the water. Two independent SCUBA divers recorded the number of eaten or rejected pellets. A pellet was considered rejected by a fish if tried and spat out 3 or more times. When a pellet was ignored, or tried by a fish just once or twice and spat out and ignored, the outcome was annulled and a new pellet of the same treatment was offered later.

Comparative deterrence quantification

A deterrence index (Becerro et al. 2003) was used for comparing sea urchin and fish deterrence in the 3 experiments. The index (DET) was defined as:

$$\mathrm{DET} = \frac{\frac{\mathrm{EC}}{\mathrm{OC}} - \frac{\mathrm{ET}}{\mathrm{OT}}}{\frac{\mathrm{EC}}{\mathrm{OC}}} \quad (1)$$

where EC is either the number of control pellets eaten by fishes or the weight losses in the control agar plates offered to sea urchins and OC is the number of control pellets offered or the initial weight of the control plate; ET is the number of treatment pellets eaten or the decrease in weight of a treatment plate and OT is the number of treatment pellets offered or the initial weight of a give treatment plate. DET varies from 0 (no deterrence) to 1 (total deterrence).

Statistical analyses

Data from the experiments in the laboratory on the sea urchin *P. lividus* and the fish *P. incognitus* were analyzed by 1-way ANOVA after rank transformation, since they did not meet the assumptions for parametric analyses. The significance values of the post hoc pairwise comparisons (Newman-Keuls test) were adjusted by the false discovery rate (FDR) correction for multiple comparisons (Yekutieli & Benjamini 1999).

Data from the sea experiment were analyzed using log-linear models for contingency tables. We tabu-

lated our data with treatment (control and treated food), fish species tested, and consumption (eaten or rejected) as factors, and the number of occurrences (pellets) in each category as observed cell frequencies (Sokal & Rohlf 1995). The statistical significance of the deviations of the observed frequencies from the expected frequencies was evaluated by Pearson's chi-squared. Statistical analyses were performed with Statistica 6 software.

RESULTS

Calcibacterial presence/abundance

Calcibacteria were present in the *Hemimycale columella* sponges used for the experiments, as proved by light and electron microscope observations. Calcibacteria spherules were extraordinarily abundant (Fig. 1A–D) in the sponge homogenates either free in suspension (due to their small size [<1 µm in diameter] and low weight) as a result of calcibacteriocyte damage or on the upper layer of the pellets within denser entire calcibacteriocytes. SEM pictures of cryofractured sponge tissue showed calcibacteria with a 100 nm coat of nanospherules arranged in a layer and with inner material corresponding to the bacteria (Fig. 1C). Images of intracellular bacteria deprived of the calcareous coat (likely due to calcium carbonate dissolution during the pH-lowering fixation process) were obtained by TEM. The intracellular vacuoles maintained the size and shape of the calcibacterial coat (Fig. 1D).

P. lividus experiment

ANOVA results on ranks showed a significant effect ($p < 0.001$) of treatments on ingested food (Table 1). Post hoc comparisons (Newman-Keuls test) after FDR correction proved significant ($p < 0.001$) differences between the 3 treatments and the 2 controls (carragenate control and acetone control), which were eaten similarly ($p = 0.22$). There were no significant differences in feeding between the calcibacteria spherules and the chemical treatment ($p = 0.29$), or between the chemical treatment and the chemical+calcibacteria spherule treatment ($p = 0.29$; Fig. 2). Thus, the crude extract, the calcibacteria spherules, and the calcibacteria+crude extract similarly deterred sea urchins from feeding on *H. columella*, but the latter combination did not deter the sea urchin in an additive or synergetic way.

Table 1. One-way ANOVA on the deterrent effect of several treatments (carragenate control, acetone control, crude extract, calcibacteria spherules, and chemical and bacterial components combined) on the sea urchin *Paracentrotus lividus*

Effect	SS	df	MS	*F*	p
Treatment	1653.00	4	413.25	17.37	<0.001
Error	594.50	25	23.78		

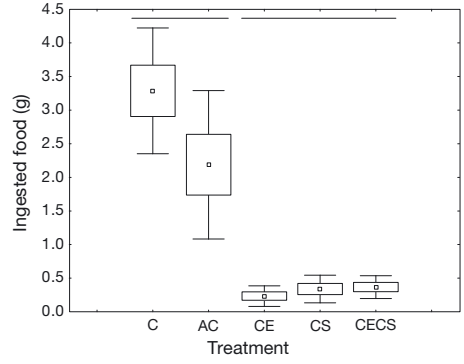

Fig. 2. Deterrent effect of treatments assayed on the sea urchin *Paracentrotus lividus* (N = 6; C: carragenate control; AC: acetone control; CE: sponge crude extract; CS: calcibacteria spherules; CECS: crude extract+calcibacteria). Horizontal bars at the top of the panel indicate no significant differences between treatments after false discovery rate correction ($p < 0.021$)

P. incognitus experiment

ANOVA on the number of pellets ingested by *P. incognitus* showed significant differences among treatments and the control (Newman-Keuls test, $p < 0.001$; Table 2). Post hoc multiple comparisons after FDR correction showed significant differences ($p < 0.001$) between the calcibacterial treatment and the control but not ($p = 0.13$) between the chemical treatment and the control (Fig. 3). Thus, only the

Table 2. One-way ANOVA on the deterrent effect of several treatments (control, crude extract, and calcibacteria spherules) on the fish *Parablenius incognitus*

Effect	SS	df	MS	*F*	p
Treatment	3548.49	2	1774.25	25.49	<0.001
Error	2853.01	41	69.59		

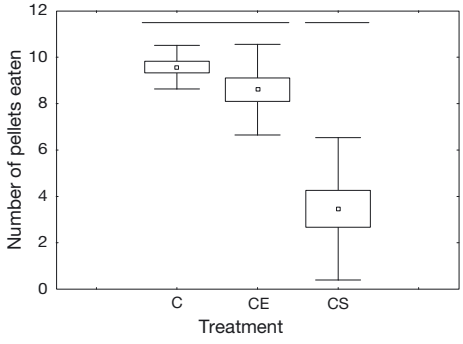

Fig. 3. Deterrent effects of treatments assayed on the fish *Parablennius incognitus* (N = 16; C: control; CE: sponge crude extract; CS: calcibacteria spherules). Horizontal bars indicate no significant differences between treatments after false discovery rate correction ($p < 0.023$). Boxes represent ±SE, bars ±SD

spherules of the symbiotic calcibacteria defended *H. columella* against predation by the small sympatric fish *P. incognitus*.

Sympatric fish experiment

The 3-way log-linear model for the contingency table with treatment, fish species, and ingested food as factors indicated that the assayed fish species, which shared habitat with the target sponge, were differently deterred from feeding by the 2 treatments assayed (Table 3; χ^2, $p < 0.001$). There were high significant differences ($p < 0.001$) between the calcibacterial treatment and the control for the 4 sympatric fishes. Conversely, the chemical (crude extract) treatment was eaten significantly less often than the control for 2 out of the 4 assayed fishes (Table 3; χ^2, $p < 0.001$).

Table 3. Frequency table from the *in situ* sympatric fish assemblage experiment that was used for contingency table analysis (143 pellets were offered per treatment). Asterisks indicate deterrent effect on ingestion (χ^2, $^{\star}p < 0.05$; $^{\star\star}p < 0.01$; ^{ns}: non-significant)

Ingested pellets	*Chromis chromis*	*Oblada melanura*	*Diplodus vulgaris*	*Coris julis*	Total
Acetone control					
Yes	31	33	27	40	131
No	4	6	1	0	11
Crude extract					
Yes	23	0	17	43	83
No	22^{ns}	$24^{\star\star}$	$14^{\star\star}$	0^{ns}	60
Calcibacteria spherules					
Yes	0		4	35	39
No	$38^{\star\star}$	$42^{\star\star}$	$18^{\star\star}$	$7^{\star}$	105
Total	118	105	81	125	429

Comparative deterrence quantification

The deterrence index (DET), which represents the relation between the consumed food and the food offered in treatments and controls, varied between treatments and among species (Fig. 4). It approached 1 for both the chemical and the calcibacterial treatments in *Paracentrotus lividus*, while it significantly varied between the calcibacterial (DET = 0.64) and chemical (DET = 0.1) treatments for *P. incognitus*.

The fish deterred most by the 2 treatments (DET = 1, crude extract and calcibacteria spherules) was the sparid *Oblada melanura*. Conversely, the labrid *Coris julis*, the pomacentrid *Chromis chromis*, and the sparid *Diplodus vulgaris* showed significantly lower deterrence indices for the chemical treatment than for the calcibacterial treatment (DET = 0, DET = 0.4, and DET = 0.4, respectively) (Fig. 4).

DISCUSSION

The various deterrence experiments performed revealed that the sponge *Hemimycale columella* combines >1 defense mechanism to dissuade potential predators. Some predators are deterred by both secondary metabolites and calcibacteria—a case example of the evolutionary fixation of redundant mechanisms of defense in a species to widen the spectrum of predators deterred. On the other hand,

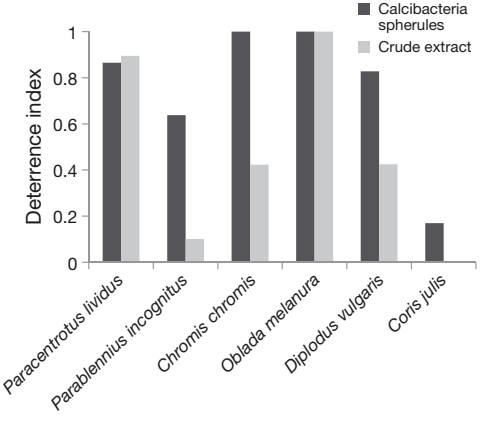

Fig. 4. Deterrence index of sponge crude extract and calcibacteria spherules for several species assayed

sponges are not the only organisms to present 2 different types of defenses; crude extracts and sclerites have also been reported to exert anti-predatory functions in gorgonians (van Alstyne & Paul 1992).

The spherules produced by the symbiotic calcibacteria significantly deterred all species assayed; thus they appear to represent a generalist defense mechanism. Conversely, the chemical extract of *H. columella* deterred some of the species assayed, but not others; thus it seems to represent a more species-specific defense mechanism.

The sea urchin *Paracentrotus lividus* was deterred from feeding on *H. columella* by both the sponge's chemical extracts and the calcibacteria spherules, as well as by a combination of both components; this finding agrees well with the observed lack of predation of *P. lividus* on *H. columella* in the field (L. Garate, A. Blanquer, M.-J. Uriz pers. obs.). Several studies reported that some sponge components deterred this sea urchin, but the outcome of the assays performed here varied as a function of the sponge species, the types of defense analyzed, and the sea urchin species used. Sponge spicules, spongin, collagen, or calcium carbonate may deter some sea urchins from predation (Pennings & Svedberg 1993, Uriz et al. 1996). Conversely, other sea urchin species feed on sponges habitually, despite the presence of siliceous spicules (De Ridder & Lawrence 1982, Santos et al. 2002). These contrasting results illustrate predator-dependent outcomes to the same type of defense.

P. lividus has been reported to feed on sponge species devoid of spicules when food resources are limited (Boudouresque & Verlaque 2007). Since *H. columella* shows a relatively poor spicule complement, predation by *P. lividus* on this sponge species would be expected, but has not been observed. Our results showed that the calcium carbonate spherules of the symbiotic calcibacteria at natural concentrations deter this sea urchin. The spherules may be unpalatable to sea urchins, but not strictly toxic (Birenheide et al. 1993), and, likely, their high concentration in sponge tissues may decrease sponge nutritional quality; thus, field sea urchins may select other more attractive food sources for optimal growth. Moreover, it has been reported that both calcite and aragonite deter some herbivore fishes from feeding (Pennings & Svedberg 1993), which has been related to a decrease in fish gut pH impairing food digestion (Schupp & Paul 1994).

Besides the symbiotic calcibacteria, *H. columella* produces secondary metabolites with demonstrated antimitotic, cytotoxic, and antibacterial activity (Amade et al. 1987, Uriz et al. 1992, Becerro et al. 1997a). Here we report on another defensive function of these secondary metabolites since they discourage the sea urchin *P. lividus* from grazing. It has also been reported that *P. lividus* is deterred by the crude extract of the sponge *Crambe crambe* (Uriz et al. 1996, Becerro et al. 1997b) and the seagrass *Posidonia oceanica* (Vergés et al. 2007), while it appears to consume the alga *Caulerpa taxifolia* during the months when it presents the lowest amount of secondary metabolites (Lemee et al. 1996). Protection from the devastating grazing by sea urchins (Guidetti & Dulčić 2007) seems to be widespread among many benthic organisms, which have developed deterrent toxicants.

The outcomes of the fish experiments differed according to the fish species assayed. In general, calcareous spherules deterred fishes more efficiently than sponge crude extract did, but, for some species, both components were similarly deterrent. Previous studies reported that sponge skeletal structures are deterrents for fishes (Burns & Ilan 2003, Jones et al. 2005, but see Chanas & Pawlik 1995, 1996). *H. columella* is a spicule-poor species, and the high concentration of calcibacteria spherules at the sponge periphery may replace spicules as deterrent elements for fishes.

The indexes formulated to compare the deterrence intensity among the potential predators assayed (DET) varied across species depending on the treatment. The calcibacteria DET index showed the highest value for *C. chromis* and *O. melanura*, followed by the sea urchin *P. lividus* (DET = 1), and exerted the lowest effect (DET = 0.19) on *C. julis.* Such differences may be due to differences in the habitual prey preferentially targeted in the field by each predator. Thus, fishes such as *C. julis*, which usually feed on invertebrates provided with an external skeleton, such as mollusks, gastropods, bivalves, and crustaceans (Kabasakal 2001, Stergiou & Karpouzi 2002), may be more adapted to encountering calcareous structures in their diet.

The DET index for the chemical treatment varied drastically with the predator species: while it reached its highest value for the sea urchin *P. lividus* and the fish *Oblada melanura*, and a medium value for *D. vulgaris* and *C. chromis*, it was close to zero for *P. incognitus* and *C. julis*.

The contrasting deterrent effects found for the crude extract may also be related to differences in the natural feeding habits of the species assayed. *O. melanura* and *D. vulgaris* are considered opportunistic predators that feed on an array of both benthic and pelagic organisms (Pallaoro et al. 2003, 2006).

Thus, they may select other, non-toxic food sources in the field. The small bleniid fish (*P. incognitus*) captures small benthic animals in the field, while grazing the surface of rocky substrata and encrusting invertebrates (Goldschmid & Kotrschal 1981); thus, it might be adapted to ingest small amounts of potentially toxic species such as *H. columella* and *C. crambe* (Becerro et al. 1997b) while capturing small invertebrates dwelling on sponges. The labrid *C. julis* is a voracious species that has been reported to predate on crustaceans and gastropod mollusks (Fasola et al. 1997, Kabasakal 2001); apparently it also tolerates, to some extent, the bioactive compounds produced by the alga *Caulerpa prolifera* (Sureda et al. 2006). Thus, the 2 latter fishes seem to show some resistance to the secondary metabolites of benthic invertebrates. On the other hand, pomacentrid fishes such as *C. chromis* are also opportunistic, omnivorous species that include sponges in their diet (Emery 1973, Emery & Thresher 1980, Horn 1989). *C. chromis*, however, has been reported to avoid artificial food containing the crude extract of the nudibranch *Discodoris indecora*, which obtains its metabolites from *Ircinia* spp. sponges (Marin et al. 1997).

Reinforcing the invertebrate periphery by mineral materials in order to make it less attractive to potential predators is the main function of mineral exoskeletons (Uriz 2006). Sponges concentrate microscleres at the periphery to form a mineral cortex (Uriz et al. 2003). Rohde & Schupp (2011) reported a higher deterrent effect of artificial food containing siliceous spicules from the sponge cortex than from the choanosome. This is likely related to the spicule sizes and may depend on the mouth size of the predator considered. Small spicules (microscleres) are densely packed in the sponge cortex (Boury-Esnault & Rützler 1997), and thus more likely to deter small predators, while protruding choanosomal megascleres (from hundreds of micrometers to millimeters) likely deter larger mouthed predators (Uriz et al. 2003). The calcified calcibacteria of *H. columella* are spherules of ca. 1 µm size that are transported by amoeboid cells (calcibacteriocytes) to the sponge sub-ectosomal zone (Uriz et al. 2012) where they form a kind of calcareous cortex. Since these calcareous spherules appear to be so efficient in deterring the potential predators assayed, their high concentration at the sponge periphery may make them very efficient in deterring an array of small-mouthed predators.

Although chemical extracts and calcibacteria deterred some potential predators individually, the deterrent effect did not increase in additive or synergistic ways when they were combined in a treatment. The few studies in which the possible synergism between structural defenses and crude extracts from sponges has been considered showed disparate results (e.g. Hay et al. 1994, Burns & Ilan 2003, Hill et al. 2005, Jones et al. 2005, Ribeiro et al. 2012).

The *Hemimycale*–calcibacteria symbiosis is not the only case in which a bacterium protects a sponge from predation. Recently, a symbiotic beta-proteobacteria of the sponge *C. crambe* has been reported to participate in the metabolic pathways (Croué et al. 2013) of 2 highly deterrent metabolites of *C. crambe* (Uriz et al. 1996). On the other hand, polyketide synthethases (PKS) of bacterial origin, with bioactive functions, have been found in many sponge–bacteria symbioses (Piel et al. 2004, Haber et al. 2011, Esteves et al. 2013). In all cases, the resulting substances produced by symbiotic microorganisms, either secondary metabolites or carbonate spherules, may be used by sponge species to their own benefit (deterrent, antibacterial, or antifouling roles; Uriz et al. 1996), thus promoting the persistence of sponge–bacteria associations.

Since the concentrations (at a volumetric proportion) of chemicals and calcibacteria used in the experiments were roughly similar to those found in natural sponges, we are confident that protection from predation is one of the benefits that *H. columella* receives from its symbiosis with calcibacteria. Its contribution to sponge survival may have helped establish this unique symbiosis between marine sponges and calcifying bacteria. However, the sponge's secondary metabolites also exert a deterrent effect against some potential sponge predators. The calcibacteria and crude extracts together do not seem to have an additive or synergetic effect on potential sponge predators, but rather may be engaged in enlarging the array of potential predators deterred. The chemical defenses of *H. columella* contribute to the complexity of Mediterranean species interactions, which supports the theory of Buss (1976), which was recently substantiated by Loh & Pawlik (2014) for sponge communities of coral reefs. In contrast, symbiotic calcibacteria unambiguously contribute to protect the sponge from generalist predators and thus favor the species' success. This is the first time that a physical defense produced by symbiotic bacteria has been documented.

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667786). All experiments complied with institutional, national, and international ethics guidelines concerning the use of animals in research. None of the species used were listed as 'Endangered'.

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