UNIVERSITAT DE BARCELONA

Patterns of genetic and morphologic diversity in Antarctic sponges

Modelos de diversidad genética y morfológicas en las esponjas antárcticas

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Patterns of genetic and morphologic diversity in Antarctic sponges

Modelos de diversidad genética y morfológica en las esponjas antárcticas

Memòria presentada per el Sr. Mirco Carella per optar al Grau de Doctor per la Universitat de Barcelona

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Centre d´Estudis Avançats de Blanes (CEAB)

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Ai miei vecchi

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DIRECTOR'S REPORT

Maria Jesús Uriz Lespe, supervisor of the Doctoral thesis by Mirco Carella entitled "Patterns of genetic and morphologic diversity in Antarctic sponges", declare that Mirco Carella has participated actively in the three chapters that constitute his Doctoral Thesis.

Chapter 1. Carella, M., Agell, G., Cárdenas, P., & Uriz, M.J. (2016). Phylogenetic reassessment of Antarctic Tetillidae (Demospongiae, Tetractinellida) reveals new genera and genetic similarity among morphologically distinct species. *PLoS One*, 11(8), e0160718.

Contribution of the candidate: in this paper, the candidate has performed the species sequencing and the taxonomic identification of problematic species by re-examining the types and other material from Museums, has conducted the molecular phylogenies and the Maximum Parsimony analysis on morphological characters and participated in the manuscript writing.

About the Journal: PlosOne has an Impact Factor of 3.54, and ranked in the 1Q of the "Multidisciplinary Sciences" section,

Chapter 2: Carella, M., & Uriz, M.J. (2018). Description of two new genera (*Antarctotetilla*, *Levantiniella*) and a new species of Tetillidae. *Zootaxa*. (Accepted, see aceptation letter).

Contribution of the candidate: in this paper, the candidate has revised the species ascribed to these two new genera, performed microscopic observation of spicules through light and SEM microscopes, performed the descriptions and illustrations, and participated in the paper redaction.

About the Journal: Zootaxa is one of the most reputed journal for publication of sponge taxonomy revisions and description of new taxa. It has an Impact factor of 0.972, ranking in 3Q of the Zoology section

Chapter 3: Carella, M., Agell, G., & Uriz, M.J. Asexual reproduction and heterozygote selection in Antarctic demosponges (*Stylocordyla chupachus*, Suberitida) approached by microsatellite analyses. *Polar Biology* (to be submitted before the thesis defense).

Contribution of the candidate: in this paper, the candidate has participated in the design and selection of the microsatellite loci from the sponge genome sequencing, amplified and genotyped the microsatellites in three populations of ca. 20 individuals each, has performed population genetic analyses and participated in the interpretation of results and redaction of the manuscript

About the Journal: The Manuscript will be published in the journal Polar Biology, which publishes first level research on polar ecosystems and organisms. The journal has an Impact Factor of 1.949 and ranked in 1Q of the Zoology section.

Blanes, May 16, 2018

Thesis Supervisor
Dra. Maria Jesus Uriz Lespe
Centro de estudios avanzados de Blanes (CEAB-CSIC)

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INTRODUCTION

Antarctic biogeographic region and Antarctic sponges

Antarctica is the southernmost continent and is surrounded by the Southern Oceans (Figure 1), which comprise about 10% of total world oceans areas. The Antarctic continent was isolated from Gondwana in the Cretaceous, about 140 million years ago (Tingey 1991; Thompson et al. 1991). Moreover, at the beginning of the Tertiary, Drake Passage opened and South America separated from West Antarctica. These geological events allowed the establishment of the Antarctic Circumpolar Current (Kennett 1982; Foster 1984; Thompson et al. 1991) that is the largest oceanic current in the world and caused isolation of many Antarctic marine benthic invertebrates from other ocean regions (Dayton et al. 1994).

Benthic invertebrate communities living in Antarctica are very rich and divers (Clarke 1992; Crame & Clarke 1997; Gray 2001). This is particularly paradigmatic as for the dense sponge beds (Ríos & Cristobo 2006) where sponges play an important role in structuring benthic habitats (Gatti 2002).

Sponge communities in the Antarctic differ from those of other seas, in many aspects (Dayton et al. 1994). In particular, they are characterized by a high degree of endemism (e.g. Dayton et al. 1970, 1974; Koltun 1968, 1970; Barthel and Gutt 1992; Sarà et al. 1992) and a eurybathic distribution of many species (Koltun 1970), characteristics that sponges share with other Antarctic benthic invertebrates. Moreover, sponge communities also harbor species with a circumpolar distribution, which embraces the Sub-Antarctic regions (e.g. Kerguelen, South Georgia) (Koltun 1969, 1970; Sarà et al. 1992), and also has a notable influence of the Magellanic region of South America and the Falkland Islands (Sarà et al. 1992).

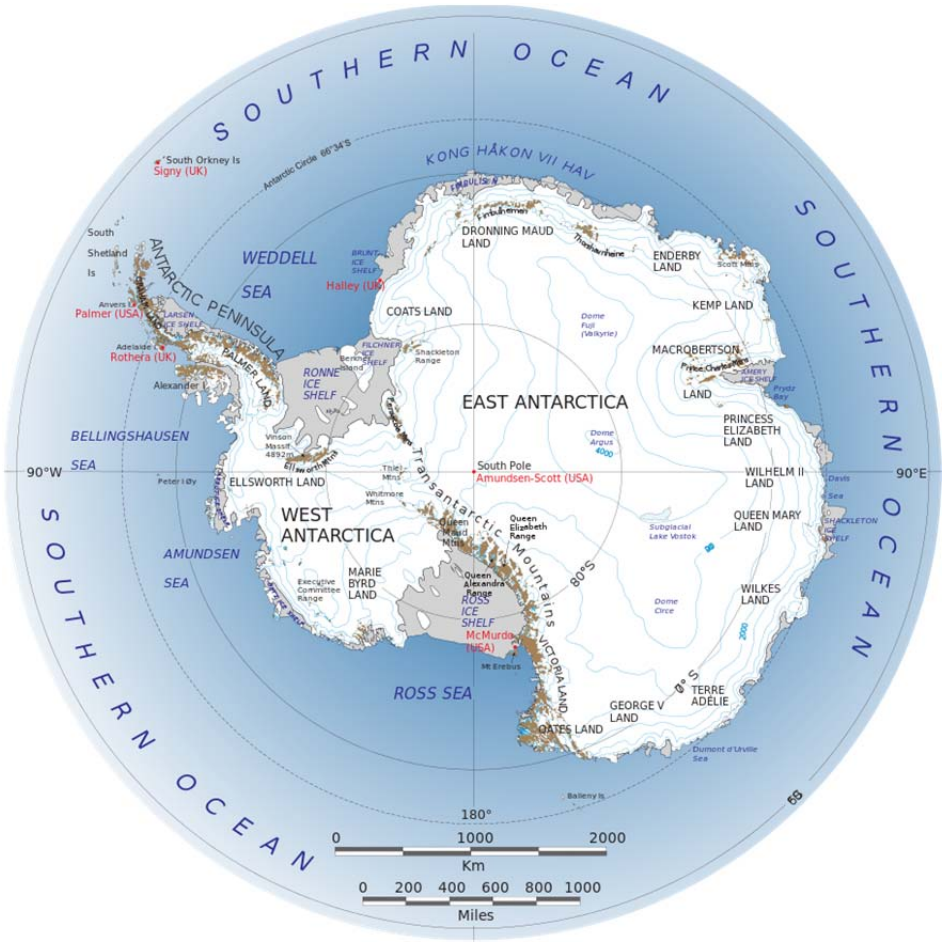

Figure 1.

Map representing the several regions of the Antarctic continent. The current study is centered in the Weddell Sea.

The latitudinal gradient of biodiversity reported for many marine species with species richness decreasing at higher latitudes (Rosenzweig 1992) cannot be applied to sponges. Conversely, the high diversity and species richness of Antarctic sponges equal or exceed that of tropical areas (van Soest 1994, McClintock 2005).

As in any marine benthic communitiy, sponges are expected to play several functions in both shallow and deep Antarctic ecosystems. They act as substrate stabilizers and bioeroders, provide refuge for a number of macro-invertebrates and fishes (Figure 2), offer substrate for other sessile organisms to settler, and contribute to the transfer of matter and energy from the pelagic to the benthic system and vive-versa, participating in the carbon cycling (McClintock et al. 2005; Bell 2008). However, functional ecology studies of Antarctic sponges are up to now scarce (Dayton et al. 1970, Dayton et al. 1994), and even descriptive studies are still largely incomplete.

Figure 2.
Hexactinellida providing refuge to a fish. Picture from Lündalv 2011.

Previous studies on Antarctic sponges

The Antarctic sponges from the continental shelf and bathyal bottoms were extensively studied during the oceanographic campaigns of the twentieth century (Lendenfeld 1907, Schulze & Kirkpatrick 1910, Hentschel 1914, Burton 1929, 1932, 1934, Kirkpatrick 1908, Koltun 1964, 1976, Topsent 1901, 1908, 1917). Many new genera and species were described based on morphological features. However, although these inventories were decisive for starting to know the Antarctic sponge fauna, many species descriptions were incomplete, as they were based on sponge fragments, or in some cases were incorrectly classified. Thus, many Antarctic species described in the past are difficult to recognize when studying new material. Posterior taxonomical surveys also described new species and reexamined past material (Boury-Esnault & Van Beveren 1982, Vacelet & Arnaud 1972) but these studies are comparatively scarce compared with other latitudes.

Additional new species are being discovered nowadays in the area (Janussen et al. 2004, Ríos & Cristobo 2006, 2007a, 2007b, Uriz et al. 2011, Plotkin et al. 2007, Göcke & Janussen 2011, 2013, Rapp et al. 2011, 2013), which suggests that the sponge biodiversity of this area is still incompletely explored and that more species remain to be revealed.

Despite the large list of traditional studies on systematics of Antarctic sponges (Lendenfeld 1907, Schulze & Kirkpatrick 1910, Hentschel 1914, Burton 1929, 1932, 1934, Kirkpatrick 1908, Koltun 1964, 1976, Topsent 1901, 1908, 1917, Boury-Esnault & Van Beveren 1982, Vacelet & Arnaud 1972, Janussen et al. 2004, Ríos & Cristobo 2006, 2007a, 2007b, Uriz et al. 2011, Plotkin et al. 2007, Göcke & Janussen 2011, 2013, Rapp et al. 2011, 2013), only recently the molecular techniques have been incorporated to the sponge taxonomy, improving species identification and the phylogenetic relationships of some genera and families.

Up to now, only two Antarctic sponge groups within classes Hexactinellida (families Rossellidae, Euplectellidae and Farreidae) and Demospongiae (family Tetillidae) benefited from molecular approaches, both without (Dohrmann et al. 2009, 2012; Vargas et. al. 2016) or in combination with morphological data (Dohrmann et al. 2008; Szitenberg et al. 2013; Carella et al. 2016; Dohrmann et al. 2017).

Despite all the above-mentioned studies, only about 2% of all sponge species reported for Antarctica had an associated sequence deposited in any of the international DNA sequence databases (Grant and Linse 2009; Grant et al. 2011; Vargas 2015).

The family Tetillidae in the Antarctica

The family Tetillidae (order Tetractinellida, suborder Spirophorina) is particularly well-represented in the Antarctic, although genera of this family have representatives distributed worldwide at different depths. They are morphologically characterized by a globular shape and a radiate/spirally arranged skeleton (Van Soest et al. 2017; Van Soest & Rützler 2002). Some species of the family have special structures, such as pore-bearing areas, called porocalices, and cortical structures. Their spicular complement is composed by megascleres, comprising protriaenes, oxeas, and anatriaenes, which often protrude the ectosomal layer, and microscleres such as sigmaspires and occasionally raphides (Van Soest & Rützler 2002). Many Tetillidae species inhabit sedimentary bottoms to which they anchor by means of long spicule bundles, which in turn represent a suitable substrate for many other hard bottom invertebrates (Gutt et al. 2013).

Szitenberg et al. (2010), in a study on mitochondrial introns of family Tetillidae suggested that many genera of this family needed to be revised. Later, Szitenberg et al. (2013) performed a phylogenetic study of this sponge group, including 88 specimens mainly from Antarctic and New Zealand waters. The molecular phylogeny was done using both nuclear and mitochondrial markers (COI M1-M6, 18S and 28S partition C1-D2) and

tried to evaluate the phylogenetic relevance of some morphological characters, such as the porocalices and the cortex. These authors retrieved five main clades: (i) *Acanthotetilla* (ii) *Cinachyrella*, *Paratetilla*, and *Amphitethya*, (iii) *Cinachyrella levantinensis*, (iv) tropical-temperate *Tetilla*, and (v) *Craniella*, *Cinachyra*, and *Fangophilina*. The genus *Craniella* was considered polyphyletic and distributed in three clades: (i) *Craniella* cf. *leptoderma* (Antarctic, New Zealand), (ii) *Craniella sagitta* (Antarctic, New Zealand) and (iii) boreo-arctic and Atlantic species mixed with New Zealand/Australian species of *Craniella*. Furthermore, Szitenberg et al. (2013) proposed to include the Antarctic *Tetilla* (*Craniella* cf. *leptoderma*), *Fangophilina,* and *Cinachyra* in *Craniella* based on the molecular markers but without an adequate morphological revision of the specimens sequenced and used in their phylogeny.

The previous study (Szitenberg et al. 2013) included a relatively few number of Antarctic specimens, and missed appropriated morphological descriptions in many cases. Thus, additional studies with a larger number of Antarctic species and accurate morphological descriptions of the specimens studied, in particular of type species, appeared necessary to improve knowledge on this family and the phylogenetic relationships of their genera distributed within and outside the Antarctic oceans.

According to Szitenberg et al. (2013) the two main morphological characters (porocalices and cortex), traditionally used for differentiating genera of the family Tetillidae (Rützler 1987, Van Soest & Hooper 2002), did not help to resolve the family phylogeny. These authors considered that the presence of the same character in different genera was due to evolutionary convergence. However, morphological characters were no analyzed formally within the frame of maximum parsimony criterion (MP) (e.g. Fitch 1971), which may cast light on their true relevance. Moreover, as the morphological diagnostic characters in the family are scarce, the use of the secondary structures of the variable (V4) region of the 18S rDNA seemed to be suitable. Molecules of rDNA tend to fold among themselves creating secondary structures (stems or loops) essential for the maintenance of their three dimensional structure, which give information on ribosomal functions as the translation process (Green & Holler, 1997; Voigt et al. 2008). These structures appeared to enhance the resolution of morphologically-based phylogenies of several sponge groups (Voigt et al. 2008) but until now, they have been rarely employed in sponge phylogenies (ej. Voigt et al. 2008; Gazave et al. 2010, 2013).

Although several molecular phylogenetic studies on Antarctic sponges using both ribosomal and mitochondrial DNA have been reported (e.g., Vargas et al. 2016), only few of them combined molecular and morphological characters (Dohrmann et al. 2008, 2017; Carella et al. 2016, this thesis). Moreover, detailed taxonomical descriptions of the sequenced species is strongly required if the phylogenetic results are aimed to be translated into taxonomy.

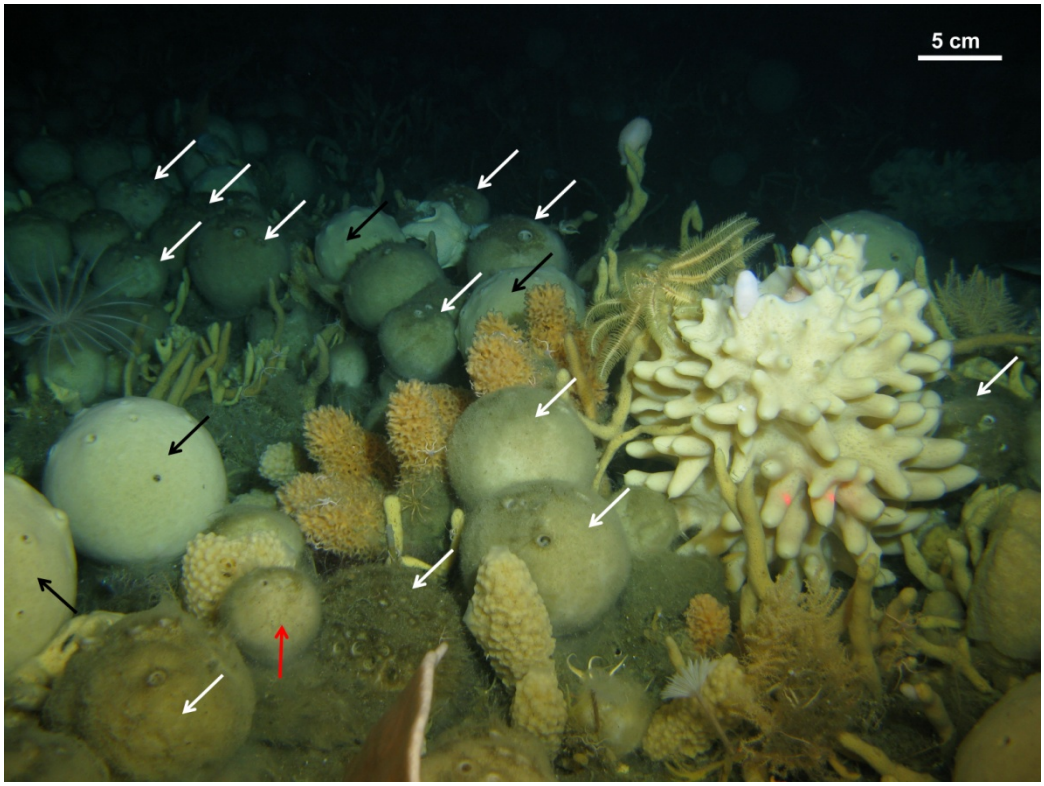

Figure 3.

Sponge beds on the continental shelf of the Weddell region (Antarctica). See the abundance of Tetillidae species (arrows), Black arrows point to *Antarctotetilla grandis*. White arrows point to *Cynachyra* spp. The red arrow points to the new Tetillidae species (*Antarctotetilla pilosa*) described in this thesis. (Picture modified from Carella et al. 2016).

Asexual reproduction in Antarctic sponges

It is widely known that Antarctic bottoms present, besides long-time geographical isolation from other continents (Thompson 1991), particular environmental conditions such as low but constant temperatures throughout the year (Clarke 1988; Gatti 2002), and limited food availability (Gatti 2002). Food limitation has been considered a greater constraint for

invertebrate development rates than the influence of low temperatures. These factors may have prompted particular reproductive strategies in sponges and other invertebrates to face the harsh but stable Antarctic environmental conditions (Clarke et al. 1980, 1990).
The crowded populations of Tetillidae, Rosellidae, Suberidae, and species from other Demospongiae families recorded in the Antarctic bottoms (Figure 3), with many examples of external buds (Figure 4), together with the reported Antarctic stability, allow expecting higher rates of clonal reproduction in Antarctic sponges than in temperate unstable seas where clonal individuals are rare (e.g. Duran et al. 2004a,b,c); Blanquer et al. 2010; Pérez-Portela et al. 2014; Guardiola et al. 2012, 2016).

Asexual reproduction seems to be a successful reproductive strategy in Antarctic bottoms, as many buds have been recorded in Hexactinellid species (*Rossella racovitzae*, *R. vanhoeffeni*, *R. nuda* and *Anoxycalyx (Scolymastra) joubini*) by Remote Operated Vehicles (ROVs), and reported in Teixidó et al. (2006).

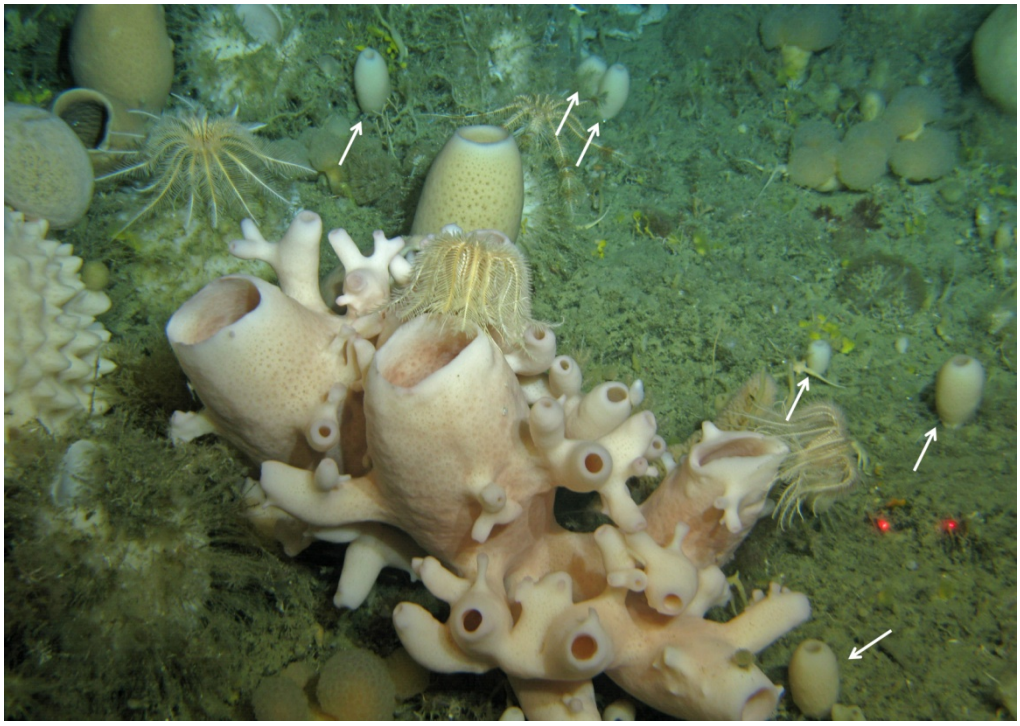

Figure 4.
Extensively budding in a *Rosella nuda* individual. See small settled individuals resulting from the species budding (arrows). Picture from Lündalv 2011.

As in Hexactinellida, high rates of clonal reproduction might also occur in Antarctic Demospongiae, in particular in species with dense, uniform in size populations (without

observable cohorts). *Stylocordyla chupachups* Uriz et al. 2011, is a Suberitida (Demospongiae), which forms this type of dense, uniform in size populations in the deep part of the Antarctic continental shelf (pers. observ.) and was selected as a case example to perform a population genetics' study, which could give us information of the extent of clonal reproduction in its populations. We used microsatellite markers, which are also named "simple sequence repeats" (SSRs) or short tandem repeats (STRs), and are among the most variable types of DNA sequence in the genome (Weber 1990; Li et al. 2002). Microsatellites have a high mutation rate due to a mismatch repair system deficiency (Ellegren 2004), and are considered useful to discriminate genotypes within a species population (Csilléry 2009). Since each microsatellite contains many mutation sites, they appear appropriate genetic markers for studies on population differentiation, gene flow, and clonality in sponges (Uriz & Turon 2012).

Many population genetics' studies were performed in marine ecosystems using microsatellites (e.g., Iglesias-Rodríguez et al. 2006; Renshaw et al. 2006; Knutsen et al. 2011), and these type of studies are sharply increasing on sponge populations in the last years (Uriz & Turon 2012; Pérez-Portela et al. 2014; González-Ramos et al. 2015; Guardiola et al. 2012, 2016; Riesgo et al. 2016; Li et al. 2018). However, up to now, no population studies employing microsatellites were done on Antarctic sponges.

The species *S. chupachups* was previously considered as *S. borealis* (Lovén, 1868), as it was though that the later species had a bipolar distribution (Sarà et al. 2002). Nevertheless, a deep morphological revision allowed confirming that Antarctic individuals of "*S. borealis"* belong indeed to a new species renamed as *Stylocordyla chupachups* (Uriz et. al 2011). The similar extreme conditions at both poles may have resulted in parallel adaptation and phenotypic convergence in the two species (Uriz et. al 2011).

Stylocordyla chupachups, as many other Antarctic species (e.g. *Rossella racovitzae*, *Cinachyra barbata*, *Antarctotetilla leptoderma*), forms dense-patchy populations (fig. 4) in some areas (Barthel & Gutt 1992; Gutt & Koltun 1995; Gatti 2002), and it is the first sponge species recolonizing extensive areas destroyed by iceberg scouring (Gutt 2000). This fast colonization of newly formed bare areas (figure 5) may likely be due to rapid development rates of the released propagula, which may consist to a high extent in both incubated, already metamorphosed individuals (Sarà et al. 2002) and asexually produced individuals.

Figure 5.

A *Stylocordyla chupachups* population on the Antarctic bottom (modified from Uriz et al. 2011).

OBJECTIVES

The main goal of this thesis is to increase the systematic and phylogenetic knowledge of a Demospongiae family (Tetillidae Sollas 1886), particularly widespread in the Antarctic bottoms (**Chapter 1** and **Chapter 2**), but which includes other species and genera distributed worldwide. The thesis also aims to approach the purported high asexual reproduction rate (**Chapter 3**) in Antarctic sponges by a population genetics' study of the species *Stylocordyla chupachups* Uriz et al. 2011, which forms dense populations in the Antarctic continental shelf. The specific objectives of each chapter are described below:

-**Chapter 1. Phylogenetic Reassessment of Antarctic Tetillidae (Demospongiae, Tetractinellida) Reveals New Genera and Genetic Similarity among Morphologically Distinct Species.** The goal of this chapter is to investigate the phylogenetic relationships between the Antarctic and tropical/temperate Tetillidae and to assess the purported endemism of its Antarctic genera. In this chapter we improve the previous molecular phylogeny (Szitenberg et al. 2013) by incorporating additional Antarctic and Sub-Antarctic specimens from geographically distant localities and a morphological based phylogeny.

-**Chapter 2. Description of two new genera (*Antarctotetilla, Levantiniella*) and a new species of Tetillidae.** The aim of the present chapter is to describe in detail the morphology of the new genera and species of Tetillidae retrieved in the phylogenetic study (Chapter 1). Moreover, we also re-examine the types of two unclear species (the syntype of *Tethya coactifera* Lendenfeld, 1907 and the holotype of *Tethya crassispicula* Lendenfeld, 1907), which clustered within the new *Antarctotetilla* genus in the previous phylogenetic study (Chapter 1). Furthermore, a new Antarctic Tetillid species, unidentified in the previous phylogeny (chapter 1), is described.

-**Chapter 3. Asexual reproduction and heterozygote selection in Antarctic demosponges approached by microsatellite analyses (*Stylocordyla chupachus*, Suberitida).** The goal of this chapter is to explore whether the high asexual reproduction recorded for several Antarctic Hexactinellid species (Teixidó et al. 2006), also extend to representatives of the Class Demospongiae. We choose *Stylocordyla chupachups* as a

case example since cohorts were hardly differentiated in its populations. For this study, we develop a set of microsatellite markers from the sponge genome and assess the extent of clonal reproduction in three populations of the target species.

CHAPTER 1

Phylogenetic Reassessment of Antarctic Tetillidae (Demospongiae, Tetractinellida) Reveals New Genera and Genetic Similarity among Morphologically Distinct Species

Abstract

Species of Tetillidae are distributed worldwide. However, some genera are unresolved and only a few genera and species of this family have been described from the Antarctic. The incorporation of 25 new COI and 18S sequences of Antarctic Tetillidae to those used recently for assessing the genera phylogeny, has allowed us to improve the resolution of some poorly resolved nodes and to confirm the monophyly of previously identified clades. Classical genera such as *Craniella* recovered their traditional diagnosis by moving the Antarctic Tetilla from *Craniella*, where they were placed in the previous family phylogeny, to *Antarctotetilla* gen. nov. The morphological re-examination of specimens used in the previous phylogeny and their comparison to the type material revealed misidentifications. The proposed monotypic new genus Levantinella had uncertain phylogenetic relationships depending on the gene partition used. Two more clades would require the inclusion of additionaspecies to be formally established as new genera. The parsimony tree based on morphological characters and the secondary structure of the 18S (V4 region) almost completely matched the COI M1-M6 and the COI+18S concatenated phylogenies. Morphological synapomorphies have been identified for the genera proposed. New 15 28S (D3-D5) and 11 COI I3-M11 partitions were exclusively sequenced for the Antarctic species subset. Remarkably, species within the Antarctic genera Cinachyra (*C. barbata* and *C. antarctica*) and *Antarctotetilla* (*A. leptoderma*, *A. grandis*, and *A. sagitta*), which are clearly distinguishable morphologically, were not genetically differentiated with any of the markers assayed. Thus, as it has been reported for other Antarctic sponges, both the mitochondrial and nuclear partitions used did not differentiate species that were well characterized morphologically. Antarctic Tetillidae offers a rare example of genetically cryptic (with the traditional markers used for sponges), morphologically distinct species.

Introduction

Sponges dominate some benthic communities in the Antarctic, in terms of both biomass (Belyaev & Ushakov 1975; Voss 1988) and diversity (Clarke & Johnston 2003). The Antarctic clockwise circumpolar current (Thomson et al. 1991) and the low water temperatures contribute to the biogeographic isolation of the Antarctic continental shelf, which partly explains the high degree of sponge endemism in the area (Sarà et al. 1992; McClintock et al. 2005; Ríos & Cristobo 2006). Taxonomic affinities between Antarctic sponges and those of the Magellanic region (South America) and the Falkland Islands have also been reported (Burton 1932; Sarà et al. 1992; Dayton et al. 1994) but the studies are still incomplete and subject to debate (Goodwin et al. 2014; Ríos & Cristobo 2006). Most of the currently known Antarctic sponge species were discovered during the oceanographic campaigns of the twentieth century (Lendenfeld 1907; Schulze & Kirkpatrick 1910; Hentschel 1914; Burton 1929, 1932, 1934; Kirkpatrick 1908; Koltun 1964, 1976; Topsent 1901, 1908, 1917; Boury-Esnault & Van Beveren 1982; Vacelet & Arnaud 1972). However, recent findings of many new species (Janussen et al. 2004; Rios & Cristobo 2006, 2007a, 2007b; Uriz et al. 2011; Plotkin et al. 2007; Göcke & Janussen 2011, 2013; Rapp et al. 2011, 2013) suggest that the sponge biodiversity of this area has not been fully explored yet and that more species remain to be discovered.

While collecting sponges during the Antarctic Polarstern ANT-XXVII/3 expedition in 2011, we realized the difficulty to identify the fairly common, well known, large conspicuous species belonging to the family Tetillidae Sollas, 1886. The World Porifera Database (WPD, http://www.marinespecies.org/porifera/) currently lists only four valid Tetillidae species from the Antarctic—*Cinachyra barbata* (Sollas, 1886), *Cinachyra antarctica* (Carter, 1872), *Tetilla leptoderma* (Sollas, 1886) and *Craniella sagitta* (Lendenfeld, 1907). Furthermore, intra-specific variations of the mitochondrial cytochrome c oxidase subunit 1 (COI), from Antarctic and New Zealand Tetillidae, also suggest that the diversity of this group is underestimated (Szitenberg et al. 2013). We also noticed that there is no consensus in the literature regarding the allocation of some Antarctic species to the genus *Craniella* or *Tetilla* (Rützler 1987; Van Soest & Rützler 2002; Szitenberg et al. 2013).

The family Tetillidae (Demospongiae, Tetractinellida, Spirophorina) contains 156 species distributed worldwide (Van Soest et al. 2016). Many of them inhabit sedimentary

bottoms to which they anchor by means of long spicule bundles, which represent a suitable substrate for many other hard bottom invertebrates (Gutt et al. 2013). Their representatives are characterized by a globular habit, a radiate skeleton composed chiefly of the following spicules: megascleres are protriaenes, oxeas, and sometimes ortho/anatriaenes or calthrops, which often protrude the ectosomal layer outward; microscleres are characteristic sigmaspires and occasionally raphides (Van Soest & Rützler 2002). To this day, the Tetillidae have no clear morphological synapomorphy, as triaenes are shared with all Tetractinellida, and sigmaspires are found in most Spirophorina families. The Tetillidae appears monophyletic with COI (Kelly & Cárdenas, 2016), but polyphyletic in 18S and 28S (C1-D2) phylogenies (Redmond et al. 2013; Schuster et al. 2015; Kelly & Cárdenas 2016).

Using the COI of 14 Tetillidae species, Szitenberg et al. (2010) suggested that most Tetillidae genera were not monophyletic. Later, Szitenberg et al. (2013), using this time a set of three molecular markers (COI, 28S and 18S) on 28 Tetillidae species belonging to eight genera, obtained five main clades (with COI and 28S): (i) *Acanthotetilla* (ii) *Cinachyrella*, *Paratetilla*, and *Amphitethya*, (iii) *Cinachyrella levantinensis*, (iv) tropical-temperate *Tetilla*, and (v) *Craniella*, *Cinachyra*, and *Fangophilina*. Results were similar with 18S, except that Acanthotetilla sequences were lacking from the NCBI genbank. One of the main issues raised by this study concerned the *Craniella*/*Cinachyra*/*Fangophilina* clade, which included all the Antarctic species. Results suggested the polyphyly of the genus *Craniella* distributed in three clades: (i) *Craniella* cf. *leptoderma* (Antarctic, New Zealand), (ii) *Craniella sagitta* (Antarctic, New Zealand) and (iii) a *Craniella* clade with boreo-arctic Atlantic species mixed with New Zealand/Australian species. Based on this polyphyly, Szitenberg et al. (2013) propose to reallocate the Antarctic *Tetilla*, *Fangophilina*, and *Cinachyra* to the genus *Craniella*, despite the absence of morphological support for such a proposal.

The goal of the current study was to investigate the relationships between the Antarctic and tropical/temperate Tetillidae to revise the taxonomy and phylogeny of the family and to assess the purported endemism of its Antarctic genera. We improved the sampling of previous phylogenies by incorporating additional Antarctic and Sub-Antarctic specimens from geographically distant localities. We used four gene partitions (mitochondrial and nuclear) to conduct molecular phylogenetic analyses. The morphology of new specimens, type species, and some specimens previously sequenced (Szitenberg et al. 2013) was examined. Finally, we also performed a maximum parsimony phylogenetic

analysis based on morphological characters and the secondary structure of the V4 region of the 18S rDNA.

Our findings confirm the monophyly of the Antarctic genera and allow us to erect two new Tetillidae genera: *Antarctotetilla* gen. nov., restricted to Antarctic and sub-Antarctic waters, and *Levantinella* gen. nov., so far limited to eastern Mediterranean. This study also resurrects a sub-Antarctic species and reveals four potential new species. The genetic homogeneity of the markers used among morphologically distinct Antarctic Tetillidae species, contrasts with the habitual finding of genetically distinct, morphologically cryptic species. A restricted geographical distribution restricted to particular regions of some Tetillidae genera has become evident.

Material and Methods

The collection of sponge samples was conducted in strict accordance with Spanish and European regulations under the rules of the Spanish National Research Council with the approval of the Directorate of Research of the Spanish Government. The study was found exempt from ethics approval by the ethics commission of the University of Barcelona since, according to article 3.1 of the European Union directive (2010/63/UE) from the 22/9/2010, no approval is needed for sponge sacrification, as they are the most primitive Animals and lack any nervous system Moreover, the collected sponges are not listed in CITES."

Sampled sponges

The majority of the samples were collected in Antarctic and sub-Antarctic regions during the Polarstern ANT-XXVII/3 expedition from Punta Arenas, Chile (February 8, 2011) to Cape Town, South Africa (18 April 2011) with Agassiz (AGT) and bottom trawl (BT) gears. During this expedition, Tetillidae were collected in South Georgia, South Orkneys Islands, and Newmayer in the Antarctic continent. A Remote Operated Vehicle (ROV) was deployed during the Polarstern cruise to gather samples between 300 and 450 meters and to photograph underwater living specimens. Given the large size (up to 30 cm in diameter) of most Antarctic Tetillidae, once the individuals were photographed, a fragment ca. 3 cm3 in size was preserved in absolute ethanol, which was changed three times before packing at -20°C for transportation and storage at the CEAB (Centre d'Estudis Avançats de Blanes, Spain). Other Tetillidae from this study were collected during the Collaborative East Antarctic Marine Census (CEAMARC) Dec. 2007-Jan. 2008 in Adélie Land, Antarctica (Beaman & O'Brien 2009; Hosie et al. 2011). The CEAMARC specimens were dredged between 170 and 1700 m; the complete specimens were bulk-fixed with ethanol (80%) in 60L metallic drums. A few samples also came from a fishery-independent biomass survey "POissons de KERguelen" (POKER II) conducted in 2010 on the Kerguelen Plateau. The complete specimens were collected using a Grande Ouverture Verticale (GOV) trawl, frozen on board and bulk-fixed in ethanol 80% at the Muséum National d'Histoire Naturelle (MNHN), Paris, France. Specimens from these two expeditions are housed at the MNHN and stored in the 'Zoothèque' at a constant 18°C temperature. Three additional samples were collected between Lavoisier and the Antarctic Peninsula between 847 and 960 m (R/V LM Gould, 2010) and were obtained from Bill Baker (University of South Florida). The samples used in this study, voucher numbers, Genbank accession numbers, and collecting localities are provided in Table 1.1.

Table 1.1. List of species used in the study, with collection reference number, accession number of the sequences stored in the Genebank, revised species name, and geographical origin. Reference numbers of individuals sequenced de novo in the current study are indicated in bold.

		Genbank accession numbers					
SPECIES	**Voucher number**	**COI**	**18S**	**COI I3-M11**	**28S (D3-D5)**	**Revised species name**	**Collection sites**
Tetilla leptoderma	ANT 27111	**KT124318**	**KT124341**	**KT124328**	**KT124362**	*Antarctotetilla leptoderma*	Sub Antarctic (South Georgia)
Tetilla leptoderma	ANT 27112	**KT124319**	**KT124343**	**KT124329**	**KT124365**	*Antarctotetilla leptoderma*	Antarctica (Newmayer)
Tetilla grandis	ANT 27123	**KT124324**	**KT124344**	**KT124330**	**KT124363**	*Antarctotetilla grandis*	Antarctica (Newmayer)
Tetilla grandis	ANT 27124	**KT124325**	**KT124346**	**KT124331**	**KT124364**	*Antarctotetilla grandis*	Antarctica (Newmayer)
Cinachyra barbata	ANT 27212	**KT124314**	**KT124356**	**KT124336**	–		Sub Antarctic (South Georgia)
Tetillidae	ANT 27211	**KT124313**	**KT124355**	–	**KT124361**	Tetillidae sp. 3	Sub Antarctic (South Orkneys)
Cinachyra antarctica	ANT 27204	**KT124317**	–	–	**KT124367**		Antarctica (Newmayer)
Tetilla leptoderma	ANT 27107	–	**KT124351**	–	**KT124358**	*Antarctotetilla leptoderma*	Antarctica (Newmayer)
Tetilla leptoderma	ANT 27108	**KT124323**	**KT124347**	–	–	*Antarctotetilla leptoderma*	Sub Antarctic (Souh Orkneys)
Tetilla leptoderma	ANT 27109	–	**KT124354**	–	–	*Antarctotetilla leptoderma*	Antarctica (Newmayer)
Cinachyra antartica	ANT 27223	–	**KT124353**	**KT124335**	**KT124368**		Antarctica (Newmayer)
Cinachyra barbata	ANT 27205	**KT124321**	**KT124340**	–	**KT124366**		Antarctica (Newmayer)
Tetilla leptoderma	ANT 27105	**KT124322**	**KT124348**	–	**KT124359**	*Antarctotetilla leptoderma*	Antarctica (Newmayer)
Tetilla leptoderma	ANT 27106	–	**KT124349**	–	**KT124360**	*Antarctotetilla leptoderma*	Antarctica (Newmayer)
Tetilla grandis	MNHN-Poker II-Chalut 32 sp.4	**KT124326**	**KT124345**	–	–	*Antarctotetilla grandis*	Sub Antarctic (Kerguelen)
Cinachyra antarctica	MC 7485	**KT124316**	**KT124339**	–	–		Between Lavoisier and Antarctica
CInachyra antarctica	MC 7486	**KT124315**	–	–	–		Between Lavoisier and Antarctica
Tetilla sagitta	MNHN-IP 2009 359	**KT124327**	–	**KT124334**	–	*Antarctotetilla sagitta*	Antarctica (Adelie Land)
Cinachyra barbata	MNHN-IP 2009 506a	**KT124312**	**KT124350**	–	–		Antarctica (Adelie Land)
Tetilla sagitta	MNHN-IP 2009 351	–	**KT124352**	**KT124333**	**KT124369**	*Antarctotetilla sagitta*	Antarctica (Adelie Land)
Cinachyra barbata	MNHN-IP 2009 387	–	**KT124342**	–	–		Antarctica (Adelie Land)
Tetilla sagitta	MNHN-IP 2009 31	**KT124320**	–	**KT124332**	**KT124370**	*Antarctotetilla sagitta*	Antarctica (Adelie Land)
Cinachyra barbata	NIWA 28877	JX177864	JX177977	–	–	*Cinachyra* cf. *barbata*	Antarctica (Oates land)
Cinachyra antarctica	NIWA 28951	JX177868	–	–	–		Antarctica (Oates land)
Cinachyra antarctica	NIWA 28957	JX177867	–	–	–		Antarctica (Oates land)
Cinachyra antarctica	QMG 311149	JX177914	–	–	–	*Cinachyra* sp.	Antarctica, Ross island (Mcmurdo base)

Craniella sagitta	NIWA 25206	JX177917	JX177981	–	–	Tetillidae sp.2	New Zealand (Chatham rise)
Craniella sagitta	NIWA 28491	JX177915	–	–	–	Tetillidae sp.2	New Zealand (Chatham rise)
Craniella sagitta	NIWA 28929	JX177863	–	–	–	Tetillidae sp.1	Antarctica (Oates land)
Craniella cf.*leptoderma*	NIWA 28910	JX177865	JX177982	–	–	*Antarctotetilla* cf. *grandis*	Antarctica (Oates land)
Craniella cf. *leptoderma*	NIWA 36097	JX177866	–	–	–	*Antarctotetilla grandis*	Antarctica (Ross island)
Craniella cf. *leptoderma*	NIWA 52077	JX177916	–	–	–	*Antarctotetilla leptoderma*	New Zealand (Chatham rise)
Craniella cf. *leptoderma*	NIWA 28496	JX177897	–	–	–	*Antarctotetilla leptoderma*	New Zealand (Chatham rise)
Craniella cf. *leptoderma*	NIWA 28524	JX177895	JX177976	_	_	*Antarctotetilla leptoderma*	New Zealand (Chatham rise)
Craniella cf.*leptoderma*	NIWA 28507	JX177896	JX177975	_	_	*Antarctotetilla leptoderma*	New Zealand (Chatham rise)
Craniella sp.	QMG 316342	HM032747	JX177983	**KT124337**	**KT124371**	*Cinachyra* sp.	Australia (South Norfolk ridge)
Craniella zetlandica	PC 252	KC122679	–	–	–		Røst reef, Norway
Craniella zetlandica	VM 14754	–	JX177986	–	–		Iceland
Craniella neocaledoniae	NIWA 28591	–	JX177984	–	–		New Zealand
Craniella sp.	QMG 318785	HM032752	JX177985	–	–		Australia (South Norfolk ridge)
Craniella sp.	BIOICE 3659	HM032750	–	–	–		Iceland
Craniella sp.	QMG 316372	HM032748	HE591469	**KT124338**	**KT124372**	*Cinachyra* sp.	Australia (South Norfolk ridge)
Craniella sp.	ZMBN 85240	HM592668	–	–	–	*Craniella* cf.*cranium*	Norway
Craniella cranium	ZMBN 85239	HM592669	–	–	–	*Craniella* aff. *zetlandica*	Norway
Fangophilina sp.	NIWA 28601	JX177919	JX177979	–	–	cf. *Fangophillina*	New Zealand (Challenger Plateau)
Fangophilina sp.	NIWA 28586	JX177918	JX177978	–	–	cf. *Fangophillina*	New Zealand (Challenger Plateau)
Fangophilina sp.	NIWA 28617	JX177912	JX177980	–	–	cf. *Fangophillina*	New Zealand (Challenger Plateau)
Paratetilla sp.	QMG 314224	HM032744	–	–	–		Australia (Curacoa Island)
Cinachyrella schulzei	QMG 320143	HM032746	–	–	–	*Cinachyrella* cf. *tenuiviolacea*	Australia (Keppel Islands)
Cinachyrella schulzei	QMG 320636	HM032745	JX177971	–	–	*Cinachyrella* cf. *tenuiviolacea*	Australia (Melanie Patches)
Cinachyrella apion		AJ843895	–	–	–		Bermuda
Cinachyrella sp.	TAU 25622	–	JX177962	–	–		Tanzania
Cinachyrella sp.	TAU 25621	HM032740	JX177964	–	–		Tanzania
Cinachyrella sp.	QMG 320270	HM032741	JX177963	–	–		Australia (Wellington point, Moreton Bay)
*Craniella*cf.*leptoderma*	QMG 315031	HM032749	JX177974	–	–	*Antarctotetilla* cf. *Sagitta*	Antartica (Casey Antartic Research Base)
Cinachyrella australiensis	QMG 321405	HM032743	–	–	–		Australia (Sunshine Coast)
Cinachyrella australiensis	QMG 320216	JX177902	JX177966	–	–		Australia (Keppel Islands)
Cinachyrella australiensis	QMG 320656	–	JX177968	–	–		Australia (Munro Reef, Coral Sea)
Cinachyrella australiensis	QMG 320656	–	JX177967	–	–		Australia

Cinachyrella apion	ZMBN 81789	HM592667	–	–	–		USA
Cinachyrella apion	SBP-B25	EF519601	–	–	–		Caribean Sea
Cinachyrella apion		FJ711645	–	–	–		Panama
Cinachyrella apion		–	AJ627186	–	–		Bermuda
Cinachyrella cf. *paterifera*	0M9H2022-P	–	KC902343	–	–		Australia
Cinachyrella sp.	USNM 1204826	–	KC901899	–	–		Panama
Cinachyrella sp.	USNM 1204829	–	KC902189	–	–		Panama (Bocas del Toro)
Cinachyrella alloclada	DH S271	JX177913	JX177965	–	–		Panama
Cinachyrella alloclada	USNM 1133831	–	KC902108	–	–		Panama
Cinachyrella alloclada	0M9G1250-W	–	KC902264	–	–		USA
Cinachyrella alloclada	TAU 25623	HM032738	–	–	–		Bahamas
Tetilla radiata	MNRJ 576	HM032742	–	–	–		Brazil (Rio De janeiro)
Tetilla murycii	UFBA 2586POR	JX177898	–	–	–		Brazil (Camamu Bay)
Cinachyrella levantinensis	TAU 25529	JX177906	JX177970	–	–	*Levantiniella levantinensis*	Lebanese Coasts
Cinachyrella levantinensis	TAU 25568	JX177904	JX177969	–	–	*Levantiniella levantinensis*	Lebanese Coasts
Cinachyrella levantinensis	MHNM 16194	JX177905	HM629803	–	–	*Levantiniella levantinensis*	Lebanese Coasts
Cinachyrella levantinensis	DH S124	JX177903	–	–	–	*Levantiniella levantinensis*	Lebanese Coasts
Cinachyrella levantinensis	TAU 25456	–	HM629802	–	–	*Levantiniella levantinensis*	Lebanese Coasts
Tetilla japonica		–	TTL18SR	–	–		Japan
Tetilla japonica	TAU 25619	JX177901	–	–	–		Japan
Cinachyrella sp.	SP.11	–	AY734439	–	–		Australia?
Cinachyrella sp.	SP.22	–	AY734437	–	–		Australia?
Cinachyrella sp.	SP.24	–	AY734438	–	–		Australia?
Cinachyrella kuekenthali	SBP-K75	EF519603	–	–	–		Caribean Sea
Cinachyrella kuekenthali	SBP-B79	EF519602	–	–	–		Caribean Sea
Cinachyrella kuekenthali		FJ711646	–	–	–		Panama
Cinachyrella kuekenthali		NC010198	–	–	–		USA
Cinachyrella kuekenthali		EU237479	–	–	–		USA
Paratetilla bacca	TAU 25620	JX177900	–	–	–		Thailand
Paratetilla bacca	LB 622	JX177894	–	–	–		Indonesia
Paratetilla bacca	LB 671	JX177893	JX177972	–	–		Indonesia
Paratetilla bacca	0M9H2290-H	–	KC902195	–	–		Australia
Amphitethya cf. *microsigma*	SAM S1189	JX177910	–	–	–	*Amphitethya microsigma*	South Australia?

Acanthotetilla celebensis	RMNH POR 2877	JX177893	–	–	–	Indonesia
Acanthotetilla walteri	UFBA 2021	JX177907	–	–	–	Brazil
Acanthotetilla seychellensis	0CDN 8107-V	–	KC902033	–	–	American Samoa
Cinachyrella kuekenthali	USNM 1133786	–	KC902290	–	–	Panama
Cinachyrella kuekenthali		–	EU702414	–	–	USA
Cinachyra sp.	0CDN 8726-T	–	KC902124	–	–	Guam
Craniella sp.	0CDN 5142-X	–	KC902265	–	–	Philippines
Geodia cydonium		–	AY348878	–	–	Mediterranean Sea
Geodia cydonium	ZMA POR 21652	HM592738	–	–	–	Portugal
Geodia neptuni		–	AY737635	–	–	Caribean Sea
Thenea levis	ZMBN 85230	HM592717	–	–	–	Norway
Theonella swinhoei	ZMA POR 16637	HM592745	–	–	–	Egypt

Abbreviations: BIOICE, The inter-Nordic BIO-Iceland project; DH, LB, personnal collections of Dorothée Huchon and Lisa Becking; MC, National Museums, Northern Ireland, Holywood; MHNM, Muséum d'Histoire Naturelle Palais Longchamp, Marseille, France; MNHM, Muséum National d'Histoire Naturelle, Paris, France; MNRJ – Museu Nacional do Rio de Janeiro, Brazil; NIWA, National Institute of Water & Atmospheric Research, New Zealand; PC, personal collection, University of Bergen, Norway; QMG, Queensland Museum, Australia; RMNH, Rijksmuseum van Natuurlijke Historie, Leiden, Nederland; SAM, South Australian Museum, Australia; SBP, Sponge Barcoding Project (http://www.palaeontologie.geo.uni-muenchen.de/SBP/);TAU, Steinhardt National Collection of Natural History, Zoological Museum at Tel Aviv University, Israel; UFBA, Universidade Federal da Bahia, Brazil; USNM, United States National Museum, U.S.A.;VM, Museum of Natural History and Archaeology, a part of the University of Science and Technology, Trondheim, Norway; ZMA, Zoölogisch Museum van de Universiteit van Amsterdam, Holland; ZMBN, Zoologisk Museum, Bergen, Norway;0CDN,0M9G, Smithsonian Institution/National Museum of Natural History, U.S.A.

Additional specimens of particular interest to obtain a more comprehensible sampling for our taxonomic study and to verify previous identifications were obtained on loan from several institutions: paratype of *Fangophilina submersa* Schmidt, 1880 (MSZ.PO160, Musée Zoologique de Strasbourg, France); paratype of *Craniella quirimure* Peixinho, Cosme, Hajdu, 2005 (MNRJ 8417,Museu nacional/UFRG, Brazil); paratype of *Tetilla radiate* Selenka, 1879 (MNRJ 576, Museu nacional/UFRG, Brazil); *Tetilla muricyi* Fernandez, Peixinho, Pinheiro, Menegola, 2011 (UFBA 2569,Museu de História Natural da Bahia, Brazil*); Craniella* sp. (QMG 316342, and QMG 316372, Queensland Museum, Brisbane, Australia); nine individuals of *Cinachyrella levantinensis* Vacelet, Bitar, Carteron, Zibrowius, Pérez, 2007 from Lebanon (06/07/2003-1 and 31/07/2003-2, Station Marine d'Endoume, Marseille) and seven specimens collected across the shore ofMa'agan Michael, Israel (courtesy of Jean Vacelet); *Craniella sagitta* Lendenfeld, 1907 (syn. *Tethya sagitta*) (NIWA 28491 and NIWA28929 National Institute of Water & Atmospheric Research, New Zealand); a small piece of the syntype of *Tethya sagitta* Lendenfeld, 1907 (ZMB Por 3504, Museum fürNaturkunde Leibniz, Germany); *Fangophilina* sp. (NIWA 28601 National Institute of Water & Atmospheric Research, New Zealand). Moreover, several individuals from Szitenberg et al. (2013) were re-examined from photographs or specimens (see Results).

Selected outgroups for the phylogenetic analyses, which mainly aimed at establishing relationships among genera, belonged to the Astrophorina (families Geodiidae and Theneidae) since previous molecular phylogenies of Demospongiae based on mitochondrial (Lavrov et al. 2008; Kelly & Cárdenas, 2016) and nuclear (Borchiellini et al. 2004; Nichols, 2005) genes placed Astrophorina either as a sister clade of the Tetillidae (COI), or sister to some Tetillidae (18S, 28S).

DNA extraction, amplification and sequencing

Genomic DNA was extracted according to the manufacturer's protocol for the DNeasy Blood & Tissue kit (Qiagen). Two mitochondrial markers were sequenced, both from COI: the M1-M6 partition, using primers LCO1490 and HCO2198 (Folmer et al. 1994) and the I3-M11 partition, using primers PorCOI2 fwd. and PorCOI2 rev. (Xavier et al. 2010). Two nuclear markers were also sequenced: 18S, using primers 1F and 1795R, (Medlin et al. 1988) and the D3-D5 partition of 28S, using primers Por28S-830Fand Por28S-1520R (Morrow et al. 2012). Different amplification protocols were performed for each marker: 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); COI I3-M11 partition (95°C, 3 min, [94°C, 30 s, 57°C, 45 s, 72°C, 90 s] x 35–40cycles, 72°C, 10 min); 18S (94°C, 5 min, [94°C, 1 min, 50–55°C, 1 min, 72°C, 1 min] x35-40 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 H20, 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. Amplifications of the COI I3-M11 partition were performed in a 50 µL volume reaction, containing 34,45 µL H20, 5 µL buffer (INVITROGEN), 0,75 µL MgCl (INVITROGEN), 2,4 µL DMSO (dimethyl sulfoxide), 2 µL BSA, 2 dNTP (Sigma), 1 µL primers forward/reverse, 0,4 Taq (INVITROGEN) and 1 µL of genomic DNA. Amplifications of 18S rRNA were performed in a 50 µL volume reaction, containing 36,85 µL H20, 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 H20, 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. Purified PCR products were sequenced in both directions using Applied Biosystems 3730xl DNA analyzers (Macrogen, South Korea).

Sequence alignment and phylogenetic reconstructions

Once the poriferan origin of the obtained sequences was verified using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), sequences were aligned using ClustalWv.1.81 (Thompson et al. 1994). In cases where the forward and reverse reads do not match, we used BioEdit v.7.2.5 (Hall 1999) and kept either the best 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 the mitochondrial and nuclear genes under the Akaike information criterium. Phylogenetic trees were constructed under 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 PhyMLv3.0 program (Guindon & Gascuel 2003, Guindon et al. 2005). We assessed the robustness of the tree clades in PhyML by a nonparametric bootstrap resampling with 1000 replicates.

Incongruence Length Difference (ILD) test (PAUP 4.0b10) was run (Swofford 2002) to verify sequence homogeneity or incongruence between the 18S and COI markers. The ILD test indicated no significant conflict ($p = 0.93$) between the two markers so a concatenated 18S-COI dataset was constructed with the species for which we had sequences for both markers.

18S rRNA secondary structure and morphological analysis

RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) (Zuker & Stiegler 1981; Zuker 2003) was used to determine the predicted secondary structure of the 18S V4 variable region for all species following Voigt et al. (2008). We used the default setting for all parameters except for the folding temperature. As the specimens came from different localities, we fixed the folding temperatures according to that of the specimen locality. However, the specimens belonging to *Geodia* spp., which were used as outgroups, lived in locations with contrasting temperatures. Only in this case, we used the default setting of 37°C (Gazave et al. 2010). The program automatically chooses the secondary structures with the lowest free energy (dG in kcal/mol) (Voigt et al. 2008). Following Gazave et al. (2013), we encoded the different parts of the predicted secondary structures as elements and treated them as binary characters (presence/absence) in the morphological matrix. As the V4 of 18S secondary structure motifs were conserved across genera, according to the species sequenced, we assumed that the few species for which the 18S sequence was not available, shared the secondary structure motifs of the genus. The morphological/secondary structure matrix, consisting of 26 morphological characters and 13 18S secondary structure motifs, is made available in S1 File. A phylogenetic-tree was built with the morphological matrix under the maximum parsimony (MP) criterion using PAUP 4.0b10 (Swofford 2002) using a heuristic search and the branch swapping method with the tree bisection and reconnection (TBR) algorithm and ACCTRAN character-state optimization.

Results

Mitochondrial COI

The COI (M1-M6 partition) dataset comprised 70 sequences (16 new) of 537 nucleotides (nt.) (158 nt. variable, of which 148 nt. were parsimony informative). Phylogenetic trees from ML and BI analyses retrieved congruent topologies, although some clades were differently supported (Figure 1.1). The Antarctic/New Zealand Tetillidae clustered in a well-supported group (93/1), which split in three well-defined clades henceforth called clades 1, 2 and 3.

Clade 1 (80/0.95, ML bootstrap supporting values /BI posterior probability) contained all the species of *Cinachyra* sequenced plus an unidentified species named Tetillidae ANT 27211.

Clade 2 (88/0.95) clustered individuals of *Tetilla grandis*, *T. sagitta*, and *T. leptoderma*, including those individuals called *Craniella leptoderma* in previous phylogenies. The position of three *Craniella sagitta* sequences (NIWA 25206, 28491, and 28929) was unresolved. These specimens were placed clearly apart from our *Tetilla sagitta* specimens from Antarctica, which morphologically conformed to the type species. Those three sequences belonged to two haplotypes with a difference of 8 nt.: NIWA 28491 and NIWA 25206 specimens from New Zealand versus NIWA 28929 from Antarctica.

Clade 3 (-/0.91) included the sequences of *Fangophilina* sp. (NIWA 28601, NIWA 28586, and NIWA 28617) and *Craniella* sp. (QMG 316342 and 316372), and was retrieved only in BI analyses.

Clade 4 (81/1) was a sister group of Antarctic sponges and included non-Antarctic/New Zealand species of *Craniella*.

Clade 5 (100/1) contained the non-Antarctic *Tetilla* (i.e. from tropical seas).

Clade 6 (98/1) included sequences of *Cinachyrella levantinensis* from the eastern Mediterranean, and was clearly apart from the rest of the *Cinachyrella* species.

Clade 7 (84/0.99) consisted of *Cinachyrella* species from tropical and subtropical waters and was divided in two well-supported sub-clades (posterior

probability 0.80 and 0.84): the first included *C. australiensis*, *C. kuekenthali*, *Amphitethya* cf. *microsigma*, and *C. apion*, while the second included *C. alloclada*, *Cinachyrella* sp., *C. schulzei*, and *Paratetilla bacca*. The latter two species clustered together (100/1).

Clade 8 (100/1), included two species of *Acanthotetilla*, and appeared as the sister group of the remaining Tetillidae.

Almost no intra-species variation was found for the M1-M6 partition for the Antarctic genera, with the notable exception of two individuals: *Cinachyra barbata* (JX177864), which differed in 3 nt. from the other *Cinachyra* sequences, and *Craniella* cf. *leptoderma* (JX177865), which differed in 2 nt. from the other *Tetilla*/*Craniella* sequences (Figure 1.1).

We obtained 11 new sequences of the COI I3-M11 partition, 614 nt. long (11 nt. Variable and parsimony informative). This partition (Figure 1.2), although it has been considered more variable than the M1-M6 partition (Erpenbeck et al. 2006), failed to reveal any difference among the Antarctic species of *Cinachyra* or *Tetilla*/*Craniella*.

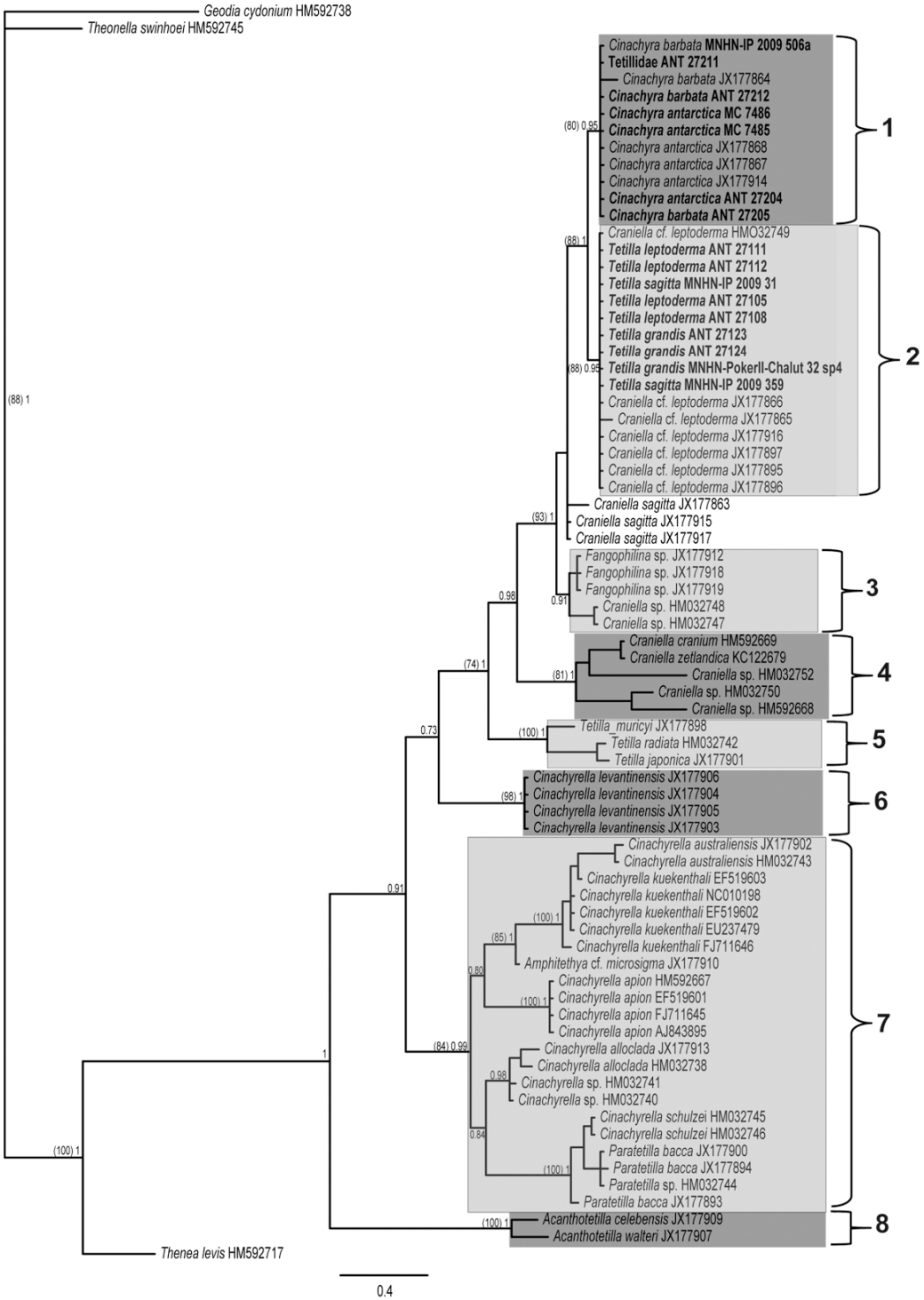

Figure 1.1. COI M1-M6, BI phylogeny of Tetillidae, which was congruent with ML tree. Species names are followed by their accession numbers (sequences downloaded from Genbank) or the specimen reference. Individuals sequenced in this study are in bold. Only supporting values higher than 70% (ML bootstrap, between parentheses on the left) or 0.75 (BI posterior probability) are represented on the tree nodes.

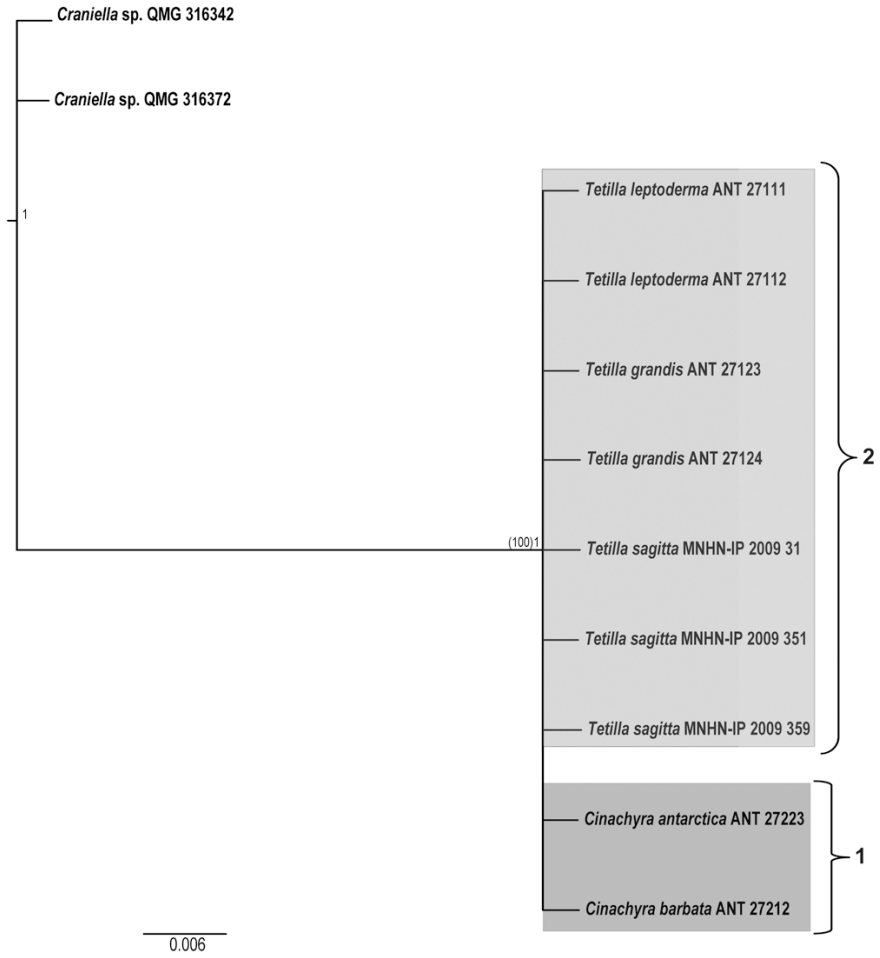

Figure 1.2. COI I3-M11 BI phylogeny of the Antarctic individuals of Tetillidae, which was congruent with ML tree, showing no clear separation between Cinachyra and Antarctotetilla. Bootstrapping and posterior probability (ML and BI, respectively) values are represented on the node of the only resulting clade. Individuals sequenced in the current study are indicated in bold.

Nuclear 18S rRNA and 28rDNA

The full-length 18S dataset comprised 48 sequences (19 new) of 1483 nt. (83 nt. variable, of which 60 nt. were parsimony informative). ML and BI analyses gave congruent topologies (Figure 1.3). These trees recovered the same clades as the COI tree, except for the absence of clade 8, since no sequences of *Acanthotetilla* were available for this marker, and clade 2. Like in the COI analyses, 18S failed to discriminate among species of the Antarctic *Tetilla* (including species named *Craniella* in previous phylogenies) or *Cinachyra*. As in the COI phylogeny, the Antarctic/New Zealand Tetillidae clustered in a clade (75/0.94) comprising *Cinachyra* spp., *Tetilla* spp, *Craniella sagitta* (NIWA 25206), *Fangophilina* spp., and *Craniella* sp. (QMG 316342 and 316372) representatives.

Clade 1 (-/0.99) contained the species that split in clades 1 and 2 in COI phylogeny. The specimen Tetillidae ANT27211 groups this time with *Tetilla* spp. and not with *Cinachyra* spp., as in the COI phylogeny.

Clade 3 (-/0.76) comprises *Fangophilina* sp. plus *Craniella* sp. (QMG 316342 and 316372) and *Craniella sagitta* (NIWA 25206) as in the COI phylogeny but without statistical support.

Clade 4 (97/1) included the same *Craniella* species as in the COI phylogeny plus *C. neocaledoniae*, a species absent from the COI sampling.

Clade 5 (100/1) encompassed non-antarctic *Tetilla* (i.e. from tropical seas).

Clade 6 (95/0.97) was formed exclusively by *C. levantinensis* sequences. This clade was sister to clade 7 whereas it was sister to clade 1–5 in the COI tree.

Clade 7 (95/0.78), as in the COI tree, clustered all *Cinachyrella* species plus *Paratetilla bacca*. The 28S rRNA gene (D3-D5 partition) comprised 15 new sequences of 650 nt. (11 nt. variables, of which 10 nt. were parsimony informative). Phylogenetic trees were consistent in ML and BI analyses (Figure 1.4). Species within any of these two genera (*Cinachyra* and *Tetilla*/*Craniella*) were not discriminated.

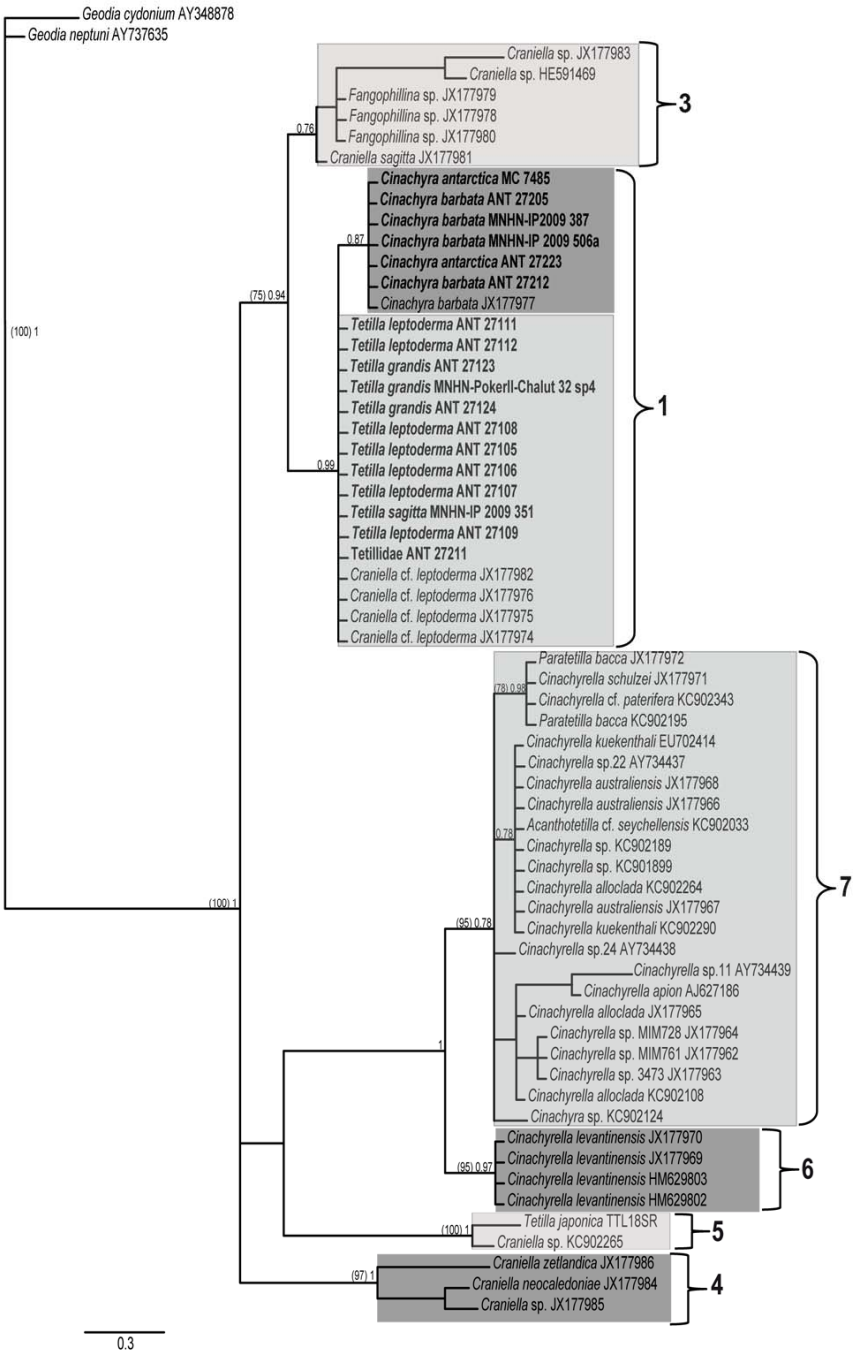

Figure 1.3. 18S rRNA BI phylogeny of Tetillidae, which was congruent with ML tree. Species names are followed by the accession numbers (sequences downloaded from Genbank) or the specimen reference. Individuals sequenced in this study are in bold). Only supporting values higher than 70% (ML bootstrap, between parentheses on the left) or 0.75 (BI posterior probability) are represented on the tree nodes.

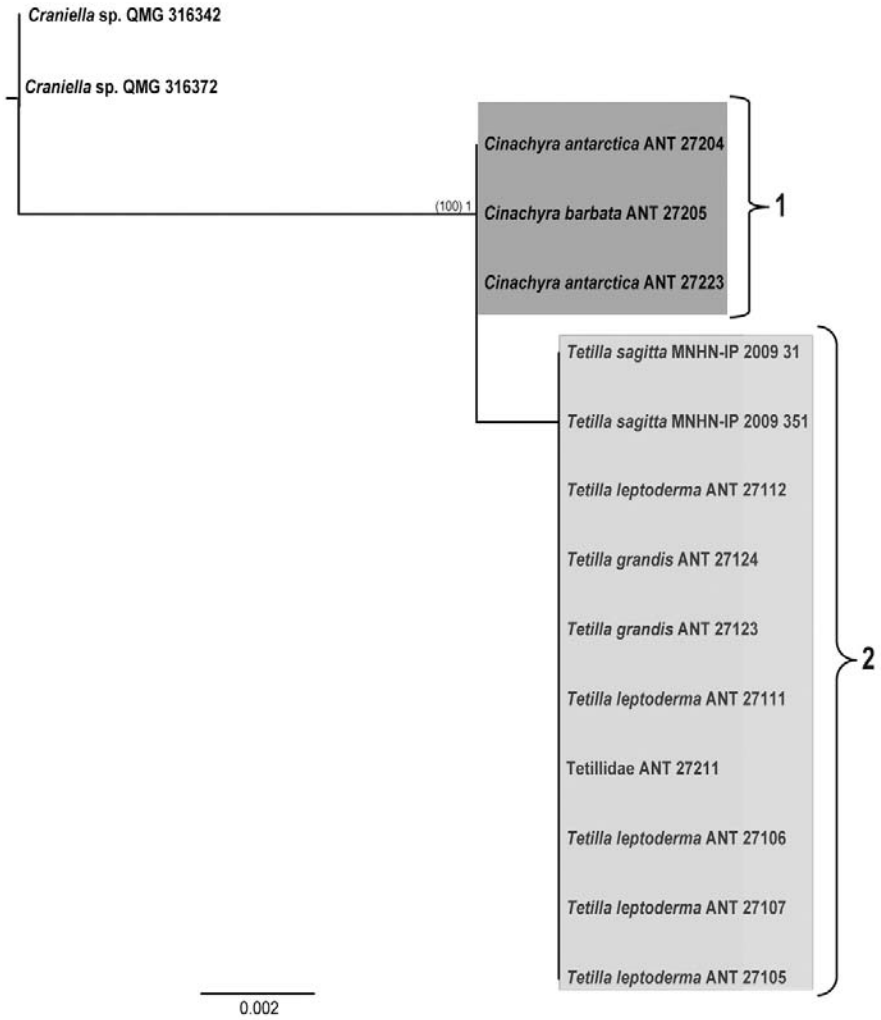

Figure 1.4. 28S (D3-D5) BI and ML phylogeny of the Antarctic individuals of Tetillidae, which was congruent with ML tree, showing no species differences within the genera *Cinachyra* and *Antarctotetilla*. Species names are followed by the accession numbers (sequences downloaded from Genbank) or the specimen reference. Individuals sequenced in this study are in bold. Only supporting values higher than 70% (ML bootstrap, between parentheses on the left) or 0.75 (BI posterior probability) are represented on the tree nodes.

Concatenated COI and 18S rRNA

The dataset for the concatenated mitochondrial and nuclear partitions (COI M1-M6 partition and 18S) comprised 39 sequences of 2019 nt. The resulting phylogenetic trees were consistent in ML and BI analyses (Figure 1.5) and were for the most part similar to the COI tree (i.e. clades 1, 3, 4, 5, and 7), except for clade 6, which was shared only with the 18S tree (Figure 1.3). On the other hand, contrarily to 18S phylogeny, clade 2 was well supported (87/0.92). Clade 3 was similar to both COI and 18S phylogenies. The supporting values of the clades slightly varied in some cases with respect to those of the previous phylogenies.

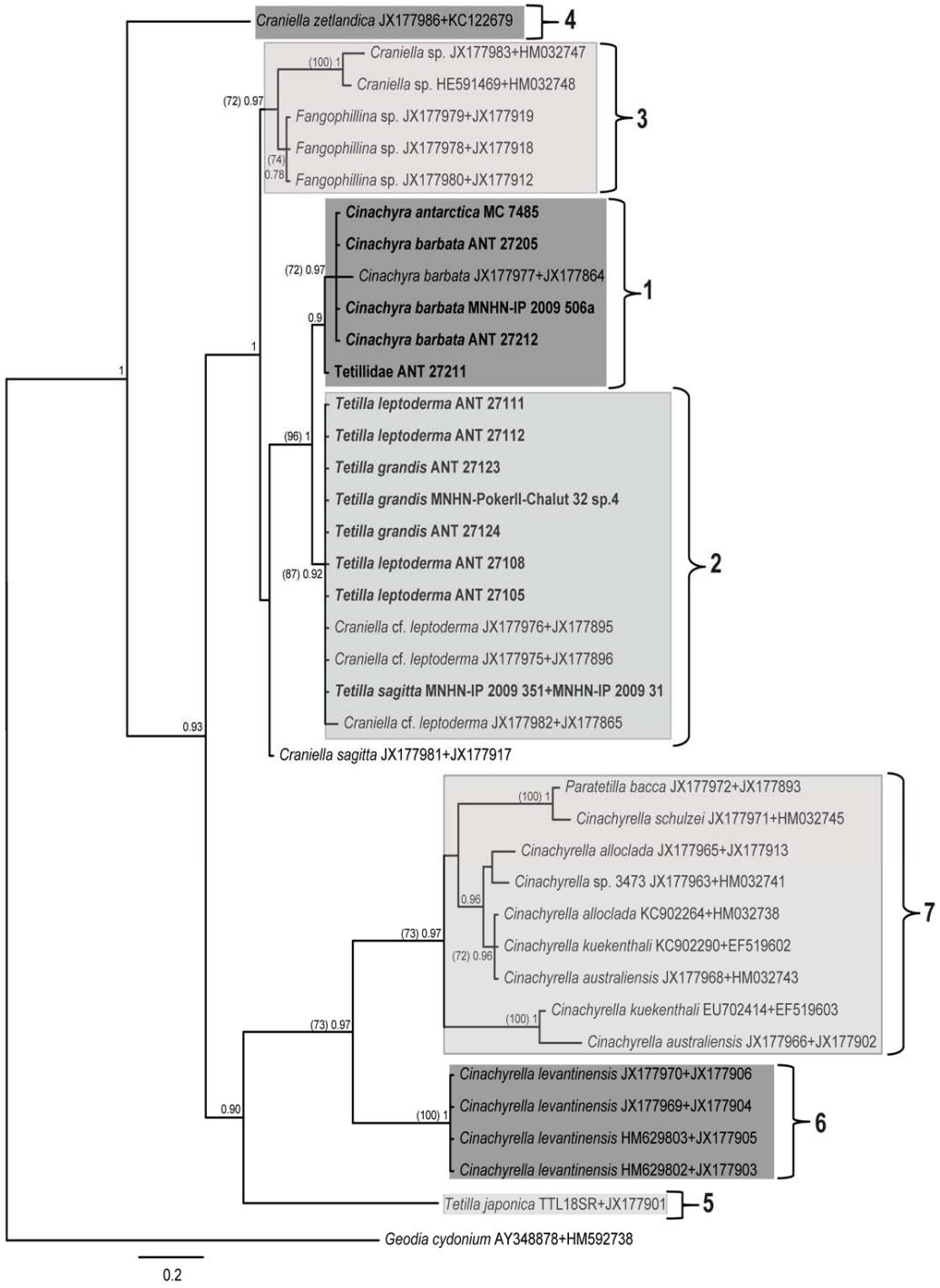

Figure 1.5. 18S rRNA–COI M1-M6 concatenate BI phylogeny of Tetillidae, which was congruent with ML tree. Species names are followed by the accession numbers (sequences downloaded from Genebank) or the specimen reference. Individuals sequenced in this study are in bold. Only supporting values higher than 70% (ML bootstrap, between parentheses on the left) or 0.75 (BI posterior probability) are represented on the tree nodes.

Morphological identifications and re-examination of specimens

To understand our phylogenetic results, which were fairly congruent with both nuclear and mitochondrial genes, we examined the morphology of our specimens, several holotypes and also some specimens previously sequenced by Szitenberg et al. (2013). The resulting decisions from our examinations are detailed below and summarized in Table 1.1 and Table 1.2.

Clade 1 comprised individuals that belonged to the *Cinachyra* genus. Most of these individuals were morphologically similar except for the specimen of *C. barbata* (NIWA 28877), and that of *Cinachyra antarctica* (QMG 311149). The former differed from the other *C. barbata* in 3 nt, and pictures of the specimen (courtesy of M. Kelly) show porocalices spread on the sponge body instead of being concentrated on the lateral zone: we decided to name it *Cinachyra* cf. *barbata* until the specimen could be studied. Underwater pictures (courtesy of J. Hooper) of *Cinachyra antarctica* (QMG 311149) show a hairy surface covered with sediments with high palisades of spicules just around the openings: we tentatively renamed it as *Cinachyra* sp.

Clade 2 included *Tetilla*/*Craniella* specimens from Antarctica/New Zealand. These specimens belonged to three species (*leptoderma*, *sagitta* and *grandis*) that had been formally placed either in the genus *Tetilla* or the genus *Craniella* by previous authors. However, these species did not have the characteristic conspicuous double-layered cortex of *Craniella* (Van Soest & Rützler 2002). Instead, they all had a loose arrangement of cortical oxeas perpendicular to the surface, which we will henceforth call 'pseudocortex' (Figures 1.6F and 1.6H and 1.7B and 1.7D). Therefore these species cannot belong to Tetilla either, which lacks a cortical specialization. Moreover, all these species have pores clustered in small, depressed areas and their oscula, single or multiple, are usually larger than in typical *Craniella*. Finally, they never harbored direct developing embryos as observed in both typical *Craniella* and some *Tetilla*. All these characteristics prompted us to erect a new genus for these three species: *Antarctotetilla* gen. nov. (see definition below). We will henceforth call these species *Antarctotetilla leptoderma*, *Antarctotetilla sagitta* and *Antarctotetilla grandis*. The latter had been synonymized with *A. leptoderma* (Burton, 1929; Boury-Esnault & Van Beveren, 1982) but it is clearly

different from *A. leptoderma* as it has several small oscula and a spherical body while *A. leptoderma* is slightly elongate/ovoid body with a sole large oscule on top. Therefore we propose to officially resurrect this species so far only recorded from the Antarctic and Sub-Antarctic.

Five out of the eight specimens of *Craniella* cf. *leptoderma* from Szitenberg et al. (2013) were confirmed morphologically to be *A. leptoderma*. Pictures of NIWA 28910 and NIWA 36097 showed that these specimens had a smooth surface and multiple small oscula, which matched the morphology of *Antarctotetilla grandis*. Finally, underwater pictures of QMG 315031 showed that the specimen had at least two oscula, which differs from the large single oscule on top, constantly present in *A. leptoderma*. QMG 315031 was therefore tentatively re-identified as *Antarctotetilla* cf. *sagitta*.

The three specimens of *Craniella sagitta* (NIWA 28491, NIWA 25206, and NIWA 28929 (Table 1.1) were examined (see pictures of NIWA 28491 in Figure 1.7F) and compared with the syntype of *Tethya sagitta* (ZMB Por 3504). These three specimens possessed a pseudocortex but lacked the main diagnostic characters of the species, such as pores grouped in sieve-like areas and oscula on the top of smooth flattened zones (Figures 1.7C and 1.7E) (Kirkpatrick, 1908). Thus, they cannot be identified with *A. sagitta* but we are unsure to which species or even genera they should belong. We could distinguish two morphotypes that corresponded to two haplotypes (differing in 8 nt.), which suggests that they represent two different species, here named Tetillidae sp. 1 and 2. Interestingly, these specimens were originally identified as two different species based on external morphology (Table 1.2).

Clade 3 included two misidentified genera: *Craniella* sp. (QMG 316342 and QMG 316372) and *Fangophilina* sp. (NIWA 28601, NIWA 28586, and NIWA 28617). The individuals called *Craniella* sp. from the Norfolk Ridge (Figure 1.8A), had porocalices and a spiculous cortex, similar to that of *Cinachyra*. They are therefore tentatively identified as *Cinachyra* sp. As for *Fangophilina* sp., its morphology was different from the species type of *Fangophilina*, *F. submersa* (Figure 1.7H). However, the small size of these specimens (ca. 1cm in diameter) prevented us to verify whether the only visible orifice was a true porocalyx or an oscule and thus they have been provisionally named cf. *Fangophilina* sp.

Clade 4 included *Craniella* species from the Boreo-Arctic Atlantic and the Norfolk ridge. We confirm that they possessed the typical double-layered cortex of *Craniella* (Figure 1.9D), and were thus considered true *Craniella*. *Craniella cranium* Müller, 1776 (ZMBN 85239) and *Craniella* sp. (ZMBN 85240) from Korsfjord-Norway (Cárdenas et al. 2011) were reexamined. ZMBN 85239 was reidentified as *Craniella* aff. *zetlandica* since it only differed from *C. zetlandica* Carter, 1872 in the presence of sigmaspires. ZMBN 85240 was re-identified as *Craniella* cf. *cranium* because it closely resembled *Craniella pilosa* Montagu, 1818, a synonym of *C. cranium* (Figures 1.8B and 1.8C). However, we keep the 'cf.' for now, until a world-wide revision of *C. cranium* can be made, this species having a long and complicated taxonomic history.

Clade 5 contained Tetilla species, which are characterized by the absence of a true cortical structure. Re-examination of a picture of the type species, *T. euplocamus* (Figure 1.8D), and the types of *T. muricyi* Fernandez, Peixinho, Pinheiro, and Menegola, 2011 (Figures 1.8E and 1.8F) and *T. radiate* Selenka, 1879, under the stereomicroscope seems to confirm previous studies of *Tetilla radiata* Santos and Hajdu, 2003 (Figures 5 and 6), and *T. muricy* Fernandez et al. 2011 (Figures 4C and 4D), which showed the absence of a cortical specialization in these Tetilla. However, a loose layer of para-tangential large oxeas below the surface may be present in some cases, as it has been reported for *T. rodriguezi* Fernandez et al. 2011 (Figure 6C).

Clade 6 only contained *Cinachyrella levantinesis*. We re-examined nine specimens of *C. levantinensis* trying to understand why they did not group with any of the other *Cinachyrella* species in the molecular trees. *C. levantinensis* lacks cortex as *Cinachyrella* (Figures 1.8H and 1.9E). Its surface is mostly covered with a dense layer of sand, which was retained by the protruding spicules (Figure 1.8G) as in some *Cinachyrella* (Figure 1.9F). However, while *Cinachyrella* species have typical large porocalices, *C. levantinensis* has numerous small rounded depressions, difficult to assign to porocalices with certainty. As stated by Vacelet et al. (2007), these depressions were not visible in specimens heavily covered by sand. These depressions are sometimes concentrated in sand free lateral areas, whereas usually porocalices are evenly distributed in typical *Cinachyrella*. All these characteristics prompted us to

officially create *Levantiniella* gen. nov. (see definition below) to welcome this species.

Clade 7 contained only specimens of *Cinachyrella*. Some individuals of dubious identification were revised (Table 1.2). *Amphitethya* cf. *microsigma* (SAM-S1189) from Szitenberg et al. (2013) showed an external morphology and spicules corresponding to the original description of *A. microsigma* Lendenfeld, 1907. Furthermore the individual SAM-S1189 was collected in southern Australia, not far from the type locality of *A. microsigma*. We therefore confirmed the species identification. Using the online SpongeMaps (http://www.spongemaps.org), we also examined underwater pictures of *Cinachyrella schulzei* Keller, 1891 (QMG 320143, QMG 320636) from Szitenberg et al. (2010, 2013). These specimens are reddish pink (QMG 320636) to pale pink (QMG 320143), and completely devoid of sand, in contrast to the typical yellowish-grey color of *C. schulzei*, which is often covered with sand (Lévi et al. 1998). These specimens may instead be conspecific with *Cinachyrella tenuiviolacea* Pulitzer-Finali, 1982, which has a typical pink color and is fairly common in Australian shallow waters and were here provisionally referred to as *C*. cf. *tenuiviolacea*.

Table 1.2. Original and revised identifications of Tetillidae voucher specimens from previous studies (Cárdenas et al., 2011, Szitenberg et al., 2010, 2013) after morphological re-examination.

Original identification	Szitenberg et al., 2013	Voucher	Locality	Depth (m)	COI Genbank accession #	External features	Identification after revision
Cinachyra* cf. *antarctica	***Craniella sagitta***	**NIWA 28929**	**Antarctica, -71.15, 171.17**	**1158-1165**	**JX177863**	**Dense hispidity.**	**Tetillidae sp. 1**
Craniella sagitta microsigma	***Craniella sagitta***	**NIWA 28491**	**New Zealand, Chatham Rise -45.04, 175.48**	**1239-1251**	**JX177915**	**Dense hispidity, oscules not visible.**	**Tetillidae sp. 2**
Craniella sagitta microsigma	*Craniella sagitta*	NIWA 25206	New Zealand, Chatham Rise -42.79, 179.98	925-1024	JX177917	Very tiny piece.	Tetillidae sp. 2
Craniella cf. *leptoderma*	*Craniella* cf. *leptoderma*	NIWA 27816*	New Zealand, Chatham Rise -43.86, 177.65	582-592	–	Single oscule on top.	*Antarctotetilla leptoderma*
Craniella cf. *leptoderma*	*Craniella* cf. *leptoderma*	NIWA 36097*	Antarctica -75.63, 169.85	525-530	JX177866	Multiple oscules on top.	*Antarctotetilla grandis*
Craniella cf. *leptoderma*	*Craniella* cf. *leptoderma*	NIWA 28910*	Antarctica 71.3°S, 170.5°E	312-323	JX177865	Multiple oscules on top.	*Antarctotetilla* cf. *grandis*
Craniella leptoderma	*Craniella* cf. *leptoderma*	NIWA 28524*	New Zealand, Chatham Rise -44.6, 178.4	1230-1241	JX177895	Single oscule on top.	*Antarctotetilla leptoderma*
Craniella leptoderma	*Craniella* cf. *leptoderma*	NIWA 28507*	New Zealand. Chatham Rise -44.4813347, 177.1430054	1230-1235	JX177896	Single oscule on top.	*Antarctotetilla leptoderma*
Craniella leptoderma	*Craniella* cf. *leptoderma*	NIWA 52077*	New Zealand, Chatham Rise -44.1, 174.4	576-578	JX177916	Single oscule on top.	*Antarctotetilla leptoderma*
Craniella leptoderma	*Craniella* cf. *leptoderma*	NIWA 28496*	New Zealand, Chatham Rise	1238-1258	JX177897	Dissociated spicule mass.	*Antarctotetilla leptoderma*

			-45.0583344, 175.4743347				
Cinachyra barbata	*Cinachyra barbata*	NIWA 28877*	Antarctica, Ross Sea -72.1279983521, 172.700668335	496-501	JX177864	Multiple oscules all over surface, cortex.	*Cinachyra* cf. *barbata*
Cinachyra antarctica	*Cinachyra antarctica*	NIWA 28951*	Antarctica -71.7, 171.14	236-240	JX177868		*Cinachyra antarctica*
Cinachyra antarctica	*Cinachyra antarctica*	NIWA 28957*	Antarctica -71.7, 170.94	127-140	JX177867		*Cinachyra antarctica*
Craniella cf. *leptoderma*	*Craniella* cf. *leptoderma*	QMG 315031*	Antarctica, Casey Antarctic Research Base.	shallow	HM032749	Globular hispid, two large oscules, no cortex.	*Antarctotetilla* cf. *sagitta*
***Craniella* sp. 3878**	***Craniella* sp. 3878**	**QMG 316342**	**Norfolk Ridge**	**400-560**	**HM032747**	**Thin cortex with a palisade of oxeas, numerous porocalices.**	***Cinachyra* sp.**
***Craniella* sp. 3878**	***Craniella* sp. 3878**	**QMG 316372**	**Norfolk Ridge**	**400-560**	**HM032748**	**Thin cortex with a palisade of oxeas, numerous porocalices.**	***Cinachyra* sp.**
Craniella sp. 3318	*Craniella* sp. 3318	QMG 318785*	Norfolk Ridge 23° 39' 26.16" S, 168° 57.184" E	302-325	HM032752	Small spherical, double-layered cortex, embryos.	*Craniella* sp. 3318
Cinachyra antarctica	*Cinachyra antarctica*	QMG 311149*	Antarctica, McMurdo Base, Ross Island	20	JX177914	Hairy, several oscules.	*Cinachyra* sp.
Fangophilina sp.	*Fangophilina* sp.	NIWA 28586	New Zealand. Challenger Plateau -37.48734, 169.4605	1152-1153	JX177918	Small sample, No porocalices.	cf. *Fangophilina* sp.
***Fangophilina* sp.**	***Fangophilina* sp.**	**NIWA 28601**	**New Zealand. Challenger Plateau**	**1145-1148**	**JX177919**	**Small sample, No porocalices.**	**cf. *Fangophilina* sp.**

			-37.1685, 167.7272				
Fangophilina sp.	*Fangophilina* sp.	NIWA 28614	New Zealand. Challenger Plateau -39.9815, 167.6933	1197-1200	–		*Fangophilina* sp.
Fangophilina sp.	*Fangophilina* sp.	NIWA 28617	New Zealand. Challenger Plateau -39.92617, 167.6933	1139-1144	JX177912	Small sample, No porocalices.	cf. *Fangophilina* sp.
Amphitethya* cf. *microsigma	***Amphitethya* cf. *microsigma***	**SAM S1189**	**South Australia, Great Australian Bight, -34.12120, 133.29300**	**100**	**JX177910**		***Amphitethya microsigma***
Cinachyrella schulzei	*Cinachyrella schulzei*	QMG 320143*	Man and Wife Island, Keppel Islands, Australia -23.12027778, 150.9902778	22	HM032746		*Cinachyrella* cf. *tenuiviolacea*
Cinachyrella schulzei	*Cinachyrella schulzei*	QMG 320636*	Mielaniie Patch, Coral Sea, Australia -14.10266685, 144.5715332	16	HM032745		*Cinachyrella* cf. *tenuiviolacea*
Craniella cranium	***Craniella cranium***	**ZMBN 85239**	**Norway, Korsfjord**	**200-400**	**HM592668**		***Craniella* aff. *zetlandica***
***Craniella* sp.**	***Craniella* sp.**	**ZMBN 85240**	**Norway, Korsfjord**	**200-400**	**HM592669**		***Craniella* cf. *cranium***

[1] but 2 bp. Difference with NIWA 36097.

Localities, depths, and Genbank accession numbers of the corresponding sequences are also listed. QMG, Queensland Museum, Brisbane, Australia; NIWA, National Institute of Water & Atmospheric Research, New Zealand; SAM, South Australia Museum; ZMBN, Zoological Museum in Bergen, Norway. In bold, specimens re-examined in this study. Specimens with (*) were only seen on pictures.

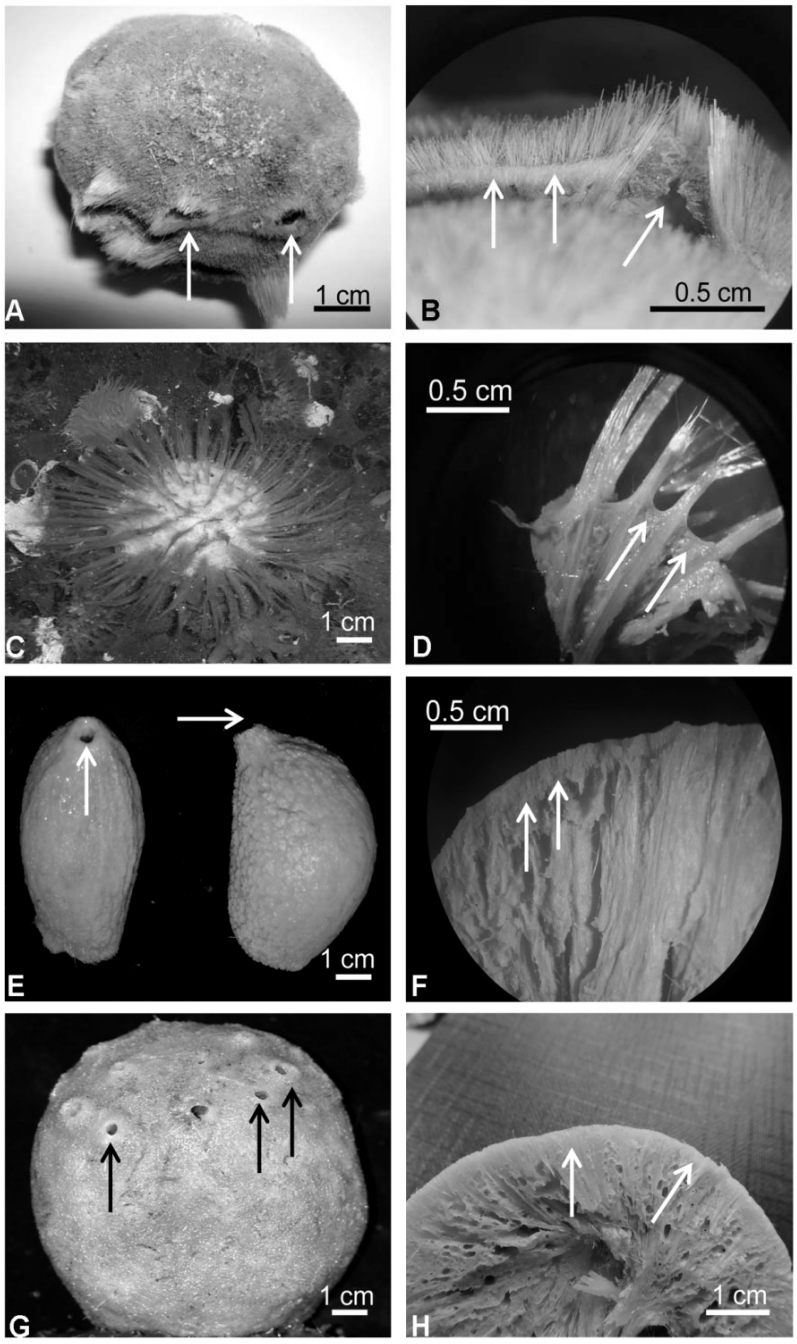

Figure 1.6. Pictures of the species of Tetillidae studied. A) *Cinachyra barbata* from Newmayer (Antarctica) arrows point to the porocalices. B) Transversal section of *C. barbata*: arrows point to the cortex and one porocalyx. C) Underwater picture of *Cinachyra antarctica* from McMurdo, (Antarctica). D) Transversal section of *C. antarctica*: arrows point to the collagenous cortex. E) *Antarctotetilla leptoderma* from South Georgia: arrows point to the unique osculum on top. F) Transversal section of *A. leptoderma*: arrows point to the dense ectosomal layer (pseudocortex). G) *Antarctotetilla grandis* from Newmayer, Antarctica: arrows point to the multiple oscula. H) Transversal section of *A. grandis*: arrows point to the slightly marked ectosomal layer. All the pictures are by the authors.

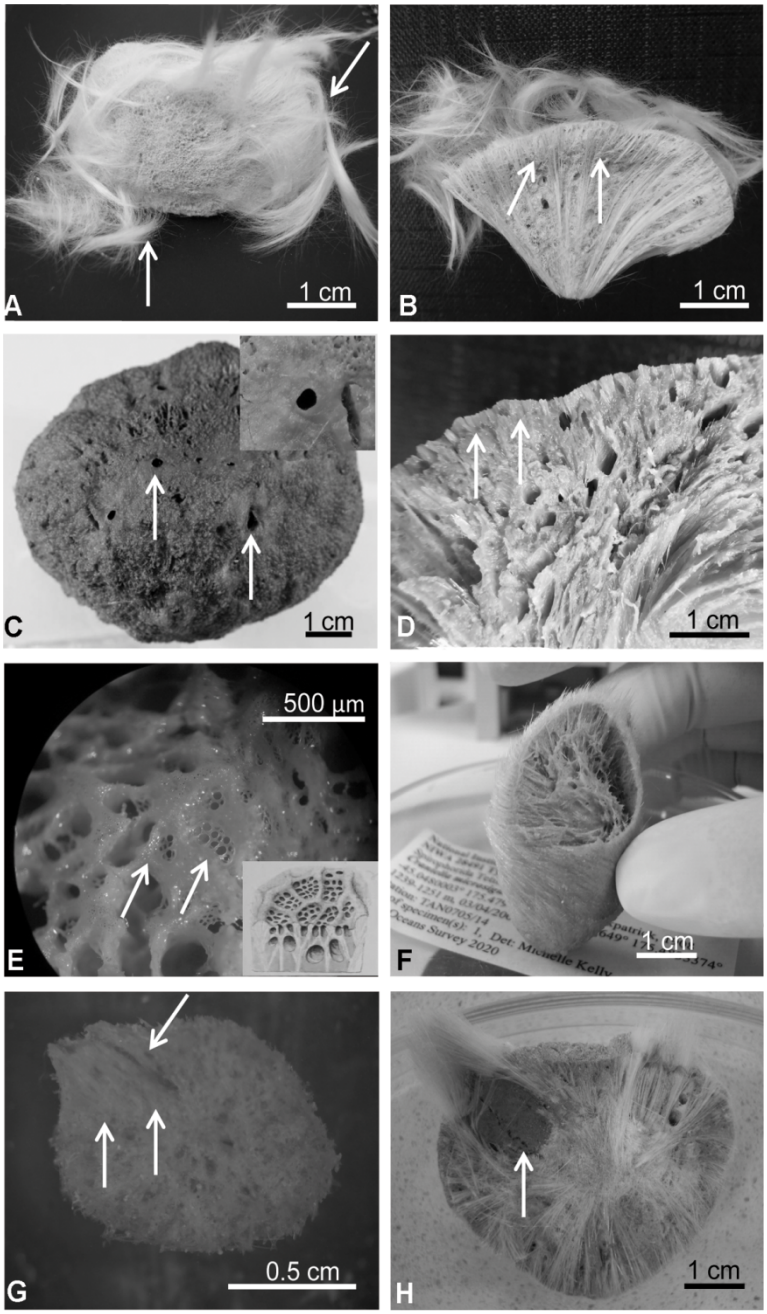

Figure 1.7. Pictures of the species of Tetillidae studied. A) Tetillidae ANT 27211 from Newmayer, Antarctica: arrows point to the hair-like hispidation pattern formed by bundles of fusiform oxeas, protriaenes and sometimes anatriaenes. B) Transversal section of Tetillidae ANT 27211: arrows point to the ectosomal layer. C) *Antarctotetilla sagitta* from Adélie Land: arrows point to the oscula; inset: detail of the even surface around the oscula. D) Transversal section of *A. sagitta*: arrows point to the ectosomal layer. E) Surface of *A. sagitta*: arrows point to the pores clustered in sieve-like areas; inset: *T. sagitta* pores in sieves by Kirkpatrick (1908). F) *Craniella sagitta* NIWA 28491 from New Zealand. G) cf. *Fangophilina* sp. NIWA 28601 from New Zealand: arrows point to the osculum. H) Lectotype of *Fangophilina submersa* MZSPO 160 from Northern Gulf of Mexico: arrow points to the porocalyx. All the pictures are by the authors except figures G, which were courtesy of Sadie Mills.

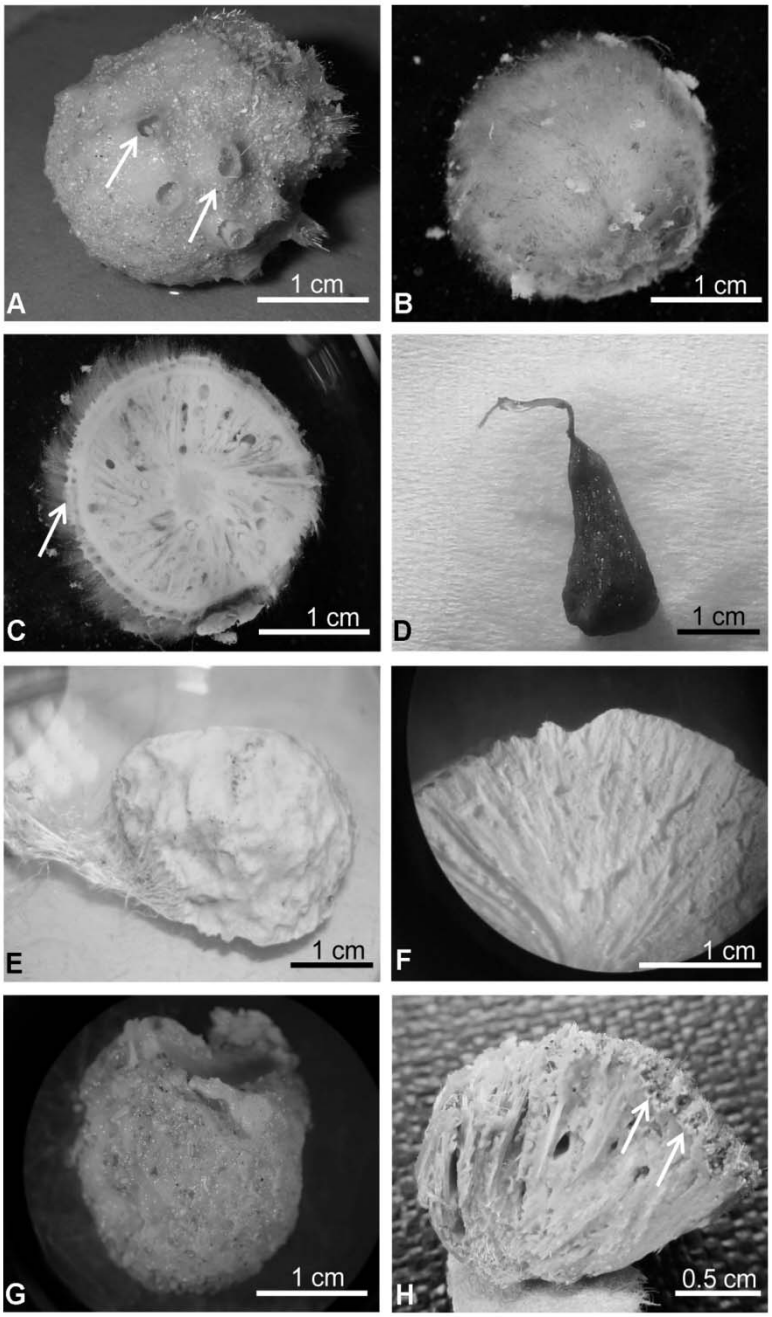

Figure 1.8. Pictures of the species of Tetillidae studied. A) *Craniella* sp. QMG 316342 from Australia: arrows point to the porocalices. B) *Craniella* cf. *cranium* ZMBN 85240 from Norway. C) Transversal section of *Craniella* cf. *cranium* ZMBN 85240: arrow points to the double-layered cortex. D) Holotype of *Tetilla euplocamos* MZSPO 206 from Brazil. E) Paratype of *Tetilla muricyi* UFBA 2569 from Brazil. F) Transversal section of the paratype of *T. muricyi* UFBA 2569. G) *Levantiniella levantinensis* from Lebanon. H) Transversal section of *L. levantinensis*: arrows point to the dense ectosomal region formed by sediment accumulation. All the pictures are by the authors except figures A, and D, which were courtesy of John Hooper, and Marie Meister, respectively.

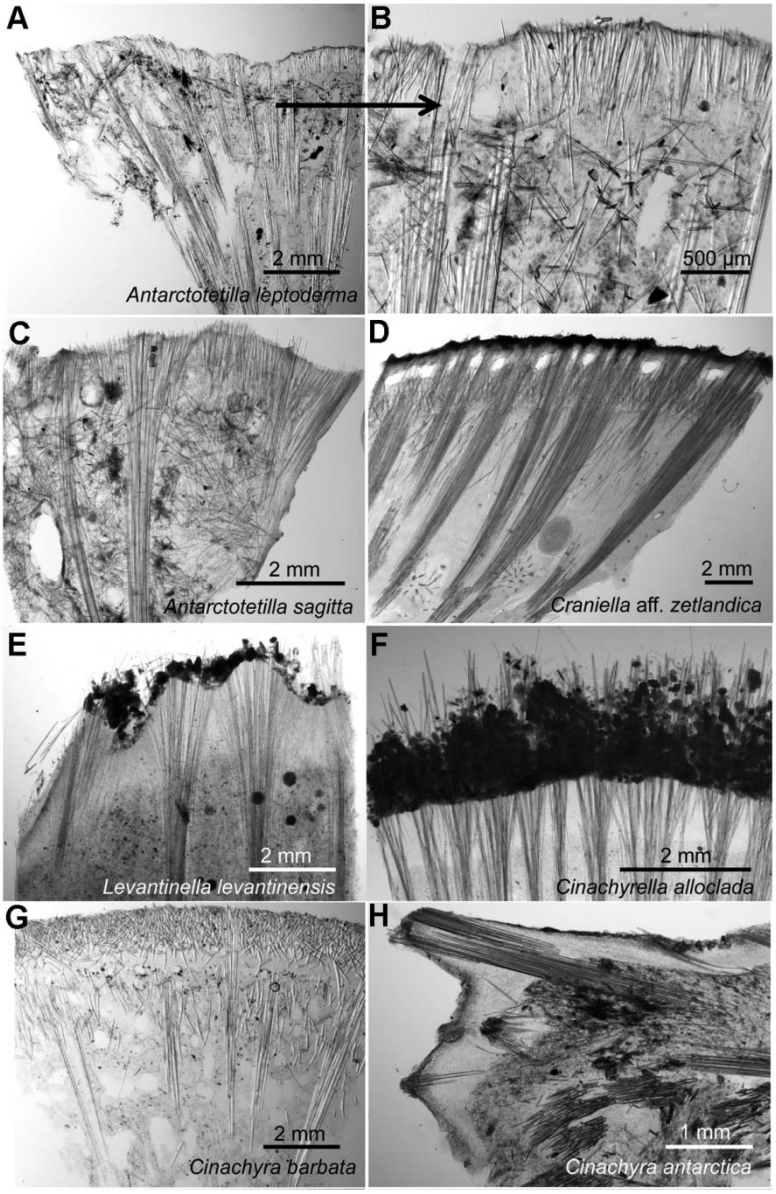

Figure 1.9. Thick sections of Tetillidae species showing differences in the ectosome or cortex structures. A) *Antarctotetilla leptoderma* from Adélie Land, Antarctica, MNHN IP-2009-544a. B) *A. leptoderma*, close-up of C. C) *Antarctotetilla sagitta* from Adélie Land, Antarctica, MNHN IP-2009-31. D) *Craniella* aff. *zetlandica* from Korsfjord,Norway, ZMBN 85239. E) *Levantiniella levantinensis* from Israel, PC 705. F) *Cinachyrella alloclada* from Bocas del Toro, Panama, ZMBN 81788. G) *Cinachyra barbata* from Adélie Land, Antarctica, MNHN IP-2009-387. H)*Cinachyra antarctica* from Adélie Land, Antarctica, MNHN IP-2009-328.

Maximum parsimony phylogeny on phenotypic characters

The MP analysis included 33 OTUs, one for each species, 26 morphological characters, and 13 motifs of the V4 18S secondary structure, which were treated as binary elements (S1 File). The astrophorin *Geodia cydonium* and *Geodia neptuni* were used as outgroups. The MP analysis retrieved 6 most parsimonious trees of 48 steps (CI = 0.684; RI = 0.887). Character states are shown at the nodes of the phylogram corresponding to tree number 1 (Figure 1.10). Clade support resulting from the majority-rule consensus tree is also shown on the phylogram (Figure 1.10).

The Tetillidae appeared divided in two strongly supported clades (100% bootstrap value), here called clade A and B. Clade A was characterized by pores grouped in several ways and was formed by two well supported sub-clades: A1 embraced two groups with the same V4 secondary structure, A1a formed by *Acanthotetilla*, with acanthoxeas, small porocalices, and a cortical region made of a palisade of acanthoxeas. The clade A1b was formed by the genus *Cinachyrella* and *Levantiniella* gen. nov. The latter species is characterized by absence of a well-formed cortex, small, little conspicuous porocalice-like structures, and a dense incorporation of sand to its surface. The genus *Cinachyrella* included *Paratetilla bacca* despite the presence of triaenes in the former, and was characterized by the absence of a defined cortex and the presence of true hemispherical large porocalices. No morphological characters could differentiate the two subgroups of *Cinachyrella* found in the molecular phylogenies (i.e. the *C. australiensis*/*C. apion* group versus the *C. alloclada*/*C.* cf. *tenuiviolacea* group).

A2 corresponded to the Antarctic clade, characterized by sharing the same V4 secondary structure. A2a included the *Cinachyra* genus, with cortex and flask-shaped porocalices as synapomorphies, and two *Cinachyra* sp. (QMG 316342 and QMG 316372), which also have cortex and porocalices but show a slightly different secondary structure. Clade A2b, with pseudocortex as a synapomorphy (Figures 1.9A, 1.9B and 1.9C), was subdivided in 2 groups: A2ba and A2bb. A2ba included *Antarctotetilla* gen. nov. with pores clustered in shallow small depressions as a synapomorphy, and Tetillidae sp.3 (ANT 27211, Figures 1.7A and 1.9B) totally covered by long hair-like spicules, without

porocalices, and with pores clustered in shallow small depressions. A2bb comprised Tetillidae sp. 1 and sp. 2 individuals (NIWA 28491, 25206 and 28929).

Clade B included species that had spread pores as a synapomorphy and was also formed by two well-supported sub-clades:

B1 included two groups. Group B1a was formed by species of Craniella with a two-layered cortex made of collagen plus spicules, and a hispid-conulose surface, and a specific V4 secondary structure. Group B1b included cf. *Fangophilina* sp, with a particular V4 secondary structure region and a hispid surface (Figure 1.7G).

B2 was divided in two groups: B2a formed by the genus *Tetilla* (tropical species) with a particular V4 secondary structure and B2b containing *Amphitethya microsigma*.

Species of the genera *Cinachyra* and *Antarctotetilla* gen. nov. were clearly differentiated, despite the fact that they shared the same V4 secondary structure: *Cinachyra antarctica* has a collagen cortex as an autopomorphy (Figures 1.6C and 1.6D and 1.9H) while *C. barbata* and the two *Cinachyra* sp. (QMG 316342 and QMG 316372) have a spicule-collagenous cortex (Figures 1.6A and 1.6B and 1.9G).

All molecular analyses suggested that the latter species was related to cf. *Fangophilina* sp. and not to *Cinachyra*. Within *Antarctotetilla* gen. nov., *A. leptoderma* and *A. sagitta* have a corrugate surface (Figures 1.6E and 1.7C), but while the former has a single large apical osculum, *A. sagitta* shows several small oscules (Figure 1.7C). On the other hand, *A. grandis* shows an even surface, spread small oscula, and pores clustered in small depressions (Figure 1.6G), while *A. sagitta* is characterized by their pores in sieves covering sub-ectosomal spaces (Figures 1.7C and 1.7E).

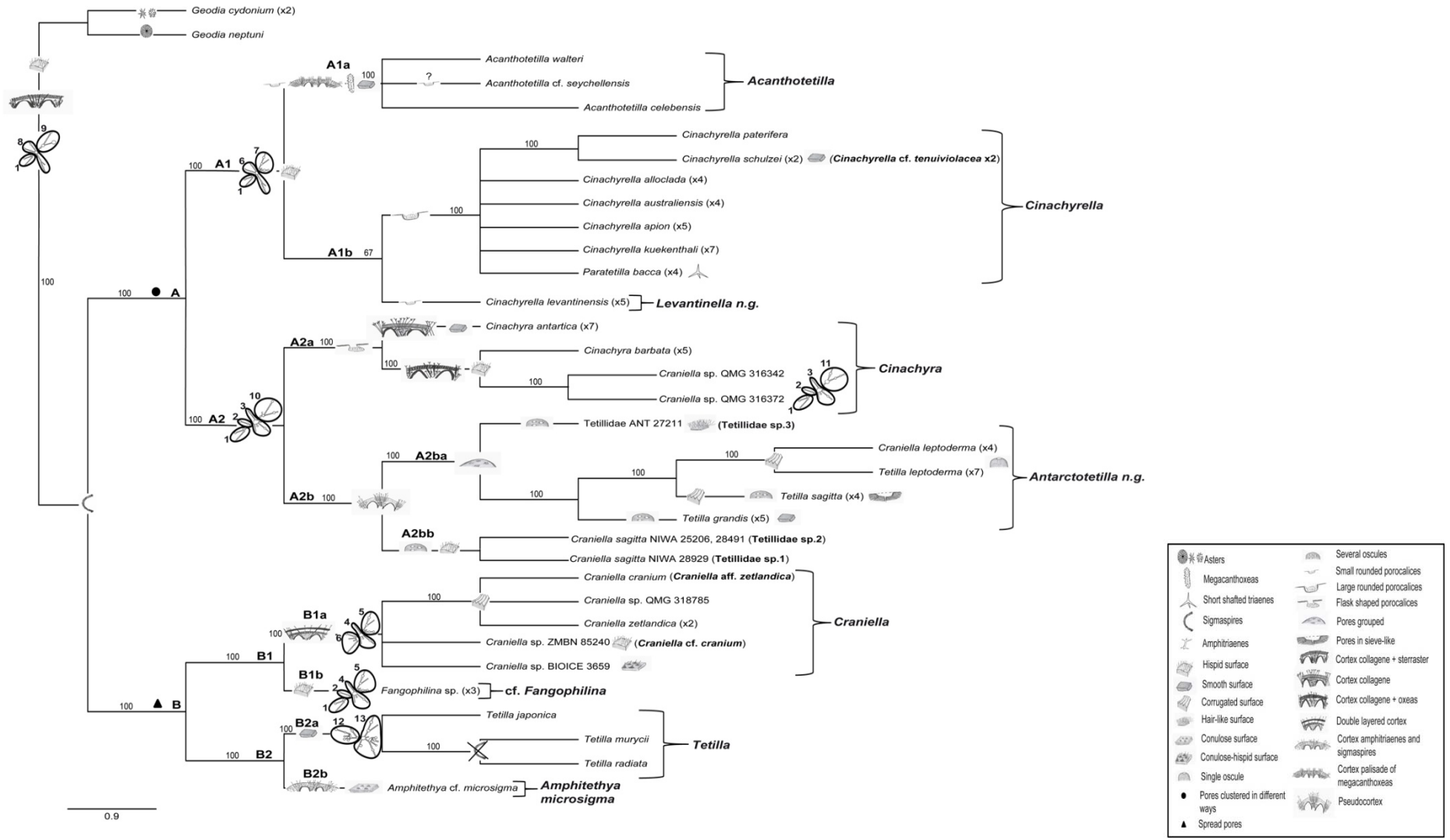
Geodia cydonium (x2)
Geodia neptuni
A1a
Acanthotetilla walteri
Acanthotetilla cf. seychellensis
Acanthotetilla celebensis
Acanthotetilla
A1
A1b
Cinachyrella paterifera
Cinachyrella schulzei (x2) (Cinachyrella cf. tenuiviolacea x2)
Cinachyrella alloclada (x4)
Cinachyrella australiensis (x4)
Cinachyrella apion (x5)
Cinachyrella kuekenthali (x7)
Paratetilla bacca (x4)
Cinachyrella
Cinachyrella levantinensis (x5)
Levantinella n.g.
A
A2a
Cinachyra antartica (x7)
Cinachyra barbata (x5)
Craniella sp. QMG 316342
Craniella sp. QMG 316372
Cinachyra
A2
Tetillidae ANT 27211 (Tetillidae sp.3)
A2ba
Craniella leptoderma (x4)
Tetilla leptoderma (x7)
Tetilla sagitta (x4)
Tetilla grandis (x5)
Antarctotetilla n.g.
A2b
A2bb
Craniella sagitta NIWA 25206, 28491 (Tetillidae sp.2)
Craniella sagitta NIWA 28929 (Tetillidae sp.1)
Craniella cranium (Craniella aff. zetlandica)
Craniella sp. QMG 318785
Craniella zetlandica (x2)
Craniella sp. ZMBN 85240 (Craniella cf. cranium)
Craniella sp. BIOICE 3659
Craniella
B1a
B
B1
B1b
Fangophilina sp. (x3)
cf. Fangophilina
B2a
Tetilla japonica
Tetilla murycii
Tetilla radiata
Tetilla
B2
B2b
Amphitethya cf. microsigma
Amphitethya microsigma
0.9
Asters
Megacanthoxeas
Short shafted triaenes
Sigmaspires
Amphitriaenes
Hispid surface
Smooth surface
Corrugated surface
Hair-like surface
Conulose surface
Conulose-hispid surface
Single oscule
Pores clustered in different ways
Spread pores
Several oscules
Small rounded porocalices
Large rounded porocalices
Flask shaped porocalices
Pores grouped
Pores in sieve-like
Cortex collagene + sterraster
Cortex collagene
Cortex collagene + oxeas
Double layered cortex
Cortex amphitriaenes and sigmaspires
Cortex palisade of megacanthoxeas
Pseudocortex

Figure 1.10. A) Phylogram of one of the most parsimonious trees on morphological characters plus the several zones identified for the V4 region of 18S secondary structure–SSRs– (numbered and encircled). Characters that represent either synapomorphies or apomorphies are depicted in the tree. The supporting bootstrap values of clades resulting from the Majority-rule consensus tree are also indicated. B) Legends to character drawings on the tree.

Formal diagnoses of the currently proposed genera

Family Tetillidae

Tetilla Schmidt, 1868
Type species: *Tetilla euplocamos* Schmidt, 1868.
Diagnosis: Tetillidae without porocalices, without cortical specialization, without auxiliary megascleres (Van Soest & Rützler 2002).

Cinachyrella Wilson, 1925
Type species: *Tetilla hirsuta* Dendy, 1889.
Diagnosis: Tetillidae usually with hemispherical porocalices (except in some stalked species), without spiculous cortical specialization; modified from (Van Soest & Rützler 2002).
Remarks: *Amphithetya* and *Paratetilla* are within the *Cinachyrella* clade in our pylogenies. The several well-supported subgroups within the *Cinachyrella* clade might correspond to subgeneres. However, a more in deep study of *Cinachyrella* by including additional species would be necessary to formally propose those subgeneres.

Levantiniella gen. nov.
Type species: *Levantiniella levantinensis* (Vacelet et al., 2007) by monotypy.
Diagnosis: Tetillidae with small porocalices formed by rounded shallow depressions, without cortex, without auxiliary megascleres.

Craniella Schmidt, 1870
Type species: *Craniella tethyoides* Schmidt, 1870.

Diagnosis: Globular sponges without porocalices and with a distinct, two-layered cortex (visible to the naked eye). The outer cortex layer often with sub-dermal cavities and the inner layer made of collagen and a tight arrangement of cortical oxeas. Presence of direct-developing embryos within the sponge tissue; modified from (Van Soest & Rützler 2002).

Cinachyra Sollas, 1886
Type species: *Cinachyra barbata* Sollas, 1886.
Diagnosis: Tetillidae with a collagenous cortex, sometimes reinforced by auxiliary oxeas, with flask-shaped porocalices; modified from (Van Soest & Rützler 2002).
Remarks: Most described *Cinachyra* have been transferred to *Cinachyrella* (see World Porifera Dadabase).

Antarctotetilla gen. nov.
Synonymy: *Tetilla* sensu Sollas, 1886 (part.), *Craniella* sensu Kirkpatrick, 1908 (part.).
Type species: *Tetilla leptoderma* Sollas, 1886 by designation.
Diagnosis: Tetillidae with single or multiple oscule(s) on top, pores clustered in shallow depressions (no porocalices) and a pseudocortex (very slight cortical differentiation) made of perpendicular oxeas, loosely arranged.
Remarks: Our molecular and morphological results suggest that that three species should be assigned to this genus: *A. leptoderma*, *A. sagitta*, and *A. grandis*. These species are only known (up to now) from the Antarctic, New Zealand, Kerguelen Islands and the Magellanic region.
Moreover, *Craniella coactifera* Lendenfeld, 1907 shows strong similarities with *A. grandis* and is likely a synonym of this species. We reallocate *C. coactifera* to *Antarctotetilla* gen. nov., and keep this species valid until its type can be compared with the type of *A. grandis*.

Discussion

Molecular markers

The molecular markers used in this study were informative enough to resolve the relationships of Tetillidae genera but did not resolve Antarctic species. Phylogenetic trees inferred with the COI M1-M6 partition (also known as the barcoding Folmer fragment) gave a better resolution of the genera, in part because of the higher number of individuals sequenced for this marker and those already available in Genbank. However, although the COIM1-M6 partition differentiated all the species of temperate and tropical genera of Tetillidae included in this study it failed to separate species within the Antarctic genera *Cinachyra* and *Antarctotetilla* gen. nov., despite clear morphological differences. Although uncommon, strictly identical COIM1-M6 sequences for different sponge species have previously been found in other demosponge groups (Schröder et al. 2003; Addis & Peterson 2005; Heim et al. 2006; Cárdenas & Rapp 2012) and also in Antarctic hexactinellid sponges: only two COI haplotypes were found among the Antarctic *Rosella* species (Vargas 2016), which had been recognized by Barthel and Tendal (1994).

Similarly, the 18S, 28S (D3–D5) and COI I3-M11 partitions did not succeed in discriminating all Antarctic species. This was to be expected from the slow evolving 18S or even from the faster evolving 28S (D3-D5), which has rather been used for inter-species relationships. However, this was rather surprising for the COI I3-M11 partition, which is considered more variable than the Folmer partition (Erpenbeck et al. 2006), and has been used in population genetics and phylogeography studies of demosponges (Lopez-Legentil & Pawlik 2009, Xavier et al. 2010, Reveillaud et al. 2011).

This may be an indication of contrasting evolutionary rates between sponge groups (Solé-Cava & Boury-Esnault 1999, Heim et al. 2006). Thus, our results suggest either a particularly slow genetic evolutionary rate of the markers 28S (D3-D5) and COI or a recent radiation with phenotypic characters evolving faster than the genes studied. Further studies on other Demospongiae families and/or more variable markers are required to shed light on the

evolutionary processes that affect Antarctic sponges, which are poorly studied so far.

Phylogeny and taxonomic actions

Insufficient knowledge on the morphological characters of Antarctic/New Zealand Tetillidae along with misidentifications biased the interpretation of previous phylogenetic studies (Szitenberg et al. 2013).The re-examination of some of these specimens as well as holotypes (Table 1.2) proved essential to understand previous puzzling results such as the polyphyly of *Craniella* (Szitenberg et al. 2013). Overall, our results improve and clarify the Tetillidae phylogeny. All COI and 18S trees, as well as the COI+18S trees were mostly congruent, except for the positions of Tetillidae sp.3 (ANT 27211), Tetillidae sp.1 ("*Craniella sagitta*" NIWA 28929), Tetillidae sp.2 ("*Craniella sagitta*" NIWA 25206 and 28491) and *Levantiniella levantinensis*.

The MP trees allowed us to identify phenotypic synapomorphies for the proposed genera. The MP phylogeny on phenotypic characters, plus the motifs of the 18S secondary structure (V4 region), differed from the molecular phylogenies only in the position of the two *Cinachyra* sp. (QMG 316342 and QMG 316372, previously wrongly identified as *Craniella* sp.): they form a strongly supported clade with cf. *Fangophilina* sp. (NIWA 28601, 28586, 28617) in molecular trees, whereas they group with *Cinachyra* in the MP tree (because of their porocalices and spiculous cortex).

According to Szitenberg et al. (2013), the presence of cortex or/and porocalices, which have been traditionally used to differentiate Tetilllidae genera (Rützler 1987, Van Soest & Hooper 2002), do not represent synapomorphies according to our molecular analyses. Instead, the types of cortex (with spicules, without spicules, with one or two layers) and/or of porocalices (e.g. deep flask-shaped, hemispherical porocalices, and small, shallow cavities) are the derived characters shared within each genus.

In the current study, we see the Tetillidae basically divided in seven well-supported clades, instead of the five recovered in Szitenberg et al. (2013). Most of these clades correspond to genera: *Antarctotetilla* gen. nov., *Cinachyra*, *Acanthotetilla*, *Tetilla*, *Cinachyrella*, *Craniella*, and *Levantiniella* gen. nov.

The new genus *Antarctotetilla* contains exclusively those Tetillidae without cortex, without porocalices, and with grouped ostia. The presence of a pseudocortex (not visible with the naked eye) instead of a clearly conspicuous cortex in those species may explain why they have been assigned to the genus *Tetilla* by previous authors (Sollas, 1886, 1888; Topsent, 1901; Boury-Esnault & Van Beveren, 1982). However, the type species of *Tetilla* (*T. euplocamos* Schmidt, 1868) and other tropical representatives that we have examined (e.g. *T. radiata*, *T. muricyi*), do not have any kind of obvious cortical specialization.

After moving the traditional Antarctic "*Tetilla*" (*Craniella* in Szitenberg et al. 2013) to *Antarctotetilla* gen. nov., the genus *Tetilla* recovers its classical diagnosis (Rützler 1987) by including those Tetillidae without cortex and without porocalices. Similarly, by moving the two misidentified *Craniella* sp. (QMG 316342 and QMG 316372) to *Cinachyra* sp., *Craniella* senso stricto (Rützler 1987; Van Soest & Hooper 2002) was recovered as a monophyletic genus, with a characteristic two-layered cortex as synapomorphy.

Szitenberg et al. (2013) proposed the inclusion of *Fangophilina* and *Cinachyra* within the genus *Craniella*, as either junior synonyms or sub-genera. However, this proposal was based on a series of misidentifications: QMG 316342 and QMG 316372 had been wrongly identified as *Craniella* sp. (now *Cinachyra* sp.), NIWA 28929, NIWA 28491 and NIWA 25206 had been wrongly identified as *Craniella sagitta* (here renamed Tetillidae sp. 1 and sp. 2). Conversely four confirmed *A. sagitta* specimens (ANT IP 31, IP 351, IP 359 and *A*. cf. *sagitta* QMG 315031) joined the *Antarctotetilla* gen. nov. clade in both molecular and morphological phylogenies.

We missed true *Fangophilina* species in our molecular analyses, since, as stated above, the three cf. *Fangophilina* sp. did not completely match the diagnostic morphological characters of the genus. The type species *F. submersa* was reported to have two opposite porocalyces: one with an inhalant function and the other exhalant (Schmidt 1880; Van Soest & Rützler 2002). The revision of the type material showed that only the cavity containing the ostia is a true porocalyx since the other one corresponded to a deep cloacal osculum. Morphological and genetic investigations on further individuals of *F. submersa*, which is known just by a single specimen, are necessary to resolve the

phylogenetic position and morphological variation of this genus, which has been considered dubious (Van Soest & Hooper 2002).

Our phylogenetic trees retrieved the type species of *Amphitethya* (*A. microsigma*) and *Paratetilla* (*P. bacca*) within the large *Cinachyrella* clade, as in previous phylogenies (Szitenberg et al. 2013). The position of *Paratetilla* within *Cinachyrella* is also recovered in the morphological tree as species of both genera have similar external morphology. Although the type species of *Cinachyrella*, *C. hirsuta* Dendy, 1889, is not included in our sampling, we are confident that it would group in the large *Cinachyrella* clade, based on its morphology (Van Soest & Hooper 2002). We would therefore have enough arguments to synonymize *Amphitethya* Lendenfeld, 1907 and *Cinachyrella* Wilson, 1925 with *Paratetilla* Dendy, 1905, the oldest genus name. However, reallocating the so far described 40species of *Cinachyrella*(Van Soest et al. 2016) to *Paratetilla* without previous reexamination would not be the most conservative option since it would hide the morphological difference we currently recognize (calthrop-like triaenes) to identify *Paratetilla*. Instead, we prefer to wait for further molecular phylogeny studies on this group to take taxonomic action. Since *Cinachyrella* species are distributed in several clades, we believe a future revision of this group with a wider sampling might indicate where to place *C. hirsuta*.

The presence of *Amphitethya* within the *Cinachyrella* clade of our molecular trees was unexpected, due to its stalked morphology and the absence of porocalices, which, conversely, placed *A. microsigma* as a sister species of *Tetilla* in the morphological tree. However, we note that the characteristic amphitriaenes of *Amphitethya* have also been occasionally observed in *Paratetilla* species: *P. aruensis* (Hentschel, 1912), and *P. merguiensis* (Topsent, 1897).

In our 18S phylogeny, a few sequences from Redmond et al. (2013) had suspicious phylogenetic affinities: *Acanthotetilla* cf. *seychellensis* (KC902033), and *Cinachyra* sp. (KC902124) clustered with *Cinachyrella* while *Craniella* sp. (KC902265) clustered with *Tetilla*. We suspect these are misidentifications but we could not re-examine the corresponding specimens.

Szitenberg et al. (2013) suggested erecting *Levantiniella* gen. nov. for the species *C. levantinensis*, which substantially diverged from the rest of the

Cinachyrella species in their study. However, no formal definition was proposed thus making *Levantiniella* a nomen nudum. We agree with these authors in that this species belongs to a new genus and we formally propose to make *Levantiniella* gen. nov. available with *C. levantinensis* as type species by monotypy. The phylogenetic affinities of *Levantiniella*, however, differed depending on the gene partition used: it appeared as a sister group of the *Tetilla*/*Craniella*/*Fangophilina*/*Antarctotetilla*/*Cinachyra* clade with COI while it was a sister group to the other *Cinachyrella* with 18S.

The family Tetillidae appeared paraphyletic or polyphyletic in previous 18S phylogenies (Redmond et al. 2013; Kelly & Cárdenas 2016) and 28S phylogenies (Schuster et al. 2015): the *Craniella*/*Cinachyra*/*Antarctotetilla* gen. nov. clade was sister to the Astrophorina. However, these results may be due to a sampling bias, a possibility further suggested by a wide COI phylogeny that recovers a monophyletic Tetillidae (Kelly & Cárdenas 2016). A more thorough worldwide study of representatives of this family is needed to further test its monophyly.

Geographical distribution of Tetillidae genera

The geographical location of the studied Tetillidae suggests a temperature related distribution of some genera (Figure 1.11). *Cinachyrella* shows a tropical–subtropical distribution, while *Craniella* species mainly inhabit temperate-cold seas. The genera *Cinachyra* and *Antarctotetilla* appear confined to the Antarctic and Sub-Antarctic regions, contributing to the reported Antarctic sponge endemism (Downey et al. 2012, Sarà et al. 1992), which underlines the importance of the Polar Front in isolating the Southern Ocean fauna. Other *Cinachyra* species, which have been reported out of the Antarctic, may have been incorrectly attributed to this genus. For example, *Cinachyra helena* Rodriguez and Muricy, 2007 from Brazil does not belong to *Cinachyra* since its purported porocalyx rather looks like a cloacal oscula with macro-orifices inside the depression (Rodriguez & Muricy 2007). Moreover, its two-layered cortex (Rodriguez & Muricy 2007) suggests that it is a *Craniella*. We therefore propose to reallocate this species to the genus *Craniella*. Other described *Cinachyra* have been moved to *Cinachyrella* posteriorly (see WPD). Current

representatives of the genus *Tetilla* are mainly living in arctic-temperate-warm seas.

In relation to depth, *Cinachyrella* is distributed in shallow-waters (<30 m depth) with few exceptions that can be found up to 100 m depth (e.g. *Cinachyrella kuekenthali*, *Amphitethya microsigma*) while other genera such as *Craniella* or *Fangophilina* are nearly restricted to the deep-sea. *Antarctotetilla* and *Cinachyra* are eurybathic inhabiting from 30 to 600 m of depth, but they are particularly abundant between 200 and 300 m of depth where they may dominate in sponge grounds.

Sponge molecular phylogenies have greatly contributed to emend the traditional sponge systematics, in particular for demosponges (Morrow et al. 2012; Cárdenas et al. 2012, Morrow & Cárdenas 2015). The sponge phylogenetic tree resolution continuously improves as new genetic markers, and more importantly, additional taxa are included in the datasets. However, a careful morphological identification of the individuals sequenced and included in the molecular phylogenies is required for a precise interpretation of the molecular results. In other words, molecular phylogenies should consistently be associated with thorough morphological studies of the specimens sequenced, as we have tried to do with the Tetillidae species.

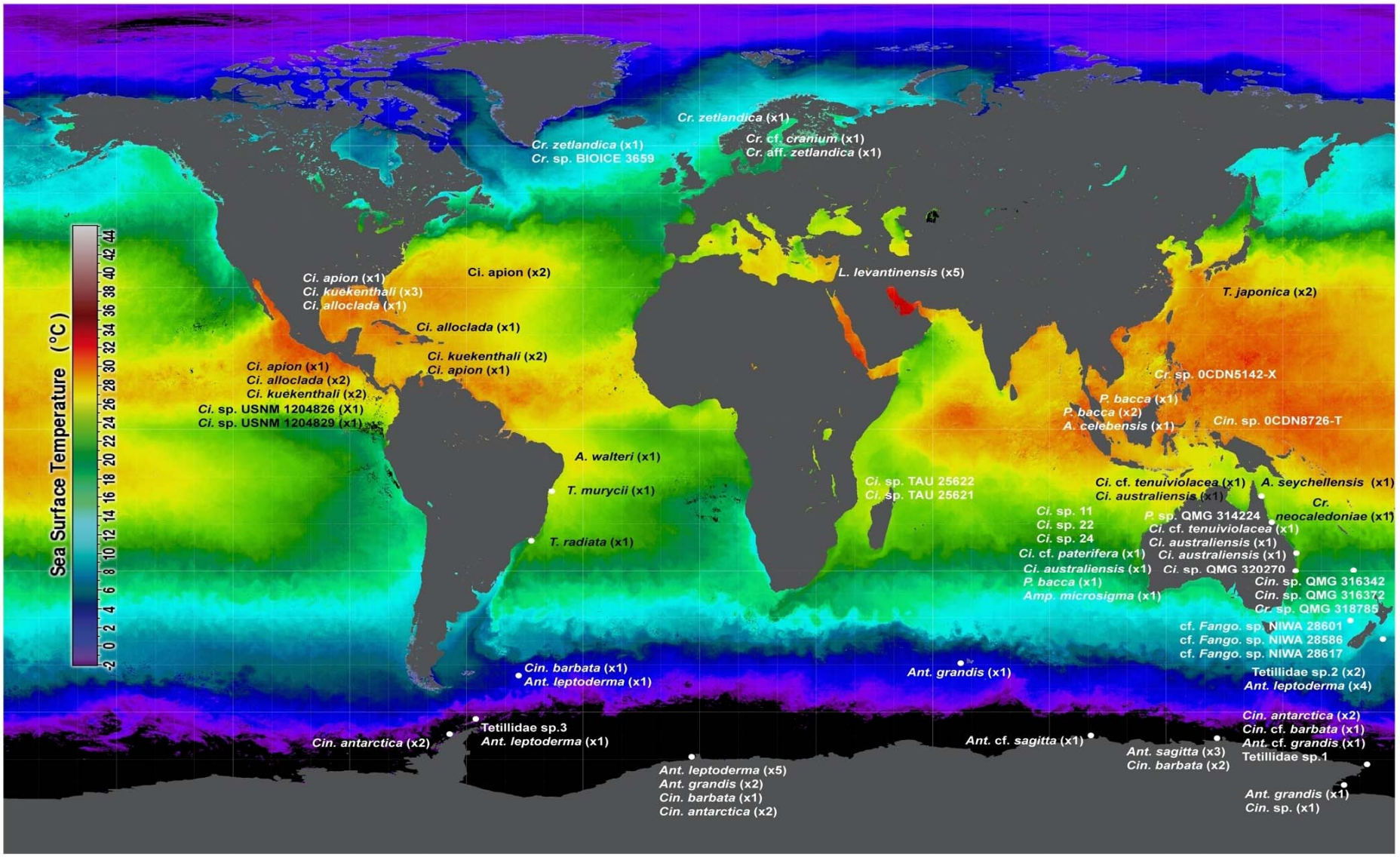
Sea Surface Temperature (°C)
-2 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Cr. zetlandica (x1)
Cr. sp. BIOICE 3659
Cr. zetlandica (x1)
Cr. cf. cranium (x1)
Cr. aff. zetlandica (x1)
Ci. apion (x1)
Ci. kuekenthali (x3)
Ci. alloclada (x1)
Ci. apion (x2)
L. levantinensis (x5)
T. japonica (x2)
Ci. alloclada (x1)
Ci. kuekenthali (x2)
Ci. apion (x1)
Ci. apion (x1)
Ci. alloclada (x2)
Ci. kuekenthali (x2)
Ci. sp. USNM 1204826 (X1)
Ci. sp. USNM 1204829 (x1)
Cr. sp. 0CDN5142-X
P. bacca (x1)
P. bacca (x2)
A. celebensis (x1)
Cin. sp. 0CDN8726-T
A. walteri (x1)
T. murycii (x1)
T. radiata (x1)
Ci. sp. TAU 25622
Ci. sp. TAU 25621
Ci. cf. tenuiviolacea (x1)
Ci. australiensis (x1)
A. seychellensis (x1)
Cr. neocaledoniae (x1)
Ci. sp. 11
Ci. sp. 22
Ci. sp. 24
P. sp. QMG 314224
Ci. cf. tenuiviolacea (x1)
Ci. australiensis (x1)
Ci. cf. paterifera (x1)
Ci. australiensis (x1)
Ci. australiensis (x1)
Ci. sp. QMG 320270
P. bacca (x1)
Amp. microsigma (x1)
Cin. sp. QMG 316342
Cin. sp. QMG 316372
Cr. sp. QMG 318785
cf. Fango. sp. NIWA 28601
cf. Fango. sp. NIWA 28586
cf. Fango. sp. NIWA 28617
Cin. barbata (x1)
Ant. leptoderma (x1)
Ant. grandis (x1)
Tetillidae sp.2 (x2)
Ant. leptoderma (x4)
Cin. antarctica (x2)
Tetillidae sp.3
Ant. leptoderma (x1)
Ant. cf. sagitta (x1)
Cin. antarctica (x2)
Cin. cf. barbata (x1)
Ant. cf. grandis (x1)
Tetillidae sp.1
Ant. sagitta (x3)
Cin. barbata (x2)
Ant. leptoderma (x5)
Ant. grandis (x2)
Cin. barbata (x1)
Cin. antarctica (x2)
Ant. grandis (x1)
Cin. sp. (x1)

Figure 1.11. Distribution of the Tetillidae species analysed in this study overlying a temperature map in South hemisphere winter (NASA Goddard Space Flight Center, Ocean Ecology Laboratory, Ocean Biology Processing Group; (2014): Sea-viewing AQUA MODIS SeaSurface Temperature, August 2013. NASA OB.DAAC. http://oceancolor.gsfc.nasa.gov/cgi/l3. Accessed on: 2015/04/29). White points represent exact sampling locations. Cr. = *Craniella*; Ci. = *Cinachyrella*; L. = *Levantiniella*; Fango. = *Fangophilina*; A. = *Acanthotetilla*; T. = *Tetilla*;Amp. = *Amphitethya*; P = *Paratetilla*; Cin. = *Cinachyra*; Ant. = *Antarctotetilla*.

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CHAPTER 2

Description of two new genera (*Antarctotetilla, Levantiniella*) and a new species of Tetillidae

Abstract

Tetillidae is a sponge family distributed all over the world but with some genera apparently endemics from the Antarctica and Sub-Antarctica (the “Antarctic clade”). Species identification results tricky due to the similarities of their morphological characters. However, molecular phylogenies have helped to resolve the family taxonomy. The last phylogenetic study on Tetillidae suggested the creation of two new genera: *Levantiniella* and *Antarctotetilla*. *Lenvantiniella*, from Middle East Mediterranean Sea, was previously classified within *Cinachyrella* from which it differs in the small rounded surface cavities, distinctive from true porocalices. *Antarctotetilla* has up to now an Antarctic distribution and harbors species wrongly classified within *Tethya*, *Craniella*, or *Tetilla* genera. The main differences of *Antarctotetilla* with other Tetillidae genera are the presence of pores grouped in small areas and a poorly-defined cortex (pseudocortex). This study aims to re-describe in detail the species of Tetillidae that belong in the two above mentioned, new genera, and to highlight that molecular phylogenies should be combined with morphological analyses to improve taxonomical decisions. We also describe a new Tetillidae species with a hair-like hispidation, which we name *Antarctotetilla pilosa* nov. sp. Furthermore, the types of *Tethya coactifera* and *Tethya crassispicula* Lendenfeld, 1907) were reexamined because of some morphological similarities with *Antarctotetilla*. The minibarcode sequences (a small COI fragment) placed them within the Antarctic clade harboring *Antarctotetilla* and *Cinachyra*, but did not resolve their genus position. A morphological revision, however, suggests placing *Tethya coactifera* in *Antarctotetilla*, while *Tethya crassispicula,* which owns porocalices and a spicule-made cortex, appeared to belong in *Cinachyra*.

Introduction

Tetillidae Sollas, 1886 is a sponge family belonging to the Sub-order Spirophorina (order Tetractinellida), which includes ten genera distributed worldwide, across a wide range of depths (Van Soest et al. 2017; Van Soest & Rützler 2002). The family Tetillidae comprise sponges with a spherical growth form, and a radially to spirally arranged skeleton. Some members have special pore-bearing structures called porocalices. The presence of a cortical structure or cortex discriminate groups of species but this character does not have phylogenetic information (Carella et al. 2016). The spicules are mainly megascleres such as protriaenes, oxeas, and anatriaenes, which often protrude the ectosomal layer; microscleres are sigmaspires and occasionally raphides (Van Soest & Rützler 2002). Many species dwell on sedimentary bottoms to which they anchor by mean of profuse long spicule bundles, which in many cases serve as a suitable substrate for other hard-bottom invertebrate to settle (Gutt et al. 2013).

The diagnostic criteria used for classifying Tetillidae genera and species include the ectosome structure, the organization of the aquiferous orifices and the spicule complement Rützler (1987). A recent study (Carella et al. 2016) on the phylogeny of Tetillidae, using nuclear and mitochondrial genes, highlighted the necessity of revising the taxonomic position of some Antarctic species such as *Tetilla leptoderma* Sollas, 1886, *Tetilla grandis* Sollas, 1886, and *Tethya sagitta* Lendenfeld, 1907, which had been assigned to wrong genera. In the above-mentioned study, Antarctic representatives of *Tetilla* Schmidt, 1868 grouped in a monophyletic clade, apart from the group of template/tropical *Tetilla*. Consequently, the new genus, *Antarctotetilla* (Carella et al. 2016) was proposed to include the Antarctic *Tetilla*. The molecular study of *Antarctotetilla* species, and other Antarctic genera such as *Cinachyra,* showed the absence of inter-species genetic differentiation with the molecular markers used. This feature contrasts with the most habitual finding of morphologically cryptic but genetically differentiated species (e.g. Solé-Cava et al. 1991; Klautau et al. 1999; Blanquer & Uriz 2007; Reveillaud et al. 2010, 2012; Xavier et al. 2010). Additionally, *Tethya coactifera* Lendenfeld, 1907 and *Tethya crassispicula* Lendenfeld, 1907 were also morphologically revised and a small COI fragment (Meusnier et al. 2008) of the type specimens was sequenced to confirm they belonged to Antarctic genera.

The aim of this study was to re-describe morphologically the species of Tetillidae that belong to the above-mentioned new genera according to the previous molecular

phylogenies, by highlighting species and genera differences. We also wanted to reassess the diagnostic value of some morphological characters, such as the cortical structures and the arrangement of the inhalant orifices, which have been traditionally used to separate genera. Furthermore, a new *Antarctotetilla* species has been described and the genus *Levantiniella* Carella et al., 2016 and the type species *Cinachyrella levantinensis* Vacelet et al. 2007 from Southeastern Mediterranean, which was also monophyletic in the above-mentioned phylogenetic studies (Szitenberg et al. 2013; Carella et al. 2016), are described in detail here.

Materials and methods

Sampling and spicule analysis

Individuals of Antarctic and Subantarctic Tetilidae were collected from several sites at depths ranging from 300 to 600 meters during two expedition cruises (Polarstern ANT-XXVII/3 -2011- and CEAMARC -2008-) employing Agassiz (AGT) and bottom trawl (BT) gears. The sponges were photographed once collected, cut into pieces of about 3 cm² and fixed in absolute alcohol and frozen for transporting. Once in the laboratory, samples were stored in a freezer at -20˚ at CEAB (Centre D'Estudis Avançat de Blanes) until processing. Additionally, two specimens of *Cinachyra australiensis* (Carter, 1886) from Alexandria (Station 101, BMNH YX1-1933), three individuals (06-07-2003-1_SLT_001) and six individuals (11-07-2003-2_JNE_001) of *Levantiniella levantinensis* (previously *Cinachyrella levantinensis*) were collected by SCUBA diving from the Lebanon coast (South-eastern Mediterranean) and loaned from Jean Vacelet of Centre d'Océanologie de Marseille, Aix-Marseille University. Sampling locations of the studied specimens are depicted in Figure. 2.1.

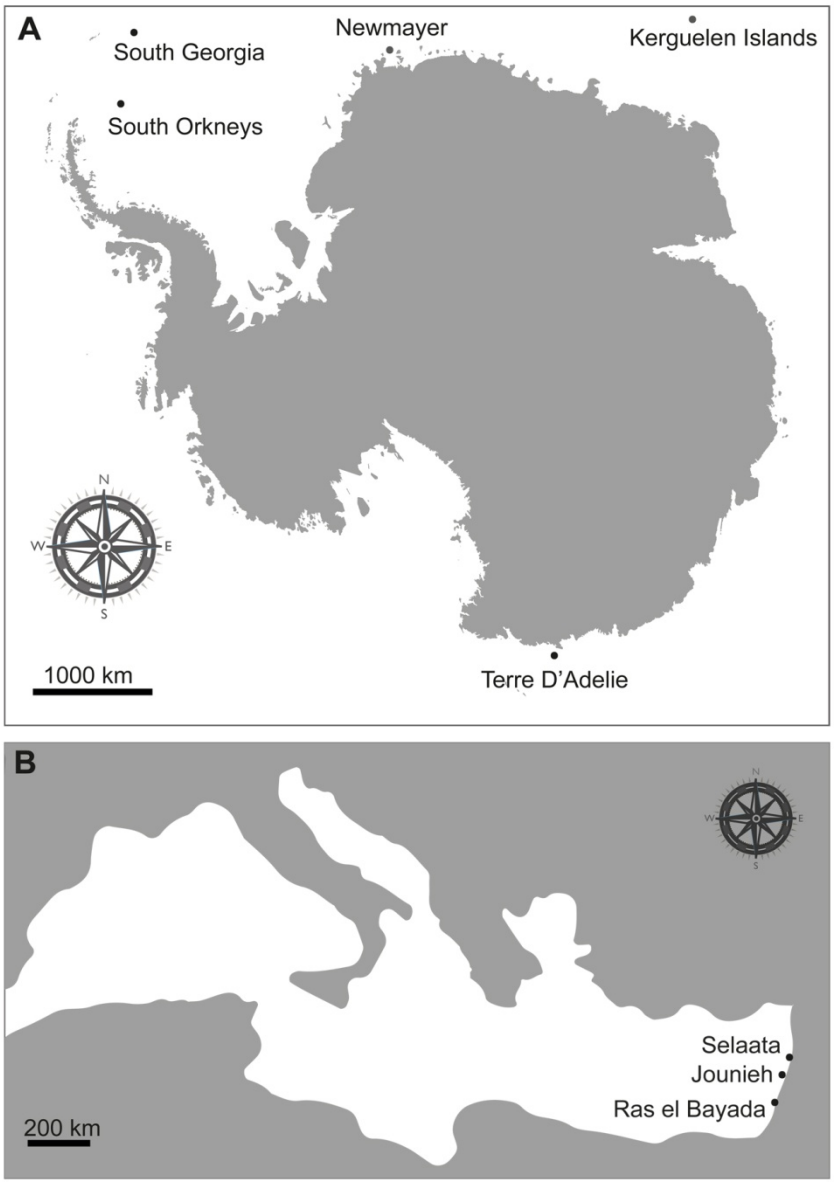

Figure 2.1. A. Map of the sampling sites of *Antarctotetilla* and *Cinachyra* in Antarctic regions: South Georgia, South Orkneys, Newmayer, Kerguelen Island and Terre D'Adelie. B. Sampling sites of *Cinachyrella levantinensis* individuals in Lebanese coast: Ras El Bayada, Selaata and Jounieh.

Besides the material above, other materials were examined too. Small fragments of the syntype of *Tethya sagitta* Lendenfeld, 1907 (ZMB Por 3504), the holotype of *Tethya crassispicula* Lendenfeld, 1907 (ZMB Por 1248), and the syntype of *Tethya coactifera* Lendenfeld, 1907 (ZMB Por 4175) were obtained on loan from Carsten Luter (Museum für Naturkunde Leibniz, Germany). The holotypes of *Tetilla leptoderma* Sollas, 1886 (NHM89.1.1.3) and *Tetilla grandis* Sollas, 1886 (NHM98.1.1.5) were loaded from the Natural History Museum, NHM, London, United Kingdom. The paratype of *Cinachyrella levantinensis* (MNHN-DJV 97) and specimens of *Antarctotetilla grandis* (voucher MNHN-IP 2011 167) and *Antarctotetilla sagitta* (vouchers MNHN-IP 2009 31; MNHN-IP 2009 366 and MNHN-IP 2009 359) were obtained from Noemie Vasset and Isabelle Domart-Coulon (Département Milieux et Peuplements Aquatiques, unité BOrEA, MNHN, Paris, France).

Spicule complements were obtained by dissolving small fragments of sponges in nitric acid, dehydrated in ethanol and mounted on microscope slides (Rützler 1978). Ectosome and choanosome sections were made either using a microtome autocut 2040 (Reichert-Jung), after inclusion in paraffin, an isomet low speed saw (Buehler), after inclusion in agar resin (Boury-Esnault et al. 2002; Cárdenas et al. 2009), or hand made using a razor blade (Hooper 1997).

Ca. 30 measurements per spicule type were performed. Minimum, average, and maximum values are given in the species descriptions (Tables 2.1 and 2.2). For scanning electron microscope (SEM) observations, small pieces of sponge tissue (about 3-5 mm) were cut and boiled in nitric acid using pyrex tubes until complete elimination of the organic matter. Spicules were cleaned with distilled water and then dehydrated in absolute alcohol. One or more drops of alcohol containing spicules were placed on carbon stubs and, after drying, metalized with gold-palladium in a sputtering Polaron SC500. Observations of spicules were performed through a Hitachi TM3000 scanning electron microscope at Centre of Advanced studies of Blanes (CEAB).

The classification of Tetillidae in this study followed Hooper & Van Soest (2002), modified according to the two recent phylogenetic studies (Szitenberg et al. 2013; Carella e tal. 2016).

Table 2.1 Spicule measurements (in µm) of the Antarctic Tetillidae species studied. Maximum and minimum values correspond to the average of the several individuals measured (from 1 to 5, depending on the species).

	Museum codes /Voucher numbers	Large fusiform oxeas	Auxiliary oxeas	Anatriaenes 1	Anatriaenes 2	Anatriaenes 3	Protriaenes 1	Protriaenes 2	Trichodal protriaenes	Sigmaspires
Antarctotetilla	CEAB.POR.BIO.500a ANT 27112	5800-7045-8400	510-1075-1600	2800-6995.8-11380	2000-3510-7450	–	2580-6301.4-11800	1600-2752.5-5780	420-750.6-1235	10-14.7-22.5
leptoderma	CEAB.POR.BIO.500b ANT 27111	X 60-71-85	X 7.5-17.6-35	X 15-22-27.5	X 8.7-14.8-20		X 12.5-21.5-37.5	X 5-10-12.5	X 2.5-4.3-5	
	CEAB.POR.BIO.500c ANT 27109 CEAB.POR.BIO.500d ANT 27108 CEAB.POR.BIO.500e ANT 27107			Clades: 120-186.2-280	Clades: 35-57.9-90		Clades: 60-158-270	Clades: 25-106.2-190	Clades: 10-34.2-105	
Antarctotetilla	CEAB.POR.BIO.501a ANT 27124	3500-5513.8-7850	600-1211.8-1900	3340-9195-14550	1650-3483.3-6000	–	4180-5217.7-8000	1700-3152.7-5400	465-931.6-1677.5	12.5-13.5-15
grandis	CEAB.POR.BIO.501b ANT 27123	X 35-54.6-78	X 6.2-20-35	X 17.5-26.3-30	X 8.7-15.6-20		X 10-28.4-42.5	X 6.2-12-18.5	X 2.5-3.3-3.7	
	MNHN-IP 2009 167			Clades: 140-220.3-320	Clades: 50-75-120		Clades: 60-152-280	Clades: 22.5-84.9-155	Clades: 12.5-57.9-92.5	
Antarctotetilla	MNHN-IP 2009 366	3700-6418.4-12250	690-1175.5-2007	4000-11357.9-19750	3150-3562.5-3900	–	3350-10993.3-17450	1500-3608.7-5540	400-958.8-2840	10-11-17.5
sagitta	MNHN-IP 2009 359	X 35-62.3-110	X 6.5-20.6-35	X 17.5-22.5-32.5	X 12.5-15.8-22.5		X 12.5-28.5-50	X 5-9.9-15	X 2.5-3.5-5	
	MNHN-IP 2009 31			Clades: 100-188.4-250	Clades: 55-74.6-100		Clades: 80-152.3-260	Clades: 20-139.3-260	Clades: 5-36-175	
Antarctotetilla		4050-7682.5-10950	630-1137-1780	7180-12460-16880	3220-3806.7-4960	4400-10265-14350	4300-6986.2-10880	2400-2767.5-3250	405-1063.6-2480	10-11.6-12.5
pilosa nov. sp.	CEAB.POR.BIO.502a ANT 27211	X 35-83.5-120	X 12.5-22.4-35	X 22.5-24.5-27.5	X 15-17.5-22.5	X 17.5-24.4-27.5	X 20-30-40	X 10-11.9-17.5	X 2-2.6-5	
				Clades: 160-190.5-210	Clades: 62.5-85.8-130	Clades: 70-117.6-140	Clades: 100-181.7-260	Clades: 80-142-240	Clades: 10-51.4-175	
**Tetillla leptoderma*[1] (now *Antarctotetilla leptoderma*)		4185-4284	measurement	6050 X 35.5	6000 x 10	–	4030 x 11.8	measurement	1162	13.8-19.7
		X 43.4-47.4	not given	Clades: 154	Clades: 118		Clades: 197	not given	Clades: 12-60	

**Tetilla grandis*[1] (now *Antarctotetilla grandis*)	5720-6070 X 75-79	measurement not given	12140 x 20 Clades: 158	Clades: 100	–	8600 X 15.8 Clades: 150	measurement not given	measurement not given	11.8
**Craniella sagitta*[2] (now *Antarctotetilla sagitta*)	5000-13000 X 60-80	1120 X 40	12000 x 20 Clades: 225	> 10000 Clades: 118	–	9000 X 52 Clades: 135	measurement not given	218 Clades: 28	12.3-13
**Tethya sagitta*[3] (now *Antarctotetilla sagitta*)	1600-1900 X 23-28-33	550-800 X 12-18	4500-5200 X 16-22 Clades: 90-120	measurement not given	–	2000-3700 X 8-18 Clades: 35-180	measurement not given	600-800 X 2.3 Clades: 10-108	17-20
**Tethya coactifera*[3] (now *Antarctotetilla coactifera*)	2850-4310-5400 X 25-40-50	470-686.6-960 X 10-16.25-22.5	7000-9400-11580 X 20-25.4-30 Clades: 150-167.7-200	2862.5-3647.8-4700 X 5-11-17.5 Clades: 20-47.5-70	3900-6620-9400 X 17.5-23.8-30 Clades: 75-110.6-140	3050-4539.4-7200 X 12.5-14.7-20 Clades: 50-121.3-200	1450-2245-2960 X 7.5-9.2-12.5 Clades: 10-61.7-112.5	425-551-675 X 2.5-2.6-3.75 Clades: 10-18.3-50	10-13.7-20
**Tethya crassispicula*[3] (now *Cinachyra crassispicula*)	5775–6890–7750 X 50–64–80	300-582.3-950 X 15-23.6-40	4500-11158-18800 X 12.5-20-27.5 Clades: 60-92.5-125	3500-4065-4650 X 7.5-9.3-12.5 Clades: 30-38.6-50	–	4470-6244.3-12360 X 10-15.4-27.5 Clades. 80-110.3-150	1450-2630-3970 X 3.75-7.26-10 Clades: 20-58.9-90	287.5-629-1400 X 1.25-2.7-5 Clades: 10-21.2-50	12.5-15-20

*Type species: [1] from Sollas, 1886; [2] from Kirkpatrick, 1908 var. microsigma and var. pachyrrhabdus; [3] from Lendenfeld, 1907.

Table 2.2 *Levantiniella levantinensis*. Spicule measurements (in µm). Maximum and minimum values are averages of the nine individuals measured.

	Museum codes /Voucher numbers	Large fusiform oxeas	Anatriaenes	Protriaenes	Spined microxeas	Sigmaspires
Levantiniella levantinensis	Three individuals (06-07-2003-1_SLT_001) Six individuals (11-07-2003-2_JNE_001)	380-3250.4-5300 X 7.5-28-50	1900-3633.8-5520 X 5-9-15 Clades: 20-57-110	1600-2763.3-7140 X 2.5-6.9-12.5 Clades: 12.5-47.5-105	55-96.6-200 X 1.25-2.5-5	10-12.7-15
**Cinachyrella levantinensis*[1]		1250-6250 × 2.5-42	6500 × 3.5-6 Clades: 7-50 × 3-5	1100-2000 Clades: 10-100 × 2.5-6	60-120 × 1-2	10–16

* Type species: [1] from Vacelet, 2007.

DNA extraction, amplification and sequencing of the minibarcode secuences.

For a better identification of the types in addition to their morphological examination, we used a universal DNA mini-barcode (a small COI fragment useful for identifying old specimens with degraded DNA). Genomic DNA was extracted with DNeasy Blood & Tissue kit (Qiagen), according to the manufacturer's protocol. The minibarcode marker was amplified using Applied Biosystems 3730xl DNA analyzers (Macrogen, South Korea) with primer forward Uni-minibarF1 (Meusnier et al. 2008) and a designed primer reverse for Tetractinellida order, Tetract-minibarR1 (Cárdenas & Moore 2017).The amplifications were performed in in a 50 µL volume reaction, containing 33,45 µL H_20, 5 µL buffer (BIOLINE), 0,75 µL MgCl (BIOLINE), 2,4 µL DMSO (dimethyl sulfoxide), 2 µL BSA, 2µL dNTP (Sigma), 1 µL primers forward/reverse, 0,4 Taq (BIOLINE) and 2 µL of genomic DNA, using the following PCR program: [5 min/94 °C; 37 cycles (15 s./94 °C, 15 s./46 °C, 15 s./72 °C); 7 min/72 °C]. Sequences were aligned using BioEdit v.7.2.5 (Hall 1999) program and the obtained minibarcodes were compared with other Tetillidae sequences using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

Systematics

Order TETRACTINELLIDA Marshall, 1876
Sub-Order SPIROPHORINA Bergquist & Hoggs, 1969
Family TETILLIDAE Sollas, 1886
Genus *Levantiniella* Carella et al., 2016
Synonymy:*Levantinella* Szitenberg et al*.,* 2013

Definition: Tetillidae with small shallow porocalyx-like structures, with spined microxeas, without auxiliary megascleres, and without cortex (Szitenberg et al*.* 2013).

Diagnosis: Globular sponges attached directly to the substrate, without root spicule tufts. Surface hispid, sometimes entirely covered by sand and debris. Porocalyx-like small, rounded structures, sometimes unconspicuous due to sand coverage; oscules inconspicuous. Skeleton composed of bundles of oxeas, anatriaenes, protriaenes, and sometimes prodiaenes spirally arranged, rising from the central zone outwards and protruding the sponge surface. No

cortical specialization visible in cross section. Hispidation caused chiefly by the strong fascicles of large oxeas that protrude the surface. Megascleres are large oxeas, protriaenes, and prodiaenes; microscleres are microxeas and sigmaspires. Species distributed in shallow waters of Southeastern Mediterranean Sea (Vacelet et al. 2007).

Remarks

The only species of this genus was placed in *Cinachyrella* on the basis of the absence of cortex and presence of porocalices (Vacelet et al. 2007). However, the revision of the paratype MNHN-DJV 97and several other specimens, which served to describe the new species, proved they have not true porocalices (as they have been defined by Boury-Esnault & Rützler 1997), but shallow open cavities. This characteristic, plus the secondary structure of the V4 region of 18S allowed retrieving a monophyletic clade separated from *Cinachyrella* in the parsimony phylogeny (Carella et al. 2016). Moreover, the molecular phylogeny based on several molecular markers (COI M1-M6, 18S and 28S D3-D5) also recovered a monophyletic group for *Cinachyrella levantinensis*, clearly separated from *Cinachyrella* (Szitenberg et al. 2013; Carella et al. 2016). All these morphological and molecular characteristics allowed us to include the species previously considered as *C. levantinensis* in the new genus *Levantiniella.*

Levantiniella levantiniensis (Vacelet et al., 2007)

Species type

Cinachyrella levantinensis Vacelet et al., 2007 by monotypy.

Synonymy: *Cinachyra cavernosa* (Lamarck, 1815 *sensu* Tsurnamal, 1969); *Chrotella cavernosa sensu* Tsurnamal, 1969; *Cinachyra australiensis sensu* Burton, 1936.

Material examined: Paratype of *Cinachyrella levantinensis* MNHN-DJV 97 Vacelet et al., 2007, Ras El Bayada, Lebanon, 33°9.969'N 35°10.853'E, 6 m depth, 12 July 2003; three individuals (registration number: 06-07-2003-1_SLT_001), Selaata, Lebanon, 34°17' 17 N, 35°39'54 E, 20 m depth, 06 July

2003 and six individuals (registration number: 11-07-2003-2_JNE_001), Jounieh, Lebanon, 34°01' 46 N, 35° 37' 18 E, 15 m depth, 11 July 2003.
GenBank accession numbers (Szitenberg et al. 2013): TAU 25529 (JX177906 and JX177970), TAU 25568 (JX177904 and JX177969), MHNM 16194 (JX177905 and HM629803), DH S124 (JX177903) and TAU 25456 (HM629802).

Description (Figure 2.2)

The species morphology matches the genus diagnosis: globular sponges directly attached to the substrate. The sponges measure 2-3 cm in diameter (Figure 2.2a). Small, rounded, shallow depressions at the sponge surface covered by sand (Figure 2.2b). Color yellow in life, brown-cream in alcohol. Transversal sections allow observing a dense ectosomal layer, which resembles a cortex, but that it is made of sediment (Figure 2.2c). In the outer part of the choanosome, many carbonate-made debris are discernible.

Spicules (Figure 2.3 ; Table 2.2)

Megascleres: oxeas (Figure 2.3a) large and fusiform: 380–3250.4–5300 µm x 7.5–28–50 µm. Anatriaenes (Figure 2.3e-f) with long, slender, or reduced to a knob clades: 20–57–110 µm; fusiform rhabdomes, progressively attenuated to a filiform termination: 1900–3633.8–5520 µm x 5–9–15 µm. Protriaenes, sometimes prodiaenes (Figure 2.3b-c), clades: 12.5–47.5–105 µm; rhabdomes thin and often flexuous: 1600–2763.3–7140 µm x 2.5–6.9–12.5 µm. Microscleres: sigmaspires (Figure 2.3g): 10–12.7–15 µm, and spined microxeas (Figure 2.3d-g): 55–96.6–200 µm x 1.25–2.5–5 µm.

Skeletal arrangement

Skeleton mainly formed by large fusiform oxeas often accompanied by protriaenes, prodiaenes, and anatriaenes grouped in bundles, which run spirally from the central part of the sponge towards the surface. Large oxeas are the most abundant spicule type and are responsible for the surface hispidation.

Microxeas and sigmaspires, distributed everywhere but mainly concentrated around the choanosome canals.

Distribution and habitat

Shallow waters, between 6 and 35 meters depth, of South-eastern Mediterranean Sea (Lebanese coast). Rocky substrates (Vacelet et al. 2007; Szitenberg et al. 2013).

Remarks

The presence of pores in the small, shallow depressions on the sponge surface were interpreted by Vacelet et al. (2007) as a kind of shallow porocalices but this could not be confirmed here in the paratype examined, because of the hard sandy layer that cover the sponge surface.

However, the other specimens examined (three individuals from Selaata, Lebanon (06-07-2003-1_SLT_001) and six individuals from Jounieh, (Lebanon 11-07-2003-2_JNE_001), presented the small, rounded, shallow depressions at the sponge surface, which resembled porocalices.

The two specimens of *C. australiensis* (Carter, 1886) from Alexandria reexamined showed similar spicule features to *Levantiniella levantinensis* (Vacelet, 2007). However, the small pieces of the Carter' samples available did not allow us finding anatriaenes, not to observe the external morphological features.

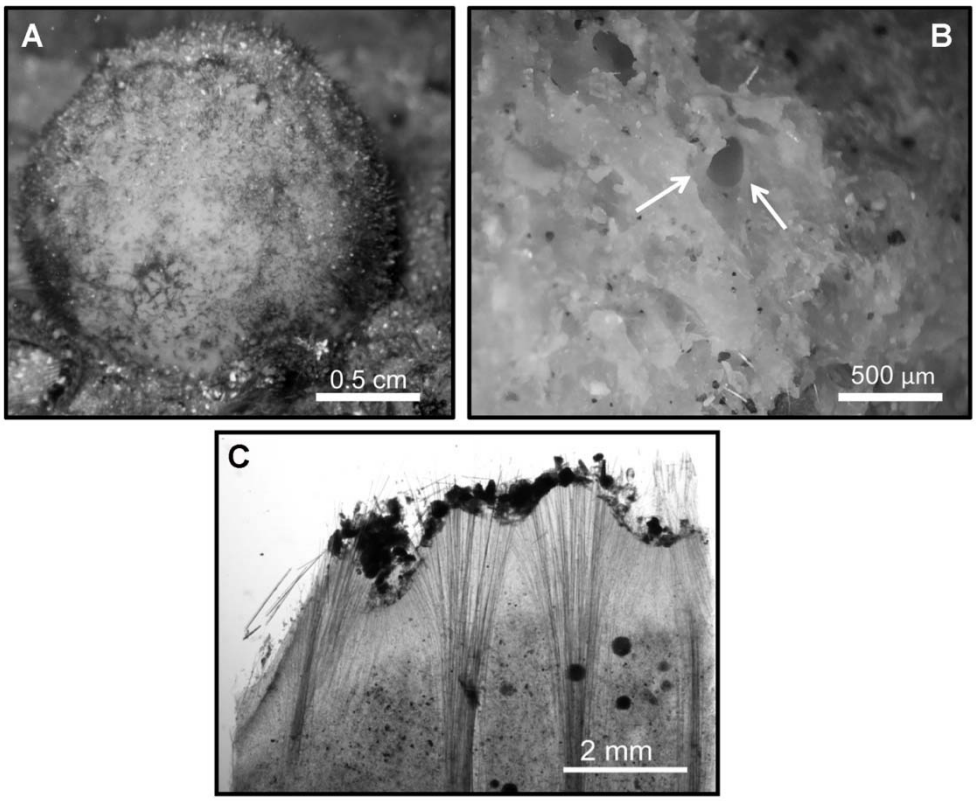

Figure 2.2. A. Underwater picture of *Levantiniella levantinensis* from the shore of Maagan Michael, Israel (courtesy of Micha Ilan, Tel Aviv University). B. Interior of *L*. *levantinensis*, arrows point to a canal. C. Transversal section of *L*. *levantinensis* (from Carella et al. 2016).

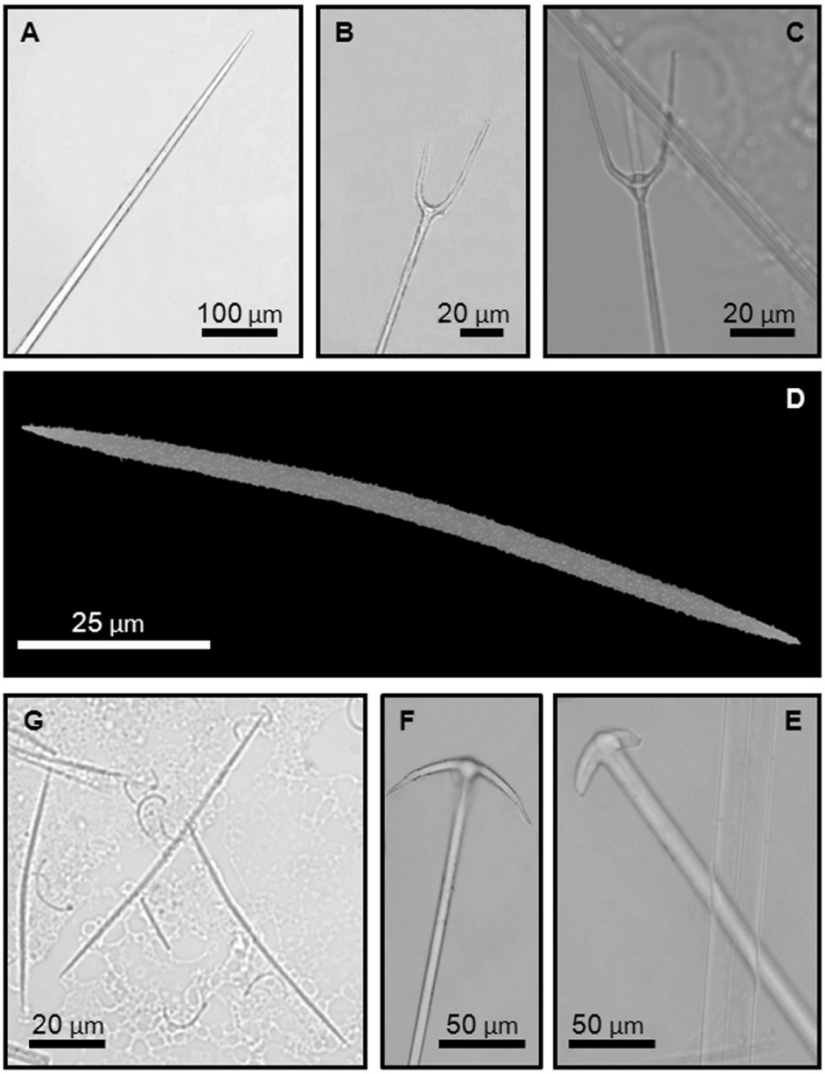

Figure 2.3. Spicules of *Levantiniella levantinensis* (six individuals from Selaata and three individuals from Jounieh, Lebanon). A. Terminal part of a large fusiform oxea. B. Protodiaene. C. Protriaene. D. Spiny microxeas. E, F. Anatriaenes. G. Microxeas and sigmaspires.

Genus *Antarctotetilla* Carella et al., 2016

Definition: Tetillidae with pores grouped in small surface depressions or in sieve-like areas, without porocalices and without a proper cortex, but with a loose arrangement of auxiliary oxeas at the sponge periphery (i.e. pseudocortex) (Carella et al. 2016).

Diagnosis: Globular or egg shaped sponges. Surface mainly smooth and without porocalices, with one or several oscules situated on top. Pores always grouped in small surface depressions or in sieve-likes overlying a subectosomal cavity. Basal bundles of spicules at the sponge base, acting as a root system. Consistency generally compact out of the water. No a real cortex, but a pseudocortex composed of few auxiliary oxeas, perpendicular to the surface mostly concentrated at the final part of the radial spicule bundles. Choanosomal skeleton consists of spiral bundles of oxeas accompanied by anatriaenes and protriaenes radiating from the sponge center towards the surface. The bundles may pierce the ectosome in some zones and thus cause a locally hispid surface. Megascleres are oxeas, protriaenes, anatriaenes and trichodal protriaenes; microscleres are sigmaspires. Distribution: mainly on deep bottoms of Antarctic, Subantarctic, and New Zealand waters (Carella *et al*. 2016)

Remarks

Antarctotetilla differs from the typical *Tetilla* by the presence of pores grouped in shallow sub-ectosomal depressions, covered by a perforate ectosome or not, and a peripheral concentration of auxiliary oxeas, irregularly arranged, forming a visually discerned layer, very different of a true cortex (e.g. that of *Craniella* or *Cinachyra*). The genus also differs from *Cinachyra* and *Cinachyrella* in the lack of porocalices. The reexamination of the species types (holotypes of *Tetilla leptoderma*, *Tetilla grandis* and syntype of *Tethya sagitta*) allowed us confirming these morphological features. The molecular and parsimony (morphological) phylogenies (Carella et al. 2016) clustered specimens previously described as *Tetilla leptoderma* Sollas, 1886*, Tetilla grandis* Sollas, 1886*, and Tethya sagitta* Lendenfeld, 1907 in a monophyletic clade that corresponds to the new genus. Furthermore, the revision of the syntype of *Tethya coactifera* as well as the new

species Tetillidae sp.3 (Carella et al. 2016), now *A. pilosa* (see below), has proved they belong in *Antarctotetilla*.

Antarctotetilla leptoderma (Sollas, 1886)

Synonymy*: Tetilla leptoderma* Sollas, 1886*; Tethya stylifera* Lendenfeld, 1907*; Tetilla grandis* Sollas, 1886*; Tetilla grandis* var.*alba* Sollas, 1886.

Material examined: holotype of *Tetilla leptoderma* NHM89.1.1.3 Sollas, 1886, Rio de la Plata, Argentine; *Antarctotetilla leptoderma*, CEAB.POR.BIO.500a ANT 27112, Newmayer, Antarctic coasts, -70° 51.52‘S, 10° 36.72‘W, 236-285 m depth, 01 April 2011; CEAB.POR.BIO.500e ANT 27107,Newmayer, Antarctic coasts, -70° 56.05‘S, 10° 30.05‘W, 211-243 m depth, 25 March 2011and CEAB.POR.BIO.500c ANT 27109, Newmayer, Antarctic coasts, -70° 47.54‘S, 10° 29.56‘W, 272-288 m depth, 28 March 2011; *Antarctotetilla leptoderma*, CEAB.POR.BIO.500d ANT 27108, South Orkneys, Subantarctic region, -61° 8.59‘S,43° 58.37‘W, 346-397 m depth, 19 February 2011; *Antarctotetilla leptoderma*, CEAB.POR.BIO.500b ANT 27111, South Georgia, Subantarctic region, -54° 26.12‘S, -35° 41.54‘W, 265-266 m depth, 16 February 2011.

GenBank accession numbers (Carella et al. 2016): CEAB.POR.BIO.500a ANT 27112 (KT124319, KT124343, KT124329 and KT124365), CEAB.POR.BIO.500e ANT 27107 (KT124351 and KT124358), CEAB.POR.BIO.500c ANT 27109 (KT124354), CEAB.POR.BIO.500d ANT 27108 (KT124323 and KT124347) andCEAB.POR.BIO.500b ANT 27111 (KT124318, KT124341, KT124328 and KT124362).

Description (Figure 2.4)

Sub-spherical or ovoid sponges with a variable size between 4 and 8 cm in diameter (Figure 2.4a). Large individuals compact; young individuals softer and more compressible. Surface conulose and corrugate, smooth to touch. Only one large oscule placed on top. Pores grouped in small depressions scattered over the entire surface (Figure 2.4c), but mostly concentrated at the equatorial zone and between the grooves formed by the conical elevations. Color dirty white or brown in living specimens, greenish in alcohol. Not a real cortex but a

pseudocortex (Figure 2.4d-e), which consists of a loosely arranged auxiliary oxeas perpendicular to the surface. Root tufts composed of anatriaenes are used as anchors to fix the sponge to the substrate.

Spicules (Figure 2.5; Table 2.1)

Megascleres: oxeas I (Figure 2.5a) large and fusiform: 5800–7045–8400 µm x 60–71–85 µm. Auxiliary small oxeas II (Figure 2.5b): 510–1075–1600 µm x 7.5–17.6–35 µm. Anatriaenes I (Figure 2.5f): 2800–6995.8–11380 µm x 15–22–27.5 µm in size (rhabdomes thickness measured at the base of clades) with long and thin clades: 120–186.2–280 µm; rhabdomes fusiform, thicker at the middle and filiform at the terminal part. Anatriaenes II: 2000–3510–7450 µm x 8.7–14.8–20 µm in size (Figure 2.5e) with short clades: 35–57.9–90 µm and fusiform rhabdomes. Protriaenes I (Figure 2.5d): 2580–6301.4–11800 µm x 12.5–21.5–37.5 µm, with clades of 60–158–270 µm long, usually one clade longer than the other two; rhabdomes tapering from the base of the clades to end in a filamentous termination. Protriaenes II (Figure 2.5c): 1600–2752.5–5780 µm x 5–10–12.5 µm, with clades: 25–106.2–190 µm in length, usually one clade longer than the other two; rhabdomes tapering to a filiform end. Trichodal protriaenes (Figure 2.5g-h) very small with filamentous rhabdomes: 420–750.6–1235 µm x 2.5–4.3–5 µm long and thin, 10–34.2–105 µm long clades. Microscleres: sigmaspires (Figure 2.5i): 10–14.7–22.5 µm in length.

Skeletal arrangement

Choanosomal skeleton made of bundles of oxeas with protriaenes and anatriaenes spirally arranged from the central part to the sponge periphery. Only oxeas and protriaenes protrude three mm the surface forming the spicular tufts of the conical elevations (Figure 2.4b). Auxiliary oxeas arranged in palisade at the sponge periphery and scattered in the choanosomal zone. Trichodal protriaenes scattered at the peripheral zone, mostly concentrated around pore and oscule areas. Sigmaspires present throughout the sponge.

Distribution and habitat

Antarctic coasts (Newmayer), Subantarctic (South Georgia and South Orkneys), Argentina (off Rio de la Plata - Sollas 1886 - Malvinas - Sarà et al. 1992),

Kerguelen (Lendenfeld 1907), Chile (Desqueyroux-Faúndez 1989), South Shetland Islands (Ríos et al. 2004), New Zealand (Szitenberg et al. 2013). The specimens live in sediment and rocky substrates from 45 meters to more than 600 meters of depth.

Remarks

Antarctotetilla leptoderma has been repeatedly put in synonymy with *A*. *grandis* (Burton 1929; Boury-Esnault & Van Beveren 1982). However both species clearly differ in several morphological characters. The main morphological differences between both species are the globular body shape, the smooth surface, and the numerous small oscules situated on short conical elevations in *A*. *grandis versus* an elongate ovoid shape, the surface small protrusions, and a single large oscule in *A*. *leptoderma*.

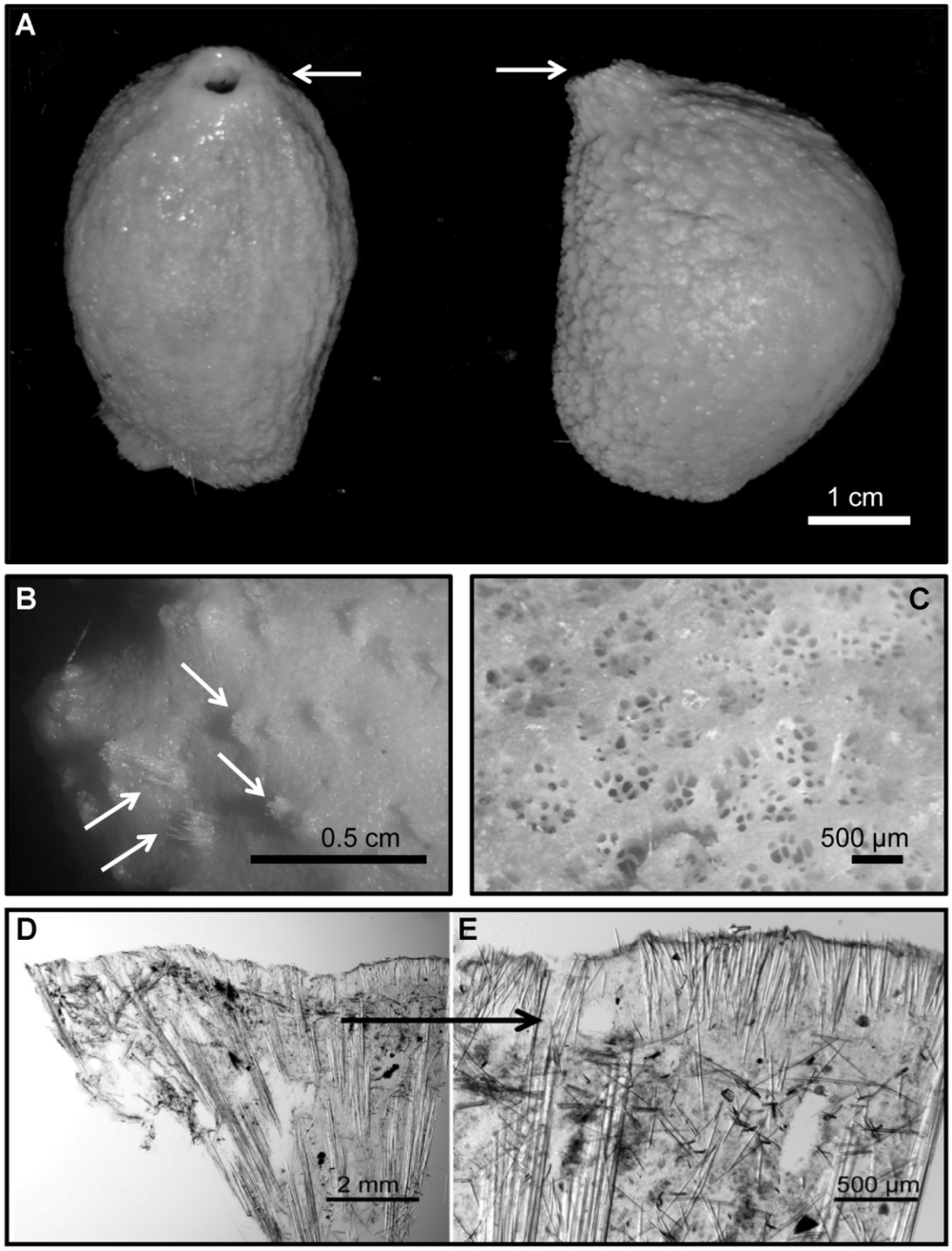

Figure 2.4. *Antarctotetilla leptoderma* from South Georgia (CEAB.POR.BIO.500b ANT 27111). A. Iexternal shape: arrows point to the osculum on top. B. Detail of the surface: arrows point to the spicular tufts that protrude the conical elevations. C. Pores grouped in small surface depressions. D, E. Transversal sections. Pictures A, D, E are from Carella et al. (2016).

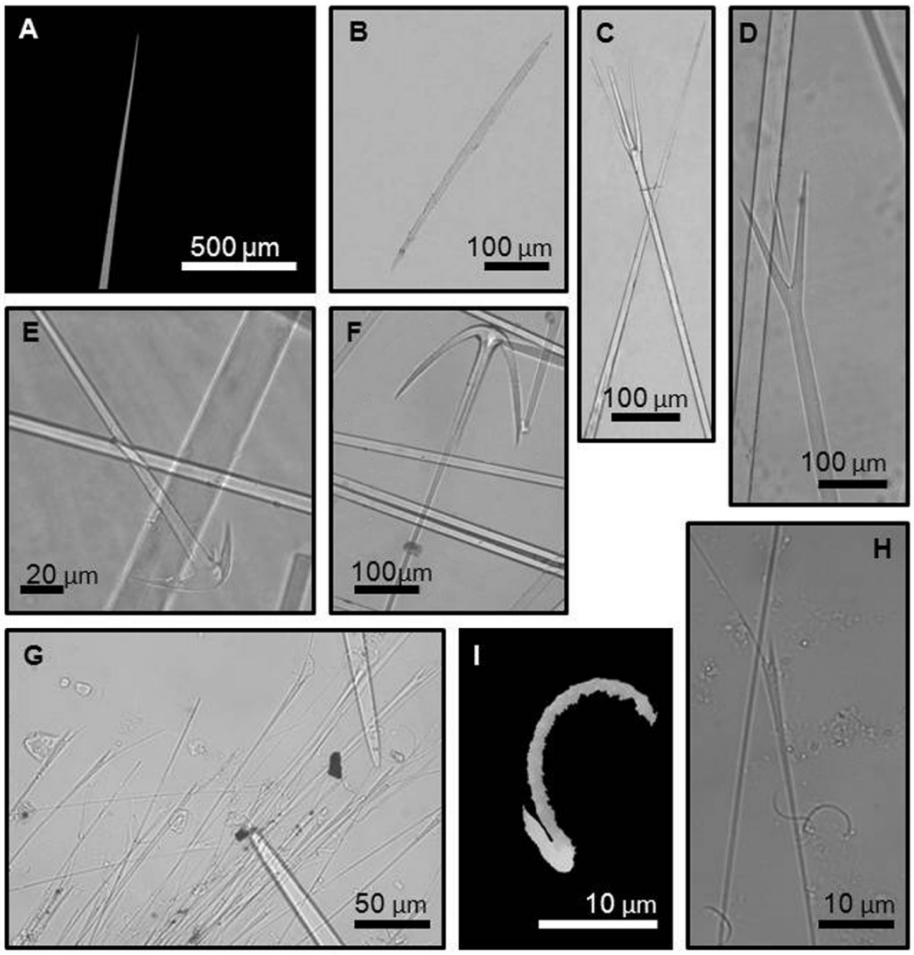

Figure 2.5. Spicules of *Antarctotetilla leptoderma* from South Georgia (CEAB.POR.BIO.500b ANT 27111). A. Terminal part of large fusiform oxea I. B. Auxiliary secondary oxea II. C. Medium size protriaene II. D. Large Protriaene I. E. Small anatriaene II. F. Large Anatriaene I. G. Trichodal protriaenes. H. Trichodal protriaenes and sigmaspires. I. Sigmaspire.

Antarctotetilla grandis (Sollas, 1886)

Synonymy: *Tetilla leptoderma* Sollas, 1886; *Tetilla grandis* var. *alba* Sollas, 1886; *Craniella coactifera* (Lendenfeld, 1907).

Material examined: holotype of *Tetilla grandis* NHM98.1.1.5 Sollas, 1886, Kerguelen, Subantarctic region; *Antarctotetilla grandis*, CEAB.POR.BIO.501b ANT 27123 and CEAB.POR.BIO.501aANT 27124, Newmayer, Antarctic coasts, -70° 52.21‘S, 10° 35.81‘W, 225-284 m depth, 01 April 2011; *Tetilla grandis*, MNHN-IP 2011 167, Terre D’Adelie, Antarctic coasts, -66°34'S, 141°204'E, 170-210 m depth, 13 January 2008.

GenBank accession numbers (Carella et al. 2016): CEAB.POR.BIO.501b ANT 27123 (KT124324, KT124344, KT124330 and KT124363) and CEAB.POR.BIO.501a ANT 27124 (KT124325, KT124346, KT124331 and KT124364).

Description (Figure 2.6)

Globular, almost spherical sponges, about 10 cm in diameter (Figure 2.6a). Surface smooth; oscules numerous on small conical elevations of the sponge surface (Figure 2.6b). Pores grouped in small depressions, widespread overall the surface (Figure 2.6c). Color beige in life, yellowish brown in alcohol. Pseudocortex (Figure 2.6d) composed of auxiliary oxeas loosely arranged perpendicularly to the surface, as in *Antarctotetilla leptoderma.* Anchoring anatriaene-made tufts.

Spicules (Figure 2.7; Table 2.1)

Megascleres: oxeas I (Figure 2.7a) large and fusiform: 3500–5513.8–7850 µm x 35–54.6–78 µm. Auxiliary small oxeas II (Figure 2.7b): 600–1211.8–1900 µm x 6.2–20–35 µm. Anatriaenes I (Figure 2.7f): 3340–9195–14550 µm x 17.5–26.3–30 µm in size with long and thin clades: 140–220.3–320 µm; rhabdomes fusiform, thicker at the middle and filiform at the terminal part. Anatriaenes II: 1650–3483.3–6000 µm x 8.7–15.6–20 µm in size (Figure 2.7e) with short clades: 50–75–120 µm and fusiform rhabdomes. Protriaenes I (Figure 2.7c): 4180–5217.7–8000 µm x 10–28.4–42.5 µm, with clades of 60–152–280 µm long, usually one clade longer than the others two; rhabdomes tapering from the

base of the clades to end in a filamentous termination. Protriaenes II (Figure 2.7d): 1700–3152.7–5400 µm x 6.2–12–18.5 µm, with clades: 22.5–84.9–155 µm in length, usually one clade longer than the other two; rhabdomes tapering to a filiform end. Trichodal protriaenes (Figure 2.7g) very small with filamentous rhabdomes: 465–931.6–1677.5 x 2.5–3.3–3.7 µm long and thin, 12.5–57.9–92.5 µm long clades. Microscleres: Sigmaspires (Figure 2.7h-i): 12.5–13.5–15 µm in length.

Skeletal arrangement

The skeletal structure is typical of Tetillidae, made of bundles of oxeas together with anatriaenes and protriaenes, which radiate upward from the central part to the periphery. Spicules protrude the ectosome only rarely and thus the surface is almost even. Auxiliary oxeas arranged in palisade at the sponge periphery and scattered in the choanosomal zone. Trichodal protriaenes have been reported at the peripheral zone (Sollas 1888), mostly around the incurrent canals under the grouped pores. Sigmaspires spread throughout the sponge.

Distribution and habitat

Antarctic coasts (Newmayer and Terre D'Adelie), Kerguelen Islands (Sollas 1886; Lendenfeld 1907), on sediment and rocky substrates, from 45 to more than 600 meters of depth.

Remarks

The variety *Tetilla grandis* var. *alba,* which lacks the anchoring basal mass described by Sollas (1886), also seems to belong in *Antarctotetilla*.

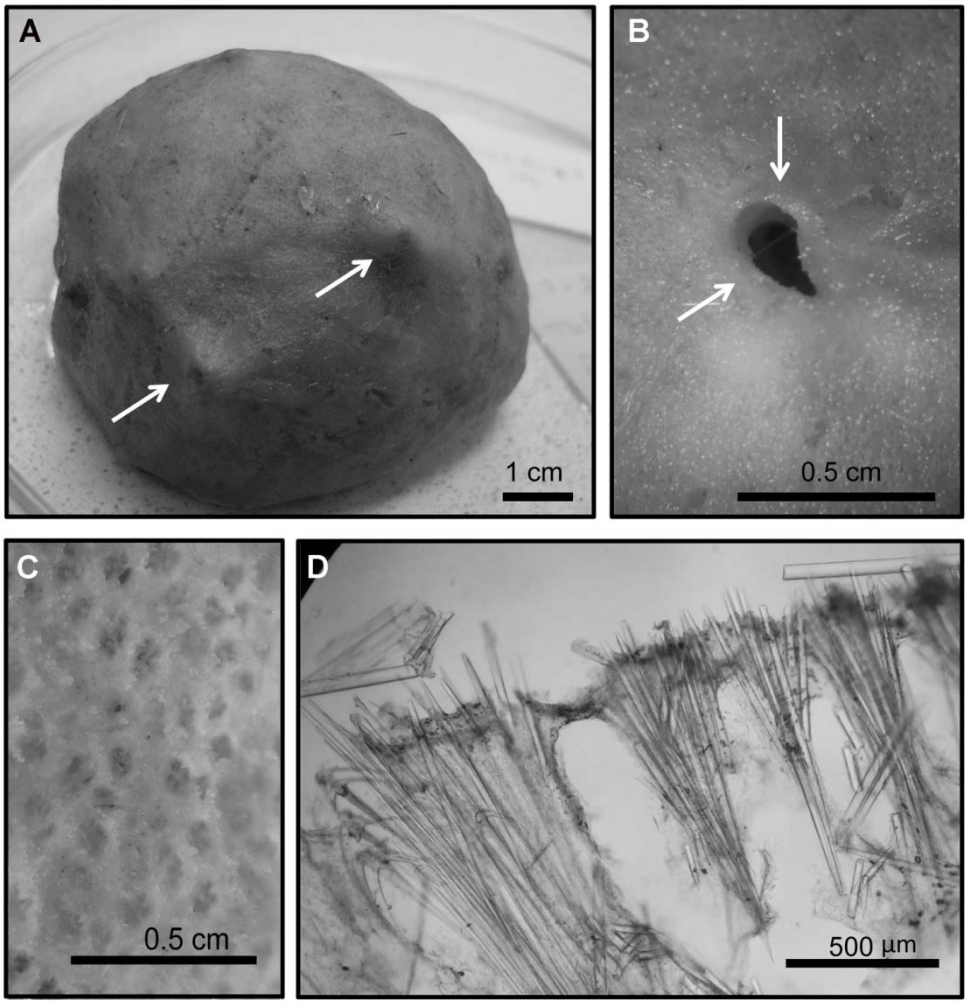

Figure 2.6. *Antarctotetilla grandis* from Terre D'Adelie (MNHN-IP 2011 167). A. Arrows point to the conical elevations. B. Summit of a conical elevation, arrows point to the oscule. C. Pores grouped in small surface depressions. D. Transversal section.

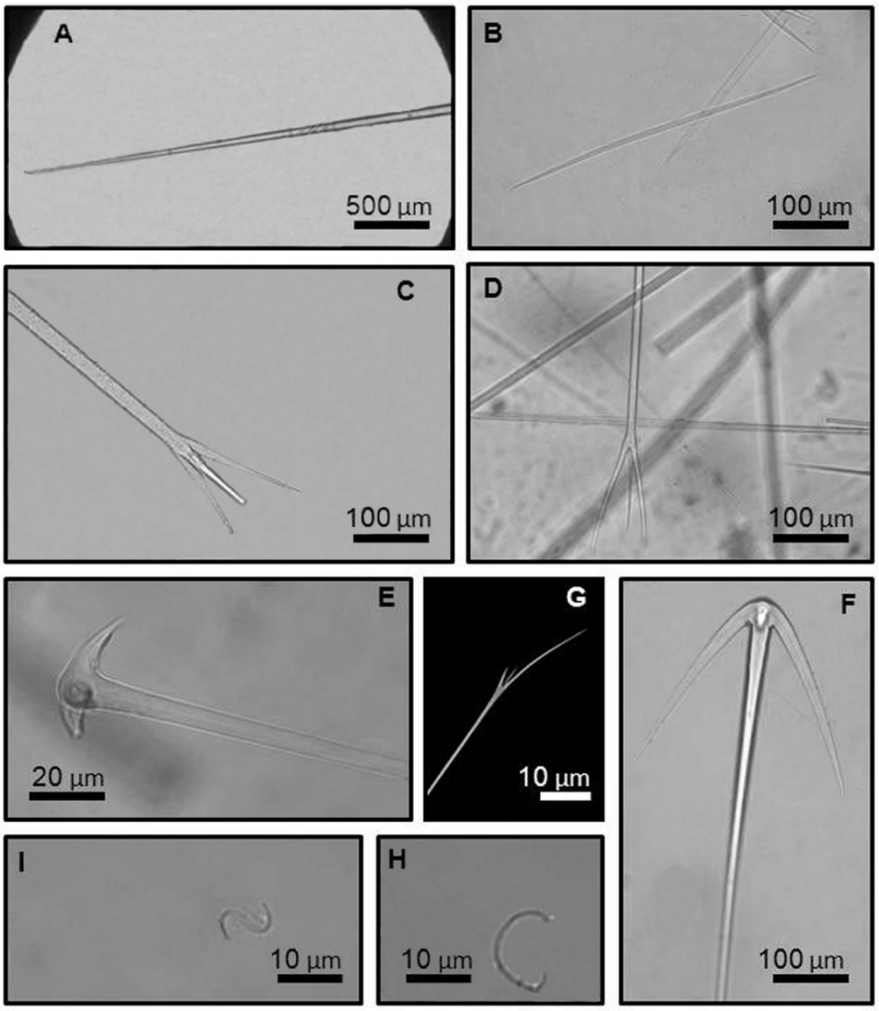

Figure 2.7. Spicules of *Antarctotetilla grandis* from Terre D'Adelie (MNHN-IP 2011 167). A. Terminal part of a large fusiform oxea I. B. Auxiliary secondary oxea II. C. Large Protriaene I. D. Medium size protriaene II. E. Small anatriaene II. F. Large Anatriaene I. G. Trichodal protriaene. H, I. Sigmaspires.

Antarctotetilla sagitta (Lendenfeld, 1907)

Synonymy*: Tethya sagitta* Lendenfeld, 1907; *Craniella sagitta* var. *microsigma* Kirkpatrick, 1908; *Craniella sagitta* var. *pachyrrhabdus* Kirkpatrick, 1908.

Material examined: A small piece of the syntype of *Tethya sagitta*, ZMB Por 3504 Lendenfeld, 1907, Kerguelen, Subantarctic region; *Antarctotetilla sagitta*, MNHN-IP 2009 31, -65°60'S, 143°03'E, 461-483 m depth, 23 December 2007; MNHN-IP 2009 366, -66°20'S, 141°20'E, 207-227 m depth, 13 January 2008 and MNHN-IP 2009 359, -65°60'S, 144°18'E, 229-237 m depth, 12 January 2008, from Terre D'Adélie, Antarctic coasts.

GenBank accession numbers (Carella et al. 2016): MNHN-IP 2009 31 (KT124320, KT124332 and KT124370), and MNHN-IP 2009 359 (KT124327 and KT124334).

Description (Figure 2.8)

Globular sponges of about 8 cm in diameter, enlarged at the equatorial region (Figure 2.8a). Surface corrugate with short projections chiefly formed by groups of oxeas, auxiliary oxeas, and protriaenes, supported by a thin collagen layer. Oscules on top, surrounded by a smooth surface area (Figure 2.8c). Pores are in sub-dermal cavities overlaid by sieve-like ectosomal areas (Fig. 8b), which are distributed mainly at the equatorial region. Sponge color brown-greenish in alcohol. Pseudocortex (Figure 2.8d-e) composed by auxiliary oxeas loosely arranged perpendicular to the sponge surface. In contrast to A. *leptoderma* and A. *grandis,* this species is easily compressible. Anchoring root-tufts composed by anatriaenes.

Spicules (Figure 2.9; Table 2.1)

Megascleres: oxeas I (Figure 2.9a) large and fusiform: 3700–6418.4–12250 µm x 35–62.3–110 µm. Auxiliary small oxeas II (Figure 2.9b): 690–1175.5–2007 µm x 6.5–20.6–35 µm. Anatriaenes I (Figure 2.9e): 4000–11357.9–19750 µm x17.5–22.5–32.5 µm in size with long thin clades: 100–188.4–250 µm in lenth; rhabdomes fusiform, thicker at the middle and filiform at the terminal part. Anatriaenes II: 3150–3562.5–3900 µm x 12.5–15.8–22.5 µm in size (Figure 2.9f) with short clades: 55–74.6–100 µm in length and fusiform rhabdomes.

Protriaenes I (Figure 2.9c): 3350–10993.3–17450 µm x 12.5 –28.5–50 µm, with clades: 80–152.3–260 µm long, usually one clade longer than the other two; rhabdomes tapering from the base of the clades to end in a filamentous termination. Protriaenes II (Figure 2.9d): 1500–3608.7–5540 µm x 5–9.9–15 µm, with clades 20–139.3–260 µm in length, usually one clade longer than the other two; rhabdomes tapering to a filiform end. Trichodal protriaenes (Figure 2.9g) very small with filamentous rhabdomes: 400–958.8–2840 µm x 2.5–3.5–5 µm long and thin, 5–36–175 µm long clades. Microscleres: sigmaspires (Figure 2.9h): 10–11–17.5 µmin length.

Skeletal arrangement

Choanosomal skeleton made of bundles of oxeas, anatriaenes, and protriaenes spiraly arranged. Auxiliary oxeas arranged in palisade at the sponge periphery and scattered throughout the choanosome. Trichodal protriaenes scattered around the oscule rim (Kirkpatrick, 1908) and, sometimes, at the external zone of the ectosome, which they perforate. Sigmaspires distributed through the sponge.

Distribution and habitat

Antarctic coasts, Terre D'Adelie; Subantarctic, Kerguelen (Kirkpatrick 1908; Lendenfeld 1907). Our specimens were collected between 228 and 483 meters of depth.

Remarks

Antarctotetilla sagitta differs from other *Antarctotetilla* species by a corrugate surface with short projections formed by groups of oxeas, auxiliary oxeas, and protriaenes. Kirkpatrick (1908) stated that the surface pile can vary considerably among specimens. The oscules are placed on top and surrounded by a smooth surface area, while pores are in sub-dermal cavities overlaid by sieve-like areas. Oxeas I, protriaenes, and anatriaenes are longer than in the other *Antarctotetilla* species examined.

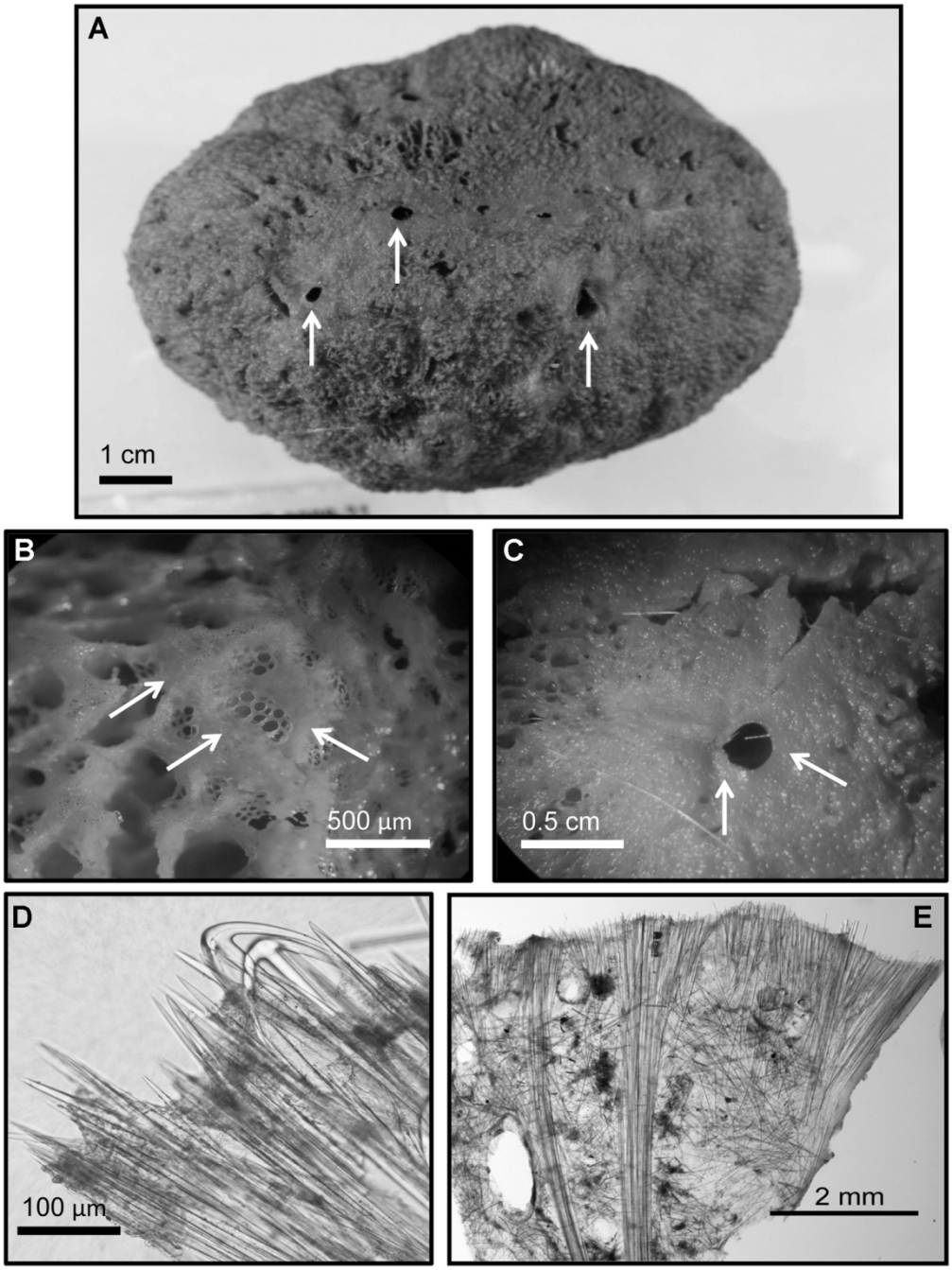

Figure 2.8. *Antarctotetilla sagitta* from Terre D'Adelie (MNHN-IP 2009 31). A. Arrows point to the oscules. B. Surface: arrows point to the pores clustered in sieve-like areas. C. Smooth area surrounding the osculum, arrows point to the osculum. D. Transversal section of the syntype of *Tethya sagitta* Lendenfeld, 1907. E. Transversal section of *A.sagitta* (MNHN-IP 2009 31). Pictures A, B, E are from Carella et al. (2016).

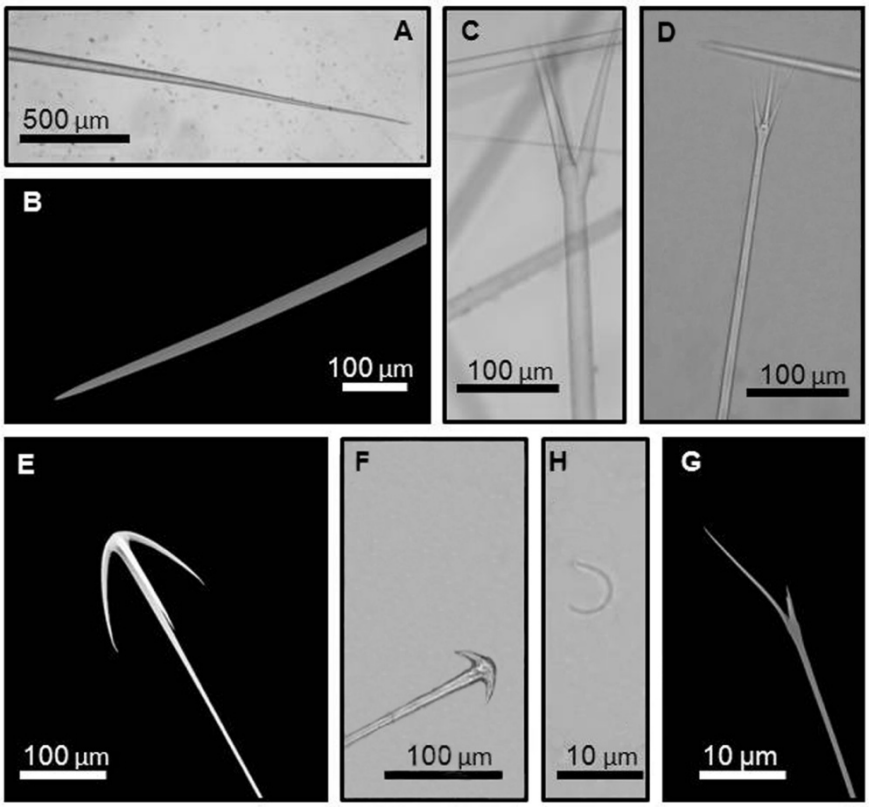

Figure 2.9. Spicules of *Antarctotetilla sagitta* (MNHN-IP 2009 31). A. Terminal part of a large fusiform oxea I. B. Auxiliary secondary oxea II. C. Large Protriaene I. D. Medium size protriaene II. E. Large Anatriaene I. F. Small anatriaene II. G. Trichodal protriaene. H. Sigmaspire.

Antarctotetilla coactifera (Lendenfeld, 1907)

Synonymy: *Tethya coactifera* Lendenfeld, 1907; *Cinachyra coactifera* (Lendenfeld, 1907); *Craniella coactifera* (Lendenfeld, 1907); *Tetilla coactifera* (Lendenfeld, 1907).

Material examined: Syntype of *Tethya coactifera*, ZMB Por 4175 Lendenfeld, 1907 from Kerguelen (Subantarctic).

GenBank accession number: Syntype of *Tethya coactifera*, ZMB Por 4175 (MF168949, S1).

Description (Figure 2.10)

Globular, 5 cm in diameter, sponge (Figure 2.10a). Surface smooth, slightly rugose. Oscules small and very scarce (Figure 2.10b). Pores grouped in slight depressions (Figure 2.10c), widespread on the sponge surface. Color yellowish brown in alcohol. Pseudocortex (Figure 2.10d) made of auxiliary oxeas, loosely arranged, perpendicularly to the sponge surface. Not conspicuous basal spicule tufts in the syntype examined.

Spicules (Figure 2.11; Table 2.1)

Megascleres: oxeas I (Figure 2.11a) large and fusiform: 2850–4310–5400 µm x 25–40–50 µm. Auxiliary small oxeas II (Figure 2.11b): 470–686.6–960 µm x 10–16.25–22.5 µm. Anatriaenes I (Figure 2.11f): 7000–9400–11580 µm x 20–25.4–30 µm in size with long and thin clades: 150–167.7–200 µm; rhabdomes fusiform, thicker at the middle and filiform at the terminal part. Anatriaenes II: 2862.5–3647.8–4700 µm x 5–11–17.5 µm in size (Figure 2.11e) with short clades: 20–47.5–70 µm and fusiform rhabdomes. Anatriaenes III (Figure 2.11g): 3900–6620–9400 µm x 17.5–23.8–30 µm in size with thick clades: 75–110.6–140 µm; rhabdomes fusiform, thicker at middle and filiform at the terminal part. Protriaenes I (Figure 2.11c): 3050– 4539.4–7200 µm x 12.5–14.7–20 µm, with clades: 50–121.3–200 µm long, usually one clade longer than the other two; rhabdomes tapering from the base of the clades to end in a filamentous termination. Protriaenes II (Figure 2.11d): 1450–2245–2960 µm x 7.5–9.2–12.5 µm, clades: 10–61.7–112.5 µm, usually one clade longer than the other two; rhabdomes tapering to a filiform end. Trichodal protriaenes (Figure 2.11h) very small with filamentous rhabdomes: 425–551–675 µm x 2.5–2.6–3.75 µm long

and thin, 10–18.3–50 μm long clades. Microscleres: Sigmaspires (Figure 2.11i): 10–13.7–20 μm in length.

Skeletal arrangement

Bundles of oxeas, anatriaenes, and protriaenes spirally arranged from the central part to the sponge periphery. Auxiliary oxeas arranged in palisade at the sponge periphery and scattered in the choanosome. Trichodal protriaenes concentrated at the peripheral zone. Sigmaspires throughout the sponge.

Distribution and habitat

Kerguelen (Lendenfeld, 1907 and Lévi, 1956).

Remarks

The type of *Tethya coactifera* Lendenfeld, 1907 also owns grouped pores, a pseudocortex made of auxiliary oxeas mainly concentrated at the peripheral zone. Thus, as suggested in Carella et al. (2016), *T. coactifera* belongs in *Antarctotetilla*. This species is very similar to *A. grandis* but does not possess the oscules on the apex of conical elevations, typical of the former species. Moreover, it has a different spicule size range than in the other known *Antarctotetilla* species: larger fusiform and auxiliary oxeas, and smaller clades of all anatriaenes and protriaenes, and also has a third type of anatriaene (anatriaenes III). The anchoring basal mass composed of anatriaenes, reported by Lendenfeld (1907) was not visible in the syntype.

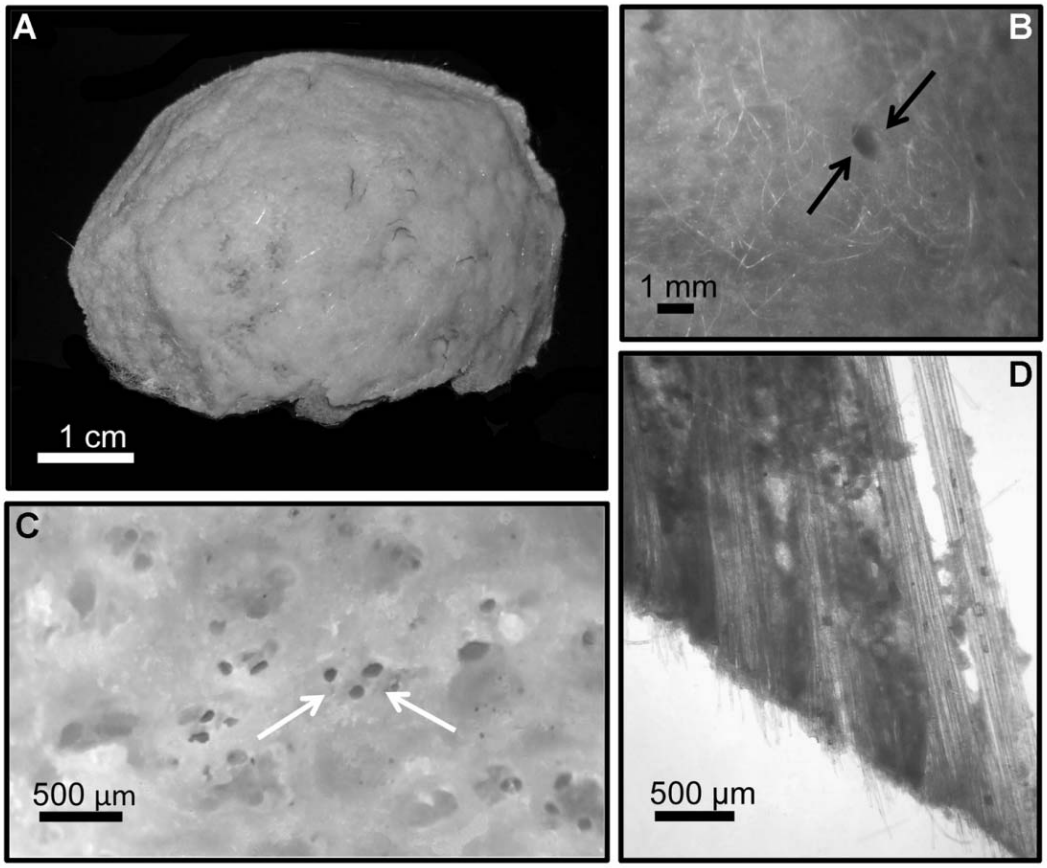

Figure 2.10. *Antarctotetilla coactifera.* A. syntype from Kerguelen (ZMB Por 4175). B. Surface, arrows point to the osculum. C. Pores grouped in small surface depressions (arrows). D. Transversal section.

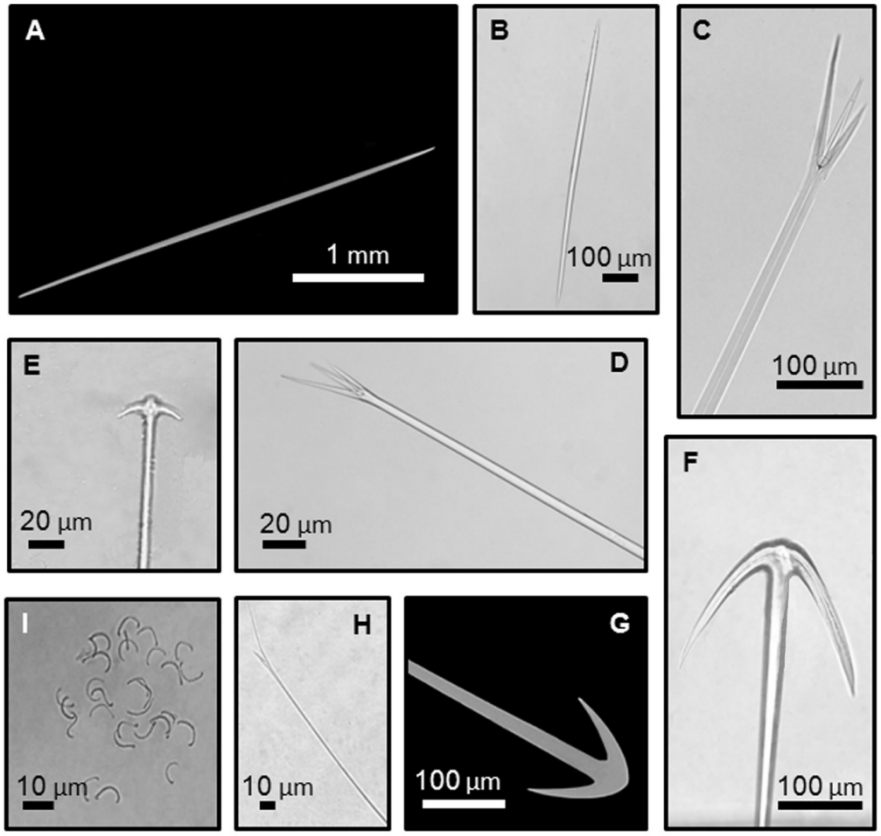

Figure 2.11. Spicules of *Antarctotetilla coactifera* (syntype, ZMB Por 4175). A. A large fusiform oxea I. B. Auxiliary secondary oxea II. C. Large Protriaene I. D. Medium size protriaene II. E. Small anatriaene II. F. Large Anatriaene I. G. Thick anatriaene III. H. Trichodal protriaene. I. Sigmaspires.

Antarctotetilla pilosa nov. sp.

Material examined: 1 individual (holotype, CEAB.POR.BIO.502a ANT 27211), South Orkneys, Subantarctic region, -61° 8.74‘S, -43° 58.15‘W, 407 m depth, 19 February 2011 (Carella et al. 2016).

GenBank accession numbers (Carella et al. 2016): CEAB.POR.BIO.502a ANT 27211(KT124313 see S1, KT124355 and KT124361).

Description (Figure 2.12)

Globular, 8 cm in diameter sponge (Figure 2.12a, b and c).Long hairy hispidation protruding up to 2 cm, throughout the surface, caused by long fusiform oxeas, protriaenes, and sometimes anatriaenes. This hispidation becomes much longer (up to 4 cm) after sponge desiccation due to flesh retraction. Oscules of several sizes spread on the sponge body (Figure 2.12d). Pores grouped in more or less deep surface depressions (Figure 2.12e). Pseudocortex (Figure 2.12f) composed of loose auxiliary oxeas perpendicular to the sponge surface. Megascleres: oxeas, protriaenes, anatriaenes, and trichodal protriaenes; microscleres: sigmaspires. A basal root-tuft system of anatriaenes.

Spicules (Figure 2.13; Table 2.1)

Megascleres: oxeas I (Figure 2.13a) large and fusiform: 4050–7682.5–10950 µm x 35–83.5–120 µm. Auxiliary small oxeas II (Figure 2.13b): 630–1137–1780 µm x 12.5–22.4–35 µm. Anatriaenes I (Figure 2.13f): 7180–12460–16880 µm x 22.5–24.5–27.5 µm in size with long and thin clades: 160–190.5–210 µm long; rhabdomes fusiform, thicker at the middle and filiform at the terminal part. Anatriaenes II: 3220–3806.7–4960 µm x 15–17.5–22.5 µm in size (Figure 2.13e) with short clades: 62.5–85.8–130 µm and fusiform rhabdomes. Anatriaenes III (Figure 2.13g): 4400–10265–14350 x 17.5–24.4–27.5 µm in size with thick, 70–117.6 –140 µm long clades; rhabdomes fusiform, thicker at middle and filiform at the terminal part. Protriaenes I (Figure 2.13d): 4300–6986.2–10880 µm x 20–30–40 µm with clades: 100–181.7–260 µm long, usually one clade longer than the other two; rhabdomes tapering from the base of the clades to end in a filamentous termination. Protriaenes II (Figure 2.13c):

2400–2767.5–3250 µm x 10–11.9–17.5 µm, with clades: 80–142–240 µm in length, usually one clade longer than the other two; rhabdomes tapering to a filiform end. Trichodal protriaenes (Figure 2.13h) very small with filamentous rhabdomes: 405–1063.6–2480 µm x 2–2.6–5 µm long and thin, 10–51.4–175 µm long clades. Microscleres: sigmaspires (Figure 2.13i): 10–11.6–12.5 µm in length.

Skeletal arrangement

Choanosomal skeleton composed by bundles of oxeas protriaenes and anatriaenes spirally arranged from the central part toward the periphery. Auxiliary oxeas either disposed in palisade at the sponge periphery or scattered in the choanosomal zone. Trichodal protriaenes distributed at the peripheral zone; sigmaspires spread through the sponge. Anatrianes III form part of the dense hair-like surface tufts.

Distribution and habitat

The only individual available was collected from the Subantarctic (South Orkneys) at a depth of 407 meters.

Remarks

Morphologically, this species fits within *Antarctotetilla* as it has their pores grouped in surface depressions and a pseudocortex. *Antarctotetilla pilosa* differs from other species of the genus by the presence of a hair-like hispidation through the whole sponge, and a third type of anatriaene with short and thick clades (anatriaene III).

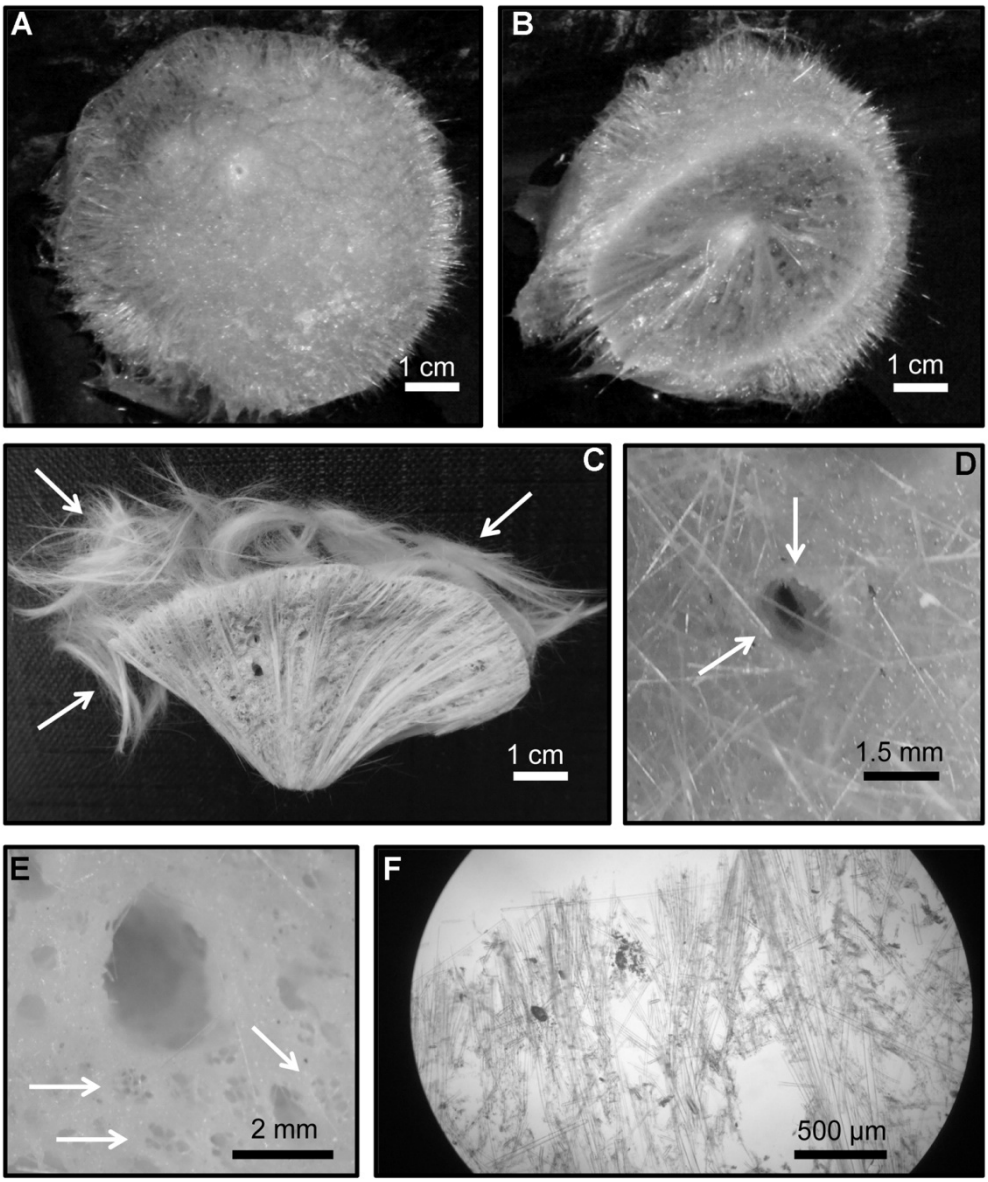

Figure 2.12. *Antarctotetilla pilosa* nov. sp. A, B. Holotype from South Orkneys (CEAB.POR.BIO.502a ANT 27211). C. *A. pilosa* after desiccation, arrows point to the long hairy hispidation caused by long fusiform oxeas, protriaenes and sometimes anatriaenes (from Carella et al. 2016). D. Area surrounding the osculum, arrows point to the osculum. E. Arrows point to the pores grouped in small surface depressions. F. Transversal section.

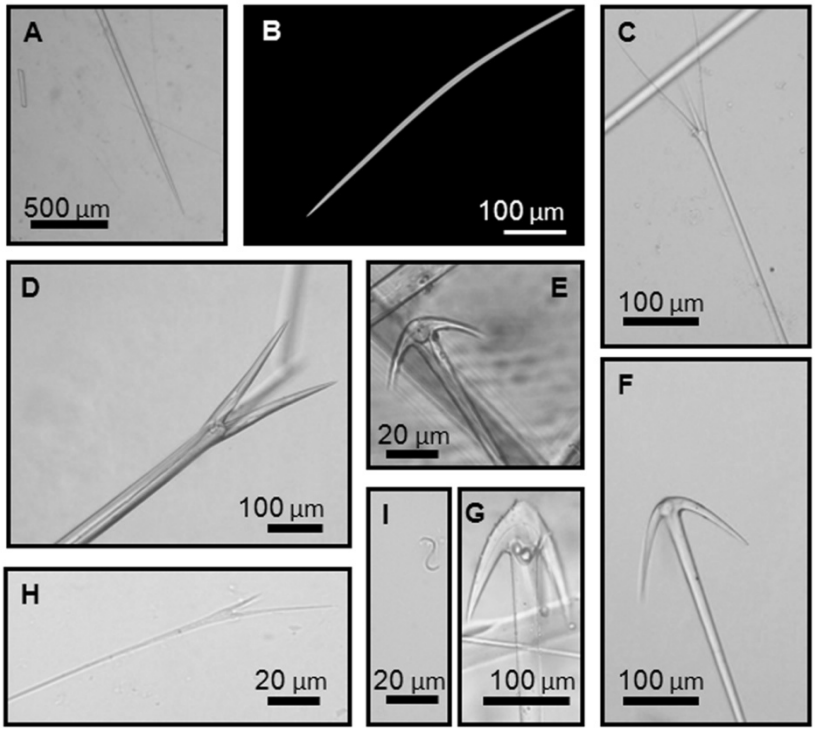

Figure 2.13. Spicules of *Antarctotetilla pilosa* nov. sp. (holotype, CEAB.POR.BIO.502a ANT 27211). A. Terminal part of large fusiform oxea I. B. Auxiliary secondary oxea II. C. Medium size protriaene II. D. Large Protriaene I. E. Small anatriaene II. F. Large anatriaene I. G. Thick anatriaene III. H. Trichodal protriaene. I. Sigmaspire.

Genus *Cinachyra* Sollas, 1886

Definition

Tetillidae with a conspicuous cortex composed either by a dense palisade of auxiliary oxeas or collagen exclusively and, with flask-shaped porocalices (Sollas 1886).

Diagnosis: Globular sponges with many large, deep, flask-shaped porocalices. Oscules scattered; large individuals present a root system of spicules at the base. Marked cortex composed either of only collagen or thick auxiliary criss-cross oxeas at the peripheral zone with variable collagen. The choanosomal skeleton consists of bundles of oxeas, anatriaenes, and protriaenes radiating spirally from the center and piercing the surface. Surface even or hispid. Megascleres are oxeas, protriaenes, anatriaenes and trichodal protriaenes; microscleres sigmaspires. Distribution: Antarctic, Subantarctic, and Australian waters, mainly in deep habitats (Carter 1872, Sollas 1886, Carella et al. 2016).

Remarks

Some species of *Cinachyra* have been repeatedly confounded with *Cinachyrella* Wilson, 1925. The latter genus also has porocalices but lacks any kind of cortical specialization (Rützler 1987).

Cinachyra crassispicula (Lendenfeld, 1907)

Synonymy*: Tethya crassispicula* Lendenfeld, 1907; *Craniella crassispicula* (Lendenfeld, 1907).

Material examined: Holotype of *Tethya crassispicula*, ZMB Por 1248 Lendenfeld, 1907 from Kerguelen (Subantarctic).

GenBank accession number: Holotype of *Tethya crassispicula*, ZMB Por 1248 MF168950. (see S1).

Description (Figure 2.14)

Globular, 8 cm in diameter, sponge (Figure 2.14a); hispid surface mainly on its apical part, produced by the protruding oxeas and protriaenes. Oscules inconspicuous. Porocalices large (up to 3 mm in diameter) and numerous with a

flask-shape pattern (Figure 2.14b), placed on the sponge lateral zones; color reddish brown in alcohol. Cortex (Figure 2.14c-d) formed by a dense layer of cris-cross, thick auxiliary oxeas together with sigmaspires. Basal anchoring tufts made of anatriaenes.

Spicules (Figure 2.15; Table 2.1)

Megascleres: oxeas I (Figure 2.15a) large and fusiform: 5775–6890–7750 µm x 50–64–80 µm. Auxiliary oxeas II small and thick (Figure 2.15b): 300–582.3–950 µm x 15–23.6–40 µm. Anatriaenes I (Figure 2.15c,d,e,f,g): 4500–11158–18800 µm x 12.5–20–27.5 µm in size with clades of different forms, sometimes deformed, more or less open: 60–92.5–125 µm in length; rhabdomes fusiform, thicker at the middle and filiform at the terminal part. Anatriaenes II: 3500–4065–4650 µm x 7.5–9.3–12.5 µm in size (Figure 2.15h) with short clades: 30–38.6–50 µm and fusiform rhabdomes. Protriaenes I (Figure 2.15i), sometimes prodiaenes: 4470–6244.3–12360 µm x 10–15.4–27.5 µm, clades with equal length: 80–110.3–150 µm long; rhabdomes tapering from the base of the clades to end in a filamentous termination. Protriaenes II (Figure 2.15j) sometimes prodiaenes: 1450–2630–3970 µm x 3.75–7.26–10 µm, clades with equal length: 20–58.9–90 µm; rhabdomes tapering from the base of the clades to end in a filamentous termination. Trichodal protriaenes (Figure 2.15l) very small with filamentous rhabdomes: 287.5–629–1400 µm x 1.25–2.7–5 µm long and thin, 10–21.2–50 µm long clades. Microscleres: sigmaspires (Figure 2.15k): 12.5–15–20 µm in length.

Skeletal arrangement

Bundles of oxeas, anatriaenes, and protriaenes radiating spirally form the center to the periphery, piercing the ectosome. Thick auxiliary oxeas mostly arranged at the sponge periphery, but also scattered in the choanosome. Trichodal protriaenes mostly present at the peripheral zone. Sigmaspires mainly accumulated at the peripheral layer of the cortex.

Distribution and habitat

Kerguelen (Lendenfeld, 1907).

Remarks

Tethya crassispicula Lendenfeld, 1907 was revised to confirm whether it belongs to any of the Antarctic genera. As it has been proved to be a *Cinachyra*, its description is included in this study. Re-examination of the type of *Tethya crassispicula* Lendenfeld, 1907 proved that this species has porocalices and a clear spicule-made cortex. These characters are typical of *Cinachyra* and thus the species is here renamed *Cinachyra crassispicula*. The COI minibarcode sequences (Meusnier et al. 2008 and Cárdenas & Moore 2017; data not shown) also suggest assigning this and *A. coactifera* to the Antarctic clade of Tetillidae.

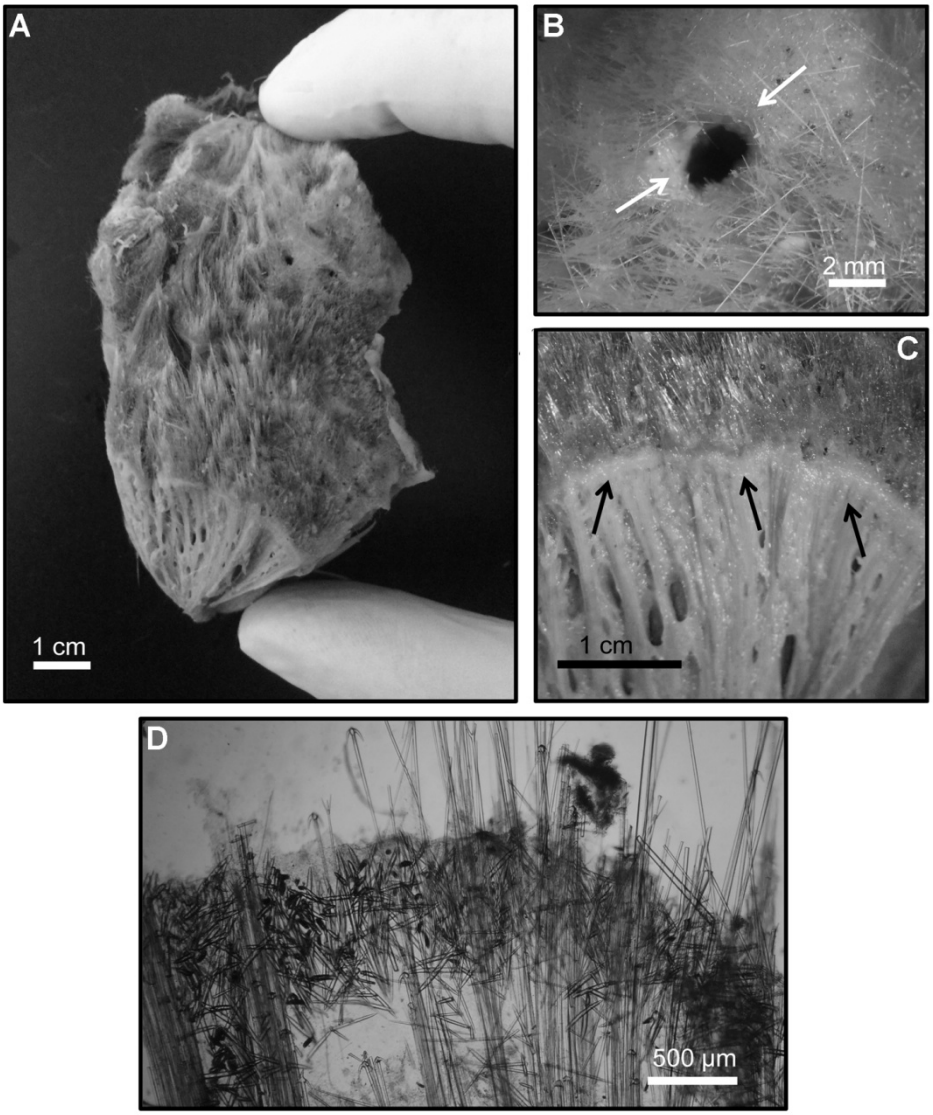

Figure 2.14. *Cinachyra crassispicula.* A. Holotype from Kerguelen (ZMB Por 1248). B. Surface around the porocalyx. C. Transversal section, arrows point to a visible cortex. D. Transversal section made by Lendenfeld, 1907.

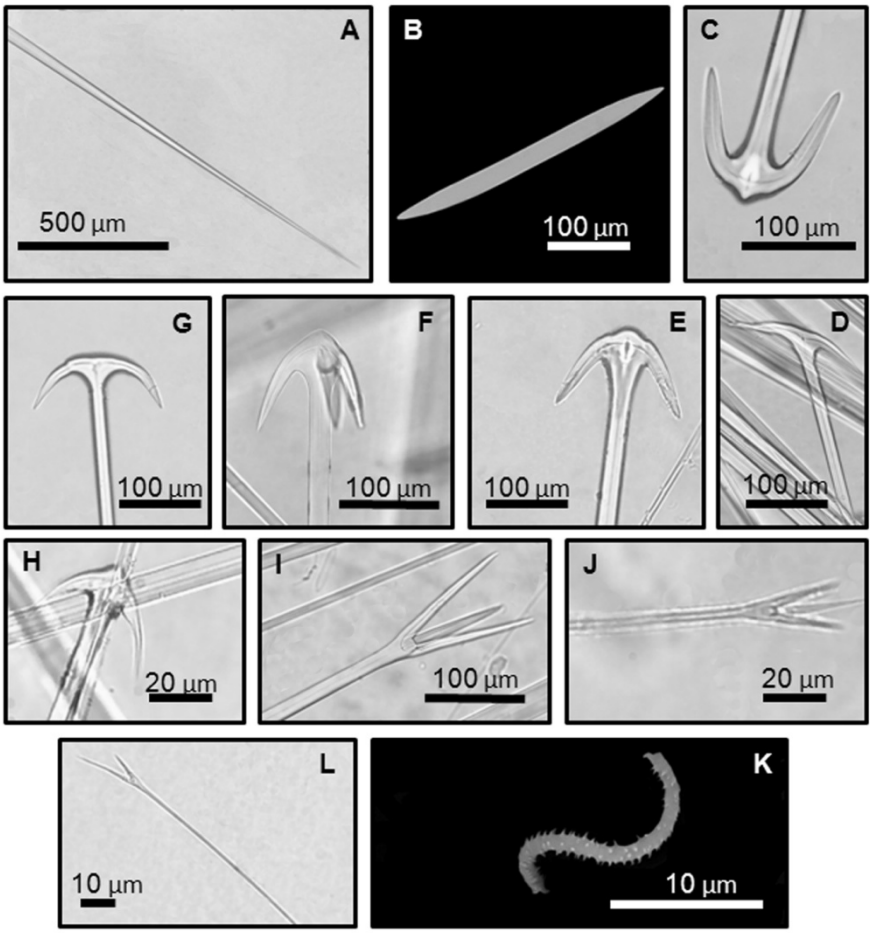

Figure 2.15. Spicules of *Cinachyra crassispicula* (holotype, ZMB Por 1248a). A. Terminal part of a large fusiform oxea I. B. Auxiliary secondary oxea II. C, D, E, F, G: Large anatriaenes I. H. Small anatriaene II. I. Large Protriaene I. J. Medium size protriaene II. K. Sigmaspire. L. Trichodal protriaene.

Key to genera

(1) Specialized pore-bearing pits ('porocalices') present 2

✓No porocalices….....5

(2) Spicules include medium-sized, heavily spined oxeas ('megacanthoxeas').....***Acanthotetilla***

✓No megacanthoxeas 3

(3) Cortex clearly visible in cross section (made of short oxeas either reinforced or not with collagen or of collagen exclusively. Flask-shaped porocalices .....***Cinachyra***

✓Without conspicuous cortical layer 4

(4) Hemispherical porocalices, (short-shafted triaenes with equal-sized rays –calthrops- may be present in some species)….....***Cinachyrella*** (including ***Paratetilla***)

✓Small shallow porocalyx-like cavities, difficult to differentiate to the naked eye (dense external sandy layer) ……..….....***Levantiniella***

✓Two oppositely placed, surface deep cavities, (previously called porocalices) with contrasting functions: one inhalant (true porocalyx) and the other exhalant (osculum) .....***Fangophillina***

(5) Stalked sponges with cortex, and different spicule arrangement in the stalk and main body: normal triaenes in the main body; triaenes with clads at both ends (amphitriaenes) in the stalk .....***Amphitethya***

✓No amphitriaenes, without a proper stiff stalk, sometimes with a thin root-like projection 6

(6) A marked cortical region with two differentiated layers, including spicules and a collagenous reinforcement .....***Craniella***

✓Without a discernible cortex……. .....***Tetilla***

✓Pores grouped in slight surface depressions; poorly differentiated cortical layer composed of a loose arrangement of auxiliary oxeas .....***Antarctotetilla***

Discussion

The morphological re-examination of the Antarctic Tetillidae, which formed a monophyletic clade in a recent phylogenetic study (Carella et al. 2016) has proved that some species previously placed either within *Tetilla* or *Craniella* Schmidt, 1870, (Sollas 1886 and 1888; Lendenfeld 1907; Kirkpatrick 1908; Koltun 1964; Boury-Esnault & Van Beveren 1982; Szitenberg et al. 2013) indeed belong to the new genus *Antarctotetilla* Carella et al., 2016. This new genus has pores grouped in slight surface depressions and a kind of light cortex, formed by auxiliary oxeas with an uneven distribution, as synapomorphies. Until now, three *Antarctotetilla* species were considered: *A*. *leptoderma*, *A*. *grandis*, previously classified within *Tetilla* (Sollas 1886 and 1888) and *A. sagitta* (Carella et al. 2016), before considered a *Craniella* (Lendenfeld 1907; Kirkpatrick 1908).

On the other hand, *A*. *leptoderma* and *A. grandis* which were considered synomymies (Lendenfeld 1907) proved to be different species. Several characters allow differentiating them: a partially conulose surface with protruding spicule tufts, and a sole large oscule on top, in *A. leptoderma*, versus a smooth surface with several small oscules on the top of short conules in *A. grandis* (Figure 2.16a, c, e, f). Moreover, *A. sagitta*, which was misclassified in *Craniella*, presents a corrugate and finely pilose surface and many oscules on top. This species does not have the typical double-layered cortex of *Craniella*, but, it seems to lack the clear collagenous layer of the latter. Conversely, as in the other *Antarctotetilla* species, *A. sagitta* has irregularly distributed, auxiliary oxeas at the peripheral zone, ("pseudocortex") mostly concentrated at the end portion of the radial spicule bundles (Figure 2.8e).This has been confirmed in the transversal sections of the holotypes of *T. leptoderma*, *T. grandis* and the syntype of *T. sagitta* (Figure 2.8d and Figure 2.16b-d).

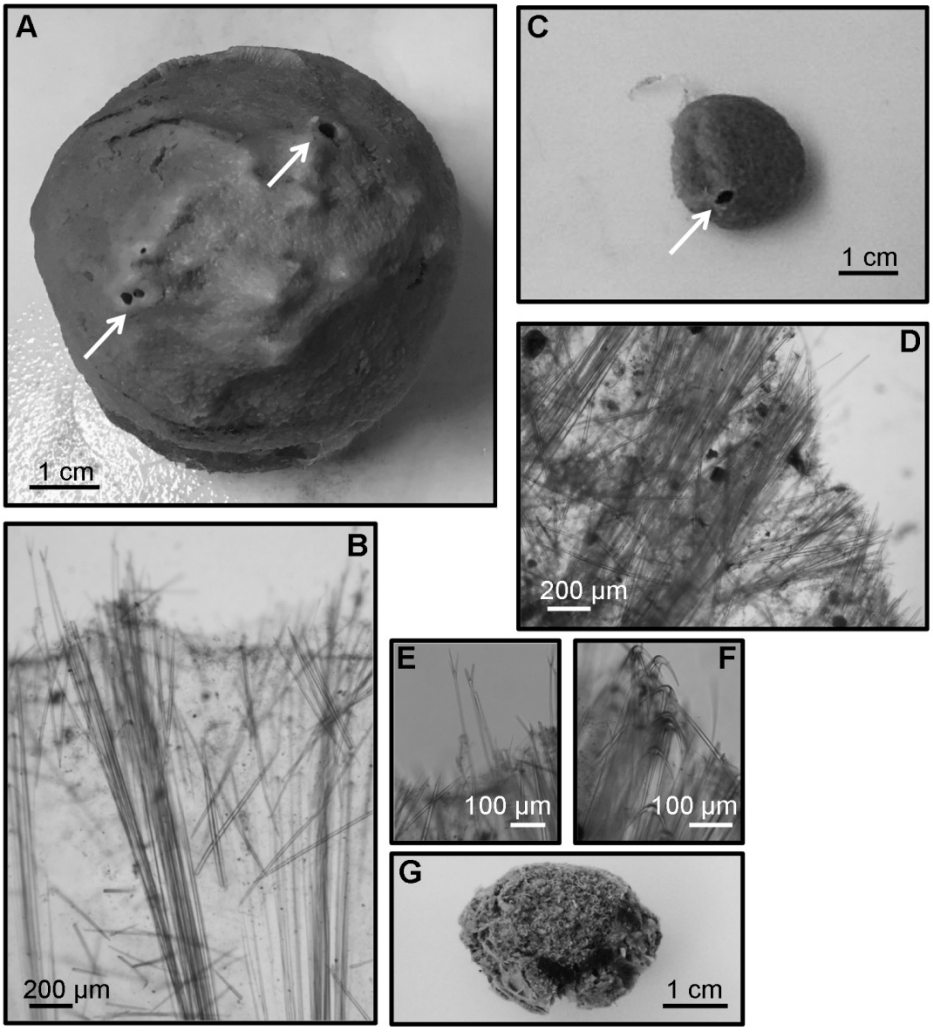

Figure 2.16. Species types revised. A. holotype of *Tetilla grandis* NHM98.1.1.5 Sollas, 1886, arrows point to oscules. B.Transversal section of the syntype of *T. grandis* NHM 98.1.1.8. C. holotype of *Tetilla leptoderma* NHM89.1.1.3 Sollas, 1886, arrow points to the osculum on top. D. Transversal section of the holotype of *T. leptoderma*. E. Protriaenes of the syntype of *T. grandis* NHM 98.1.1.8. piercing the ectosomal region. F. Anatriaenes of the holotype of *T. leptoderma* NHM 89.1.1.3.concentrated at the ectosomal region. G. paratype of *Cinachyrella levantinensis* MNHN-DJV 97 Vacelet et al., 2007.

Spicule types and sizes hardly differentiate species in Antarctic Tetillidae. *A. leptoderma* and *A. grandis* have the same types of spicules, also similar in their size range. However, *A. sagitta* has the fusiform oxeas and the rhabdomes of trichodal protriaenes, protriaenes I, and anatriaenes I significantly longer than *A. leptoderma* and *A. grandis*, We also found an additional type of protriaenes of an intermediate size (protriaenes II, Table 2.1) in all the species of *Antarctotetilla* examined, in contrast to the two clear-cut types of protriaenes

described in Antarctic Tetillidae species by past authors (Sollas 1886; Lendenfeld, 1907; Kirkpatrick 1908; Boury-Esnault & Van Beveren 1982).

The new species of Antarctic Tetillidae with a hair-like hispidation here described (*A. pilosa,* formerly Tetillidae sp.3) appeared either within *Cinachyra* (mitochondrial COI marker, S1) or within *Antarctotetilla* (nuclear 18S, 28S markers) in previous phylogeny reconstructions (Carella et al. 2016). However, we realized that the COI sequence of this species used in the previous study was wrong. Consequently, a new phylogeny reconstruction using the right COI sequence was obtained, and the species clustered within the genus *Antarctotetilla*, as it occurred with the nuclear markers. Thus, the new species is here renamed *Antarctotetilla pilosa* nov. sp. Morphologically, the species also fits within *Antarctotetilla* but differs from the other species in the very long, dense spicule cover throughout its surface, and because of a particular type of anatriaene with short thick clades.

The two additional Tetillidae species revised (*Tethya coactifera* Lendenfeld, 1907 and *Tethya crassispicula* Lendenfeld, 1907), which were not included in the previous phylogenetic study (Carella et al. 2016) are here moved to different genera. *T.coactifera* Lendenfeld, 1907 has grouped pores, a pseudocortex and auxiliary oxeas mainly concentrated at the peripheral zone. Thus, as suggested in Carella et al. (2016), it belongs to *Antarctotetilla*. This species differs from *A. grandis* in the absence of oscules on the top of conical elevations. On the other hand, *T. crassispicula* Lendenfeld, 1907 has porocalices and a spicule-made cortex, which confirms that this species is a *Cinachyra* (*C. crassispicula*). The minibarcode was not informative at the genus or species levels due to its short length and the low nucleotide variability of COI in the Antarctic Tellidae species (Carella et al. 2016).

Despite the spicule similarity between the new Antarctic genus *Antarctotetilla* and the temperate/tropical genus *Tetilla*, other morphological characters allow differentiating them*; Tetilla* is cortex-free, has spread micropores, and sometimes lacks microspined sigmaspires (e.g. *Tetilla muricyi* Fernandez et al. 2011, *Tetilla radiata* Selenka, 1879, and the type species of the genus, *Tetilla euplocamos* Schmidt, 1868, lack sigmaspires). Conversely,

sigmaspires were very abundant in all *Antarctotetilla* species examined. These microscleres are considered to have a diagnostic value for Spirophorina (Rützler 1987), but sigmaspires might have been lost several times along the evolutionary history of the family Tetillidae as not all their genera show them.

Levantiniella levantinensis (Vacelet et al., 2007) formed a monophyletic group separate from *Cinachyrella* in the previous phylogenetic studies (Szitenberg et al. 2013; Carella et al. 2016) and thus it was considered to be a new genus, named *Levantinella* Szitenberg et al., 2013 and renamed *Levantiniella* Carella et al., 2016. *Levantiniella levantinensis* differs from *Cinachyrella* by the small rounded depressions, sometimes inconspicuous because of a thick sand cover, (Figure 2.16g), which are difficult to assign to porocalices with certainty. The thick sand coverage may be confounded with a cortex but individual sections prove that no cortex is discernible. The only *Cinachyrella* species in the Mediterranean, *Cinachyrella tarentina* (Pulitzer-Finali, 1983) differs from the new genus *Levantiniella* in the presence of true porocalices, plagiotriaenes, and the absence of spined microxeas.

To summarize, this study describes in detail the new genera *Levantiniella* and *Antarctotetilla* and suggests relocating two additional species in *Antarctotetilla* (*A. pilosa* and *A*. *coactifera*) and one species in *Cinachyra* (*C*. *crassispicula*). Many other species remain to be described and some others from old campaigns need taxonomical updates. Genera, such as *Craniella*, *Cinachyrella*, *Fangophilina*, *Amphitethya*, and the misidentified Antarctic complex "*Craniella sagitta*" deserve further morphological and molecular revisions (Szitenberg et al. 2010 and 2013, Carella et al. 2016).

With the knowledge at hand, the family Tetillidae comprises currently 10 genera and 160 species. Based on the known species, some Tetillidae genera seem to have a restricted geographical distribution (Carella et al. 2016). For instance, *Antarctotetilla* seems to be only distributed through the Antarctic and Subantarctic waters, while no true *Tetilla* species have been recorded from the Antarctic. On the other hand, *Levantiniella* appears up to now to be confined to the eastern Mediterranean.

This study confirms that molecular phylogenies should be combined with detailed morphological observation of the specimens sequenced, what has

been named integrative taxonomy, for a precise interpretation of the results (Dohrmann et al. 2008; Szitenberg et al. 2013; Carella et al. 2016; Dohrmann et al. 2017).

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CHAPTER 3

Asexual reproduction and heterozygote selection in Antarctic demosponges (*Stylocordyla chupachus*, Suberitida) approached by microsatellite analyses

Abstract

Antarctic bottoms harbor stable, benthic communities, subjected to constantly low temperatures. Environmental stability may promote the asexual (clonal) reproduction of sponges to maintain adapted genotypes to those particular conditions. Stylocordyla chupachups (formerly S. borealis) forms dense, patchy populations across the Antarctic continental shelf. Individuals are mostly similar in size without distinct cohorts, which indicates fast growth and suggest a possible relevance of clonal reproduction. To analyze the weight of clonal reproduction in its populations, a genetic study was performed on three close populations using eight polymorphic microsatellites loci that were designed from massive sequencing. The number of alleles ranged from 2 to 6 in the three populations. The Fst, Dst estimators, AMOVA analyses and the presence of private alleles indicated low but significant population structure in the three populations. Seven identical multilocus genotypes (MLG) were found. The program MLGsim indicated an asexual origin for five of them (ca. 25% of the individuals). An excess of heterozygotes was found in all the three populations, which suggests a positive selection mechanism for heterozygotes, as other alternative explanations are unlike. The relatively high rates of asexual reproduction may be the result of adaptation to the environment stability, while heterozygote selection would help maintaining some genetic diversity in the populations despite the high level of clonal reproduction. S. chupachups has been reported to be one of the first sponge species recolonizing bare areas resulting from iceberg scouring, which indicates a high species fitness and adaptation to Antarctic bottoms. Two out of the three study populations showed bottleneck, which may indicate a founder effect and confirms the pioneer nature of this species.

Introduction

Sponges are one of the most important taxa in terms of diversity and abundance in marine benthic ecosystems (Gili & Coma 1998). They are still more abundant in the Antarctic region, where sponges dominate many benthic communities between 100 and 200 m of depth, and account for ca. 75% of the benthic biomass (Belyaev and Ushakov 1975).

Antarctic benthic communities have remained isolated from the rest of continents since Antarctica was separated from Gondwana ca. 40 millions of years ago, due to the formation of the circumpolar current (Dayton 1990; Thompson 1991). Moreover, the Antarctic continental shelf presents extreme constantly low temperatures. These features have contributed to the biogeographic isolation of many Antarctic invertebrates, promoting species and genera endemism of sponges in the area (Sarà 1992; McClintock 2005; Ríos 2006). However, morphological convergence between species as a result of adaptation to similar environmental conditions, such as low temperature and stability has caused misidentification of foreign species in the Antarctic. Stylocordyla borealis from the Norwegian coasts was recorded from several Antarctic localities, but later identified as a new Antarctic species named Stylocordyla chupachups (Uriz et. al 2011). Some constant, at first sight cryptic, differences allowed differentiating both species. S. borealis is smooth and has a flat apical zone surrounded by a spicule fringe while S. chupachups is hispid and almost spherical without any spicule fringe (Uriz et al. 2011).

Patchy distributions are common for Antarctic sponges (Barthel & Gutt 1992; Gutt and Koltun 1995; Gatti 2002). Some species form dense populations in some areas while they are absent from many others despite apparently similar environmental conditions (e.g. Rossella racovitzae, Cinachyra barbata, Antarctotetilla leptoderma, and Stylocordyla chupachups).

The later species behaves as a pioneer species in the colonization of disturbed areas by iceberg scouring (Gutt 2000). The recorded populations of S. chupachups are mostly formed by individuals of the same size (i.e. no cohorts were clearly differentiated), what permitted us hypothesizing a high rate of asexual reproduction in this species. To assess the genetic traits population and

the extent of clonal reproduction in this species we performed a population genetics' study using microsatellite markers.

Microsatellites are among the most variable types of DNA sequences in the genome (Weber 1990). As each microsatellite contains many mutation sites, they have been considered suitable markers for studies of population genetics (Csilléry 2009), in particular where only small samples are available (Haasl & Payseur 2011) as often occurs in sponges (Duran et al. 2004a, Blanquer et al. 2009, Blanquer & Uriz 2010, Dailianis et al. 2011, Guardiola et al. 2012, Pérez-Portela et al. 2014).

In the present study we developed a set of microsatellite markers from sequencing a part of the Stylocordyla chupachups genome (Guardiola et al. 2016), analyzed their suitability for a population genetics' study of and used these markers for assessing the extent of clonal reproduction in this sponge.

Materials and methods

SAMPLING

The samples of Stylocordyla chupachups were collected during the Polarstern ANT-XXVII/3 expedition in the Antarctic region (-70°50'33.0"S, -10°35'21.6"W, Figure 3.1). Three populations: Ant1 (18 individuals), Ant2 (20 individuals) and Ant3 (20 individuals) summarizing 58 individuals, were collected from an area of ca. 1,8 Km of diameter, between 238 and 268 meters of depth, during three separate trawling operations (Agasiz trawl). Fragments of the samples were fixed in ethanol on board immediately after collection, placed in hermetic plastic bowls, transported in a freezer to the CEAB (Centre d'Estudis Avançats de Blanes, Spain) at -20 ºC and stored there at −20°C until DNA extraction.

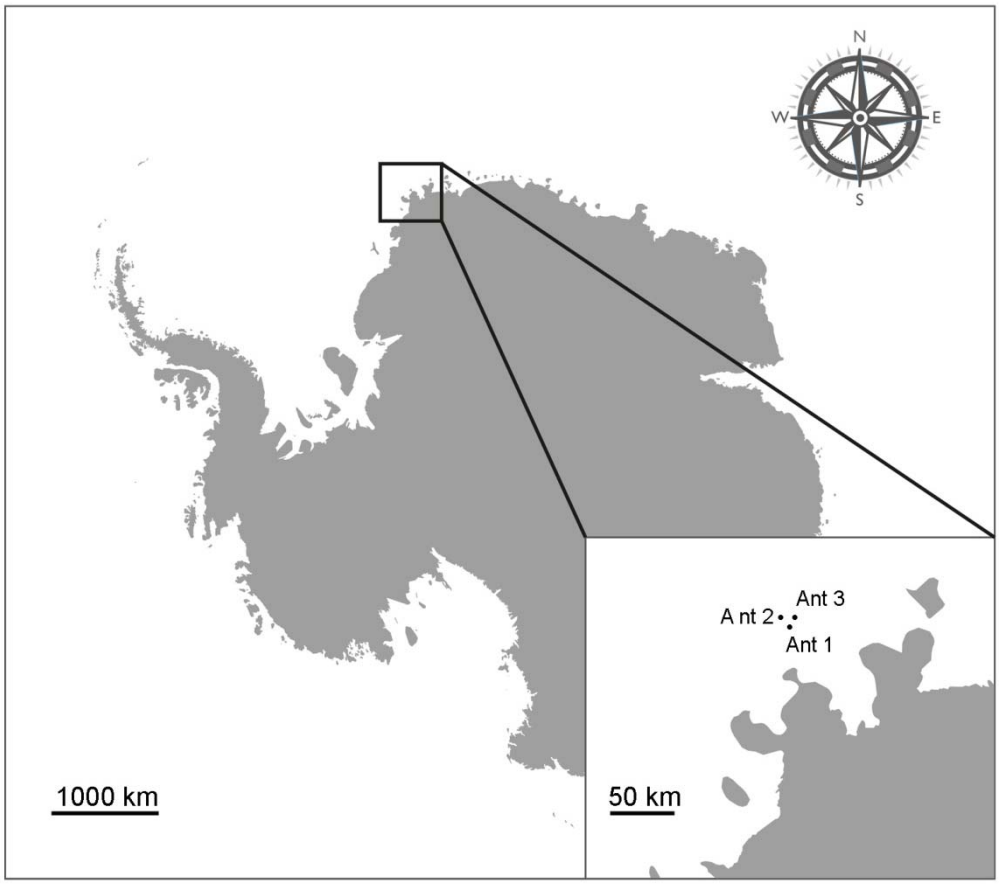

Figure 3.1. Sampling locations near Newmayer, Antarctica. The inset image indicates the sampling places of the three study populations: Ant1, Ant2, and Ant3.

DNA EXTRACTION, PYROSEQUENCING AND MICROSATELLITE SELECTION

The DNA of Stylocordyla chupachups was extracted for pyrosequencing with QIAmp DNA stool kit (Qiagen). DNA concentration and quality were assessed using a Qubit fluorometer (Invitrogen) and a 2100 Bioanalyzer (Agilent Technologies), respectively. Sequencing (1/2 run) was performed in a 454 GS-FLX sequencer (Roche) at the Scientific and Technological Center of the University of Barcelona (CCiTUB). Sequences were analyzed with the open-access program QDD v.2.1 (Meglécz et al. 2010), which is a useful tool for the discovery and selection of microsatellites, and for primer design. The QDD package integrates the software programs BLAST, ClustalW (Larkin et al. 2007) and Primer 3-1.1.4 (Rozen & Skaletsky 2000), and works in 3 steps: sequence

cleaning and microsatellite recognition, detection of sequence similarity, and primer design. Among, the best 100 microsatellites, which were selected taking into account product size (120 to 285 bp), suitable flanking region, self-complementarity, guanine–cytosine (GC) content (50 to 60%), and GC clamp regions, ten were finally selected: Stylo_A, Stylo_D, Stylo_H, Stylo_K, Stylo_G, Stylo_M, Stylo_N, Stylo_R, Stylo_S and Stylo_T.

AMPLIFICATION AND MICROSATELLITE GENOTYPING

From the ten selected loci, Stylo_R and Stylo_T failed to amplify. Thus, all the analyses were performed with the remaining 8 microsatellites (Stylo_A, Stylo_D, Stylo_H, Stylo_K, Stylo_G, Stylo_M, Stylo_N, and Stylo_S) (Table 3.1). The forward primers of each locus were labelled with fluorescent dyes (NED, PET, VIC, 6-FAM; Applied Biosystems) for screening and were then amplified in a final volume of 25 µl (10–30 ng of DNA), which contained 17,15 µL H20, 2,5 µL 10X buffer (BIO LINE), 2 mmol L^1 µL MgCl (BIO LINE), 1,2 µL DMSO (dimethyl sulfoxide), 1 µL BSA, 0.25 mmol L^1 of dNTP mix (Sigma-Aldrich), 0.25 µmol L^1 of each primer, 1 U Taq (BIO LINE) 1 µl of DNA template.

Amplification was performed in Bio Applied and Bio-Rad PCRs with the following parameters: 1 min denaturation at 94°C, followed by 40 cycles of 30 s at 94°C, 40 s at a locus-specific annealing temperature (temperature varies from 52°C to 63 °C), 50 s at 72°C, followed by an extension cycle of 3 min at 72°C. The resulting PCR products were then visualized on 1.3% agarose gel stained with GelRed (Biotium) and then genotyped in an ABI Prism 3700 automated sequencer (Applied Biosystems) at the CCiTUB. The length of the PCR products was estimated relative to the internal size standard GeneScan 500LIZ and determined using GeneMapper and Peak Scanner software. The raw data generated were reviewed using AutoBin v.0.9 (Excel macro written in Microsoft Visual Basic, F. Salin unpubl.), in order to automatically detect relevant gaps in allele size. Moreover, 3 independent readers checked the AutoBin results to ensure a lack of scoring errors.

Table 3.1. Genetic information on the eight microsatellite loci for each population of *Stylocordyla chupachups*. Na= number of alleles, He= expected heterozygosity, Ho= observed heterozygosity, *F*is= inbreeding coefficient, Pa= number of private alleles per population. Significant values: *p < 0.05; **p < 0.01; ***p < 0.001.

Locus	**Supopulations**			
Stylo_A	**Ant1**	**Ant2**	**Ant3**	**Mean Na Locus**
Na	3	1	2	2
He	0.476	0	0.188	
Ho	0.647	0	0.211	
***F*is**	-0.36	0	-0.118	
Stylo_D				
Na	3	3	4	3,3
He	0.678	0.565	0.594	
Ho	0.823	1	0.736	
***F*is**	-0.214***	-0.769***	-0.24	
Stylo_H				
Na	6	3	5	4,6
He	0.668	0.565	0.573	
Ho	1	1	1	
***F*is**	-0.497***	-0.769***	-0.744***	
Stylo_K				
Na	4	4	4	4
He	0.756	0.721	0.552	
Ho	0.471	0.615	0.368	
***F*is**	0.377	0.147**	0.333	
Stylo_G				
Na	3	2	3	2,6
He	0.469	0.473	0.386	
Ho	0.353	0.307	0.368	
***F*is**	0.247	0.35	0.046	
Stylo_M				
Na	2	2	2	2
He	0.553	0.5	0.498	
Ho	0.941	1	0.947	
***F*is**	-0.7***	-1***	-0.9***	
Stylo_N				
Na	2	3	6	3,6
He	0.498	0.535	0.655	
Ho	0.941	1	0.789	
***F*is**	-0.888***	-0.867***	-0.205	
Stylo_S				
Na	2	2	2	2
He	0.484	0.311	0.361	
Ho	0.117	0.231	0.263	
***F*is**	0.757**	0.257	0.272	
Pa	2	1	4	
Mean Na\ population	3,125	2,5	3,5	
Mean He	0.572	0.458	0.476	
Mean Ho	0.662	0.644	0.585	
Mean Fis	-0.316	-0.557	-0.356	

DATA ANALYSIS

Linkage disequilibrium, departure from Hardy-Weinberg equilibrium, heterozygote deficit or excess, allele frequencies, number of alleles per loci, the expected (He) and observed (Ho) heterozygosity, genotypic frequency, number of private alleles, and the inbreeding coefficient (FIS) (Weir & Cockerham 1984) for each locus individually, as well as for all loci combined, were calculated with GENEPOP, web version 4.0 (Raymond & Rousset 1995, Rousset 2008). The inbreeding coefficient was also calculated with F-STAT version 2.9.3 (Goudet 1995, 2001) to confirm the results obtained by GENEPOP. The presence of null alleles was assessed with MicroChecker v.2.2.3 (Van Oosterhout et al. 2004). To evaluate the extent of clonal reproduction, we recorded the presence of identical multi-locus genotypes (MLGs) in our populations. To assess whether individuals sharing MLGs could have resulted from sexual or asexual reproduction we used the program MLGsim (10000 simulations; Stenberg et al. 2003).

The P-values of all analyses involving multiple comparisons were corrected by the False Discovery Corrections (FDR) (Benjamini and Yekutieli 2001).

POPULATION DATA ANALYSES

The genetic differentiation of the three populations was assessed by two estimators: the differentiation index D, which is independent of within-population heterozygosity (Jost 2008) and the FST statistic, which measures allele fixation (Weir & Cockerham 1984). The Pair-wise FST estimator, which ranges from 0 to 1 (0 = no differentiation; 1 = absolute differentiation) was assessed with ARLEQUIN (Excoffier et al. 2005), while D values were calculated with DEMEtics (Gerlach et al. 2010). The molecular variance among populations, within populations, and within individuals was analyzed by AMOVA, using ARLEQUIN (Excoffier et al. 2005). Whether populations deviate or not from mutation/drift equilibrium, was analyzed Bottleneck vs 1.2.02 (Cornuet & Luikart, 1996; Piry et al. 1999). The Wilcoxon test was chosen because it has

been reported to be the most powerful and robust for populations with few polymorphic loci (Piry et al. 1999).

Results

Among the microsatellites selected, two loci (Stylo_D and Stylo_H) were perfect and six loci (Stylo_A, Stylo_G, Stylo_K, Stylo_S, Stylo_M and Stylo_N) were compound or imperfect. Null alleles were found for locus Stylo_S, exclusively. Analyses were repeated removing locus Stylo_S and analogous results were obtained.

All loci showed low polymorphism, with a mean number of alleles per locus ranging from 2 to 6. No loci showed linkage disequilibrium after False Discovery Rate correction (FDR) for multiple comparisons.

The observed heterozygosity (Ho) was higher than that expected one in five out of the eight loci analyzed in the three populations. The exact test for Hardy-Weinberg equilibrium indicated significant deviations from equilibrium with mean Fis values negative in the three populations, when all loci were considered together (Table 3.1). The heterozygote loci represented 71.43% of all loci in the clonal genotypes and 66.97% in the sexually produced genotypes. Two private alleles were found in population ANT1, one in ANT2 and four in ANT3 (Table 3.1). The mean frequency of private alleles for the 8 loci, in the three populations was 0.08.

Seven identical multilocus genotypes, accounting for 18 individuals, were found in the three populations analyzed. The MLGsim test indicated that five (MLG1 to MLG5, Table 3.2) of them were produced by asexual reproduction, as their P-values, which inform on the chance to have been produced sexually, were significant ($p < 0.001$ in two cases, $p < 0.05$ in other two cases and < 0.01 in one case) and then the null hypothesis on their sexual origin has to be rejected. However, two identical multilocus genotypes (MLG6 and MLG7) could be the result of sexual reproduction as no significant values were retrieved by the MLGsim analyses.

Table 3.2. Identical multi-locus genotypes (MLGs) identified in *Stylocordyla chupachups* populations.

	Genotypes								Population/	MLGsim
	Stylo_A	**Stylo_D**	**Stylo_H**	**Stylo_K**	**Stylo_G**	**Stylo_M**	**Stylo_N**	**Stylo_S**	**individuals**	
MLG1	122/127	239/264	160/166	278/302	149/164	117/120	260/263	255/255	ANT 1 IND. 13	***
									ANT 1 IND. 18	***
MLG2	127/127	239/264	160/166	278/302	164/164	117/120	260/263	255/255	ANT 1 IND. 16	*
									ANT 2 IND. 11	*
MLG3	127/127	239/264	160/166	285/302	157/157	117/120	260/263	255/255	ANT 2 IND. 10	**
									ANT 2 IND. 18	**
MLG4	127/127	239/264	160/166	285/302	157/164	117/120	260/263	255/255	ANT 2 IND. 7	***
									ANT 2 IND. 8	***
									ANT 2 IND. 9	***
									ANT 2 IND. 12	***
									ANT 2 IND. 13	***
									ANT 2 IND. 14	***
MLG5	127/127	244/264	160/166	285/302	164/164	117/120	260/263	255/255	ANT 2 IND. 2	*
									ANT 2 IND. 3	*
MLG6	127/127	239/264	160/166	285/302	164/164	117/120	260/263	255/255	ANT 1 IND. 12	ns
									ANT 2 IND. 5	ns
MLG7	127/127	244/264	160/166	000/000	157/164	117/120	260/263	255/255	ANT 3 IND. 8	ns
									ANT 3 IND. 13	ns

Seven MLGs were found in the three populations. Significant p-values (i.e. <0.05) in the MLGsim analysis allowed rejecting the null hypothesis of a sexual origin for all the MLGs except MLG6 and MLG7 (significant values: * <0.05, **<0.01, ***<0.001)

From these groups of identical MLG, four (MLG1, MLG3, MLG4 and MLG5) were found in the same population, while MLG2 was found in two separate populations (Table 3.2).

Genetic differentiation was low among the three target populations as indicated by the estimators FST and D. These estimators were low but significant between populations Ant1 and Ant2 (FST 0.073, p< 0.001 and D 0.032, p< 0.05), between Ant1 and Ant3 (FST 0.029 p<0.05 and D 0.018 n.s.) and between Ant2 and Ant3 (FST 0.031 p<0.01 and D 0.026 p<0.05). The

hierarchical AMOVA confirmed the low but significant genetic differentiation among populations (FST= 0.074, p<0.001).

Bottleneck analyses indicated that population ANT2 deviated significantly (Wilcoxon test, p< 0.05) from mutation/drift equilibrium under the three mutation models (IAM, TPM, SMM) and population ANT1 did it just under IAM and TPM models. However ANT3 did not deviate from the mutation/drift equilibrium (Wilcoxon test, p-value> 0,05).

Discussion

The microsatellites assayed were selected among the best 100 loci from the sponge genome, taking into account the most suitable characteristics advised by the software developers (Primer 3-1.1.4, Rozen and Skaletsky, 2000). However, they showed a very low polymorphism in the study populations, compared with microsatellites genotyped in sponges from other latitudes (e.g. Dailianis et al. 2011; González-Ramos et al. 2015). Some mechanisms of DNA reparation might be operating in these stable Antarctic environments.

The three study populations showed a low genetic diversity. Bottleneck analyses indicate a founder effect in ANT 1 and ANT 2 populations, as they proved to deviate from mutation/drift equilibrium model, but not in ANT3. S. chupachups seems to be a pioneer species in the Antarctic shelf, as it has been recorded to form monospecific beds in areas subjected to recent iceberg scouring (Gutt 1996, 2000), and ANT1 and ANT2 populations may represent examples of colonization events.

The three study populations showed moderate to low genetic structure, according to the FST and D estimators, and the presence of some private alleles in all of them indicates, that they are currently poorly connected. Restricted gene flow even at short geographical distances seems to be the rule in marine sponges (Duran et al. 2004a, b, c; Nichols and Barnes, 2005; Calderon et al. 2007; Blanquer et al., 2009; López-Legentil and Pawlik, 2009; Blanquer and Uriz, 2010; Dailianis et al. 2011) as a result of low dispersal larvae (Mariani et al. 2005), and this pattern is also showed in S. chupachups.

The Antarctic shelf harbors theoretically stable, low impacted (Halpern et al. 2008) benthic communities, subjected to constantly low temperatures (Clarke 1988). This environmental stability could have promoted in sponges and other marine invertebrates, asexual (clonal) reproduction for the prevalence of suitable (adapted) genotypes to the Antarctic particular conditions. As we hypothesized, a relatively high asexual reproduction rate (ca. 25% of the study individuals) was detected in the targeted populations. Some clones were found in populations separated at ca. 2 km. Fragment rafting caused by the action of currents, iceberg scouring, and trawling operations of research and fishery vessels, may be possible explanations for the presence of clones even relatively far from each other.

Antarctic low dispersal invertebrates, such as the sponges, have been isolated from the rest of continents for several millions of years (Dayton 1990; Thompson 1991). Isolation and stability on evolutionary time scales allow envisaging that some Antarctic invertebrates might have resulted from ancient asexual lineages, which perpetuate suitable genotypes, well adapted to the Antarctic particular conditions. Although population genetics' studies are lacking for other Antarctic sponges, a huge production of external buds has been reported for several species of hexactinellid sponges (Teixidó et al. 2006), which allows predicting a high rate of clonal individuals in their populations.

Asexual reproduction may prompt colonization of new substrates as the resulting individuals already have the adult internal organization and may grow faster than the small recruits resulting from larval settling. Stylocordyla chupachups has been reported to incubate mature small individuals of sexual origin (Bergquist 1972; Sarà et al. 2002, as S. borealis), which together with the high percentage of clones may accelerate individual growth and colonization success once released. Releasing functional sponges, might explain why Stylocordyla chupachups is a pioneer species in colonizing newly bare areas originated from iceberg scouring (Gutt 1996, 2000).

Inbreeding (i.e. positive FIS values) is common among marine invertebrates (Addison and Hart, 2005), and has been attributed to several causes, such as restricted dispersal and population structure (Carlon, 1999 and Grosberg, 1987)

genetic drift, bottleneck, and a decline in the effective population size (Perez-Portela, 2014). Also sponge populations usually show homozygote excess (Duran et al. 2004a, b, c; Nichols and Barnes, 2005; Calderon et al. 2007; Blanquer et al. 2009; López-Legentil and Pawlik, 2009; Blanquer and Uriz, 2010; Dailianis et al. 2011). Conversely, heterozygote excess, as revealed by negative FIS values, was found in the three study populations of S. chupachups.

Heterozygote excess has been poorly studied (Stoeckels et al. 2006) and the potential causes for the negative FIS values observed in S. chupachups populations can be only speculated. Negative assortative mating or active avoidance of self and consanguineous mating (Storz et al. 2001) may favor heterozygosity, particularly in small-size populations but it has never been reported for sponges.

Over-dominant selection of loci with heterozygotic advantage (heterosis) might be proposed, as it may induce an excess of heterozygotes at other neutral loci closely linked to those under selection (Strobeck 1979; Nei 1987; Coulson et al. 1998). If there is a fitness advantage to heterozygosity, as it cannot be lost through sexual recombination in clonal individuals, the population should become more heterozygous with time. (Welch & Meselson 2000).

However the percentage of heterozygotes in S. chupachups was slightly but not significantly higher in clonal individuals than in those resulting from sexual reproduction. Thus, several mechanisms might also be operating.

To summarize, relatively elevated rates of clonal reproduction and heterozygote excess found in the target populations are traits rarely found in sponge species from other latitudes and may be related to the particular environmental characteristics and the evolutionary history of the Antarctic. Clonal reproduction may represent a mechanism of genotype adaptation to the particularly stable Antarctic conditions and may underlie the low genetic diversity found in the targeted populations of S. chupachups. A founder effect was found in two of the study populations, which agrees with the pioneer nature reported for the

species and the observations of monospecific beds after iceberg scouring. At first sight, low genetic diversity resulting from a high rate of clonal reproduction would suggest fragility for sponge populations in the Antarctic ecosystems. However, compensatory genetic mechanisms, such an ancestral selection for heterozygotes, may be acting, and together with sexual reproduction, may preserve the minimal genetic diversity in S. chupachups populations to success in the Antarctic. Similar genetic strategies might be shared with other Antarctic sponge species with high rates of clonal reproduction, such as the hexactinellids of the genera Rosella (Carter) and Anoxycalyx (Topsent), but studies on population genetics of those species are still missing.

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GENERAL DISCUSSION

During the last 20 years, the taxonomic and ecological studies of the marine benthos in general and of sponges in particular have benefited from the use of molecular tools (Uriz & Turon 2012). For instance, the availability of massive sequencing techniques promoted studies on population genetics by facilitating the design of suitable markers (Uriz & Turon 2012). Moreover, mitochondrial and nuclear markers have been increasingly used to improve the traditional sponge systematics and phylogeny of Demospongiae (Morrow et al. 2012; Cárdenas et al. 2012; Morrow & Cárdenas 2015), Hexactinellida (Dohrmann et al. 2008, 2009, 2012, 2017; Vargas et al. 2016) and Calcarea (Solé-Cava et al. 1991; Klautau et al. 1999). Sponge molecular phylogenies are in continuous improvement, as additional taxa are being included in the datasets. However, for an accurate translation into taxonomy of the phylogenetic, molecular-based trees, a careful morphological identification of the species/individuals considered in those studies is also required.

Phylogeny of Tetillidae family with particular emphasis on Antarctic genera

Increasing the number of species has been considered more decisive than increasing the number of genes for resolving phylogenetic reconstructions (Carella et al. 2016, this thesis). The study of the Tetillidae conducted by Szitenberg et al. (2010, 2013) failed to resolve some parts of the family phylogenetic tree. Our approach, which included a larger number of Antarctic species, has improved the resolution of the family phylogeny, with respect to previous studies (Szitenberg et al. 2010, 2013).

However, the four molecular markers used in this thesis (COI M1-M6 partition, COI I3-M11 partition, 18S and 28S D3-D5 partition) did not discriminate species within some Antarctic genera such as *Cinachyra* or *Antarctotetilla,* although these species show strong morphological differences. The lack of variation also in the I3-M11 partition of COI, among morphologically distinct species was particularly amazing, as this marker is considered to be more variable than the Folmer partition (Erpenbeck et al. 2006), and has been used for detecting intra-species variation in population genetics and phylogeographic studies of

demosponges (López-Legentil & Pawlik 2009, Xavier et al. 2010, Reveillaud et al. 2011). This lack of variation does not occur in other Tetillidae from tropical and temperate latitudes (e.g. Carella et al. 2016). Thus, the Antarctic representatives of this family seem to present particular genetic traits, which might be explained by either a slow genetic evolutionary rate of the markers used or a recent radiation of the clade containing these two genera, with phenotypic characters evolving faster than the mitochondrial and molecular genes utilized (Carella et a. 2016, this thesis).

A lack of variation in the COI M1-M6 was also found in other sponge classes such as Hexactinellida. Species of *Rossella* showed no differences in COI M1-M6 sequences (Vargas 2016). However, this lack of variation in some markers is not exclusive of Antarctic sponges, as similar cases have also been reported for COI sequences of the freshwater Demospongiae *Lubomirskia*, *Baikalospongia* (Schröder et al. 2003; Heim et al. 2006). In the same way, species of *Thenea* (Demospongiae, Astrophorina) from the North Atlantic did not differ in the COI M1-M6 and 28S C1-D2 partitions (Cárdenas & Rapp, 2012).

The molecular phylogenetic reconstructions retrieved in our study, together with the morphological re-examination of some specimens misidentified, which were used in the previous Tetillidae study (Szitenberg et al. 2013) and several holotypes of type species, helped us to clarify some unresolved parts of the previous phylogeny, such as the polyphyly of *Craniella* (Szitenberg et al. 2013). All COI and 18S trees, as well as the COI+18S concatenated trees were mostly congruent, except for the positions of Tetillidae sp.3 (ANT 27211), Tetillidae sp.1 ("*Craniella sagitta*" NIWA 28929), Tetillidae sp.2 ("*Craniella sagitta*" NIWA 25206 and 28491) and the clade containing *Cinachyrella levantinensis* individuals.

The maximum parsimony phylogeny (MP) on phenotypic characters, plus the motifs of the 18S secondary structure (V4 region), recovered similar tree topologies for the targeted Tetillidae than the molecular phylogenies except for the position of two *Craniella* sp., and *Amphitethya* cf. *microsigma*, which grouped either with *Fangophilina* sp. or *Cinachyrella*, respectively, in the

molecular trees, and with *Cinachyra* or as a sister species to *Tetilla*, respectively, in the morphological tree.

Sigmaspires, cortex, and porocalices have been traditionally considered the main morphological characters for Tetillidae genus and species. Sigmaspires are microscleres with a diagnostic value for the suborder Spirophorina (Rützler 1987) but they appear to have been lost several times along the evolutionary history of the family as they are absent from some genera.

According to Szitenberg et al. (2013), the two main morphological characters (presence of cortex and porocalices), which have been used to differentiate Tetillidae genera (Rützler 1987, Van Soest & Hooper 2002), do not contain information to resolve the phylogeny of our targeted sponges. Conversely, the combination of both molecular and maximum parsimony phylogenies performed in our study (Carella et al. 2016) proved that the cortex structure (i.e. with spicules, without spicules, with one or two layers) and the shape (deep flask-shaped, hemispherical, and small, shallow) of porocalices are diagnostic characters for several genera.

In contrast, to the five clades indicated in Szitenberg et al. (2013), we retrieved seven clades, which corresponded to the genera: *Antarctotetilla* gen. nov., *Cinachyra*, *Acanthotetilla*, *Tetilla*, *Cinachyrella*, *Craniella*, and *Levantinella* gen. nov. The first two genera (*Antarctotetilla* and *Cinachyra*) clustered in a strongly supported clade in all phylogenies and represent the “Antarctic Tetillidae”. *Antarctotetilla* is a new genus that contains those Tetillidae without porocalices but with grouped ostia without a real cortex but a light cortical structure called pseudocortex (Carella et al. 2016). The later trait may explain why previous authors (Sollas, 1886, 1888; Topsent, 1901; Boury-Esnault & Van Beveren, 1982) assigned specimens of this new sponge group to *Tetilla* genus. However, the type species of *Tetilla* (*T. euplocamos* Schmidt, 1868) and other representatives here examined (*T. radiata* and *T. muricyi*) have spread micropores, lack sigmaspires and do not have any kind of cortical specialization. Thus the Antarctic “Tetilla” (*Craniella* in Szitenberg et al. 2013) are clearly different from tropical and temperate Tetilla. After moving the Antarctic ‘’*Tetilla*’’ to *Antarctotetilla* gen. nov., the genus *Tetilla* recovers its classical diagnosis (Rützler 1987).

Cinachyra genus comprises species with a collagenous cortex, sometimes with thick auxiliary oxeas, and flask shaped porocalices. However, many *Cinachyra* species, which have been reported in the literature, might have been incorrectly attributed to this genus. For example in *Cinachyra helena* Rodriguez and Muricy, 2007, the presence of true porocalices is unsure and its two-layered cortex (Rodriguez & Muricy, 2007) suggests that it rather belongs to *Craniella*.

Szitenberg et al. (2013) proposed the inclusion of *Fangophilina* and *Cinachyra* within the genus *Craniella,* either as junior synonyms or sub-genera, and subdivided *Craniella* in two subclades: the first including *Craniella zetlandica*, *C. cranium*, *Craniella* sp. 3318 and *Craniella* sp. Bioice 3659, and the second comprising the remaining *Craniella* species as well as *Fangophilina* and *Cinachyra*. However*,* the *Craniella* genus as proposed by Szitenberg et al. (2013) comprised two wrongly identified species, which have been now reallocated in *Cinachyra* genus because of their flask shaped porocalices, a collagen-made cortex with auxiliary oxeas in a criss-cross arrangement.

On the other hand, three *Craniella sagitta* from New Zealand did not match the diagnostic morphological characters of the genus. Since their position was not resolved in the molecular phylogenies they are provisionally renamed Tetillidae sp. 1 and Tetillidae sp. 2. Moreover, the four re-examined *A. sagitta* joined the *Antarctotetilla* gen. nov. clade in both molecular and morphological phylogenies. Thus, *Craniella senso stricto* (Rützler 1987, Van Soest & Hooper 2002) was recovered as a monophyletic genus, with the characteristic two-layered cortex as a synapomorphy.

Both our phylogenetic studies (Carella et al. 2016) and those in Szitenberg et al. (2013) missed true *Fangophillina* species, since the three cf*. Fangophillina* sp. from New Zealand used do not match the diagnostic morphological characters of the genus. The type species *F. submersa* was reported to have two opposite porocalyces: one with an inhalant function and the other exhalant (Schmidt 1880; Van Soest & Rützler 2002). However, the revision of the type material showed that only the cavity containing the ostia is a true porocalyx while the other one corresponds to a deep cloacal osculum. Additional morphological and genetic investigations on individuals of *F. submersa* are required to resolve its

phylogenetic position, which have been considered dubious (Van Soest & Hooper 2002).

In agreement with Szitenberg et al. (2013), in the three phylogenetic reconstructions (COI, 18S, 28S), the large clade of *Cinachyrella* encompasses *Amphitethya microsigma* and several species of *Paratetilla*, which differ morphologically from the type species *Cinachyrella hirsuta* Dendy. This polyphyletic clade includes genera with different morphology, such as presence of porocalices in *C. hirsuta*, calthrop-like triaenes in *Paratetilla* and amphytriaenes and a stalked morphology in *A. microsigma*). However, many *Cinachyrella* species still remain morphologically and molecularly poorly studied. Thus, we prefer not to consider *Amphitethya* Lendenfeld, 1907 and *Cinachyrella* Wilson, 1925 as synonymies of *Paratetilla* Dendy, 1905, the oldest genus name, until further, in-deep studies on this sponge group will be performed.

Szitenberg et al. (2013) proposed creating the monotypic genus *Levantinella* to embrace *Cinachyrella levantinensis* on the basis of his molecular phylogenies. Our morphological and molecular phylogenies confirm this clade, which is well supported and clearly separated from *Cinachyrella*. However, the name *Levantinella* was not valid because it was in homonymy with a genus name of a fossil Foraminifera (Fourcade, Mouty & Teherani, 1997) and thus, we changed it for *Levantiniella*.

The family Tetillidae appeared paraphyletic in 18S (Redmond et al. 2013) and 28S (Schuster et al. 2015) reconstructions or polyphyletic (Kelly & Cárdenas, 2016) in an 18S phylogeny, while the later authors reported monophyly for this family using COI. However, as the taxon sets used for the 18S and 28S phylogenies were widely incomplete, further studies on a wider Tetillidae dataset are expected to recover this family monophyletic also with nuclear genes (Kelly & Cárdenas 2016).

Two additional species (*Tethya coactifera* Lendenfeld, 1907 and *Tethya crassispicula* Lendenfeld, 1907), which could not be amplified in our phylogenetic study, presented morphological similarities with the Antarctic Tetillidae (both *Antarctotetilla* and *Cinachyra*). As the type material was inadequately fixed for amplification of normal COI primers, they were further

analysed using COI minibarcode primers (Meusnier et al. 2008). NCBI blast of these short sequences fully matched larger COI sequences of species belonging to the Antarctic clade. However, the minibarcode was not informative at genus and species levels due to its short length and the low variability of COI in the Antarctic clade of Tetillidae.

The several genera recovered in our phylogenetic reconstructions mostly show a disjoint distribution. Water temperature seems to be the main underlying factor affecting their distribution. For instance, *Cinachyra* and *Antarctotetilla* are endemic of the Antarctic and Subantarctic waters (deep bottoms), while no true *Tetilla* species have been recorded from the Antarctic. Moreover, *Cinachyrella* (plus *Amphitethya* and *Paratetilla*) are widespread along the tropical/subtropical belt across oceans and *Levantiniella* seems up to now (only one species is known) to be confined to the eastern Mediterranean (shallow waters). On the other hand, species of *Craniella* and *Fangophilina* mainly inhabit deep regions of temperate-cold seas.

Taxonomic analysis of new Tetillidae recovered in the phylogenetic study

Two new genera were erected (*Antarctotetilla* and *Levantiniella*) but not described in the previous phylogenetic studies of family Tetillidae (Szitenberg et al. 2013 and Carella et al. 2016). Therefore, they were considered *nomen nudum* and their description was provided in the second chapter of the thesis (Carella et al. 2018).

Antarctotetilla comprises some Antarctic species previously included in *Tetilla* Schmidt, 1868 (Sollas 1886 and 1888, Boury-Esnault & Van Beveren 1982), *Craniella* Schmidt, 1870 (Kirkpatrick 1908, Koltun 1964, Szitenberg et al. 2013) and *Tethya* Lamarck, 1815 (Lendenfeld 1907). The morphological features that differentiate this genus from other Tetillidae genera are the pores grouped in small surface depressions or in sieve-like areas and a poorly defined cortical region, named ‘’pseudocortex’’. Five species are included in *Antarctotetilla*: *A*. *leptoderma*, *A*. *grandis*, *A*. *sagitta*, *A*. *pilosa* nov. sp., and *A*. *coactifera*.

A. *leptoderma* and *A*. *grandis* were previously considered synonymies (Lendenfeld 1907) but remarkable morphological differences allow us to

differentiated them: a partially conulose surface with protruding spicule tufts and a sole large oscule on top in *A. leptoderma*, *versus* a smooth surface with many small, slightly elevated oscules in *A. grandis*.

A. sagitta has been considered up to now to be a *Craniella* Schmidt, 1870 (Kirkpatrick 1908) or a *Tethya* Lamarck, 1815 (Lendenfeld 1907). However it presents a slight cortex (pseudocortex), which is completely different from the thick cortex of the genera *Craniella* or *Tethya*.

The new species *A. pilosa*, formerly named Tetillidae sp.3 (Carella et al. 2016), appeared in the phylogenetic study either within *Cinachyra* (COI) or within *Antarctotetilla* (18S and 28S D3-D5 partition). However, we realized that the COI sequence of this species used in the previous phylogeny was wrong. A new phylogenetic analysis conducted using the right COI sequence show that this species clustered within the genus *Antarctotetilla,* as it occurred with the nuclear markers*. A. pilosa* morphologically fits within *Antarctotetilla* but differs from the other species of this genus in the presence of a hair-like hispidation through the whole surface and a third type of anatriaene with short and thick clades.

Furthermore, after a morphological review of the types of *Tethya coactifera* Ledenfeld, 1907 (syntype) and *Tethya crassispicula* Ledenfeld, 1907 (holotype), species that proved to belong to the “Antarctic clade” with the COI minibarcode, we can confirm that *T. coactifera* is an *Antarctotetilla*, as it has pores grouped in slight depressions and a pseudocortex. This species is very similar to *A. grandis* but does not have oscules on the apex of conical elevations. Moreover, its spicules show different sizes range than those the other known *Antarctotetilla* species: larger fusiform and auxiliary oxeas, smaller clades of anatriaenes and protriaenes, and a third type of anatriaene with short and thick clades, as in *A. pilosa*. On the other hand, *T. crassispicula* belongs to *Cinachyra* because of its flask shaped porocalices and a spicule-made cortex.

Levantiniella levantiniensis was formerly considered a *Cinachyrella* (Vacelet et al. 2007) but differs from the latter by the small rounded depressions, difficult to assign to porocalices with certainty. The only *Cinachyrella* present in Mediterranean coasts *Cinachyrella tarentina* (Pulitzer-Finali, 1983) differs from

Levantiniella in the presence of true porocalices, plagiotriaenes and the absence of spined microxeas.

Despite many phylogenetic and taxonomic studies on Tetillidae (Szitenberg *et al*. 2010, 2013; Carella et al. 2016 and 2018, this thesis), some genera of this sponge family such as *Craniella*, *Cinachyrella*, *Fangophilina*, *Amphitethya*, and the Antarctic complex "*Craniella sagitta*" still require further morphological and molecular revisions.

The extent of asexual reproduction in Antarctic demosponges: the case of *Stylocordyla chupachups* (Suberitida).

Antarctic benthic communities live under theoretically stable environment conditions, with constantly low temperatures (Clarke 1988), and scarce anthropic impacts (Halpern et al. 2008). Moreover, they have been isolated from the rest of continents for several millions of years (Dayton 1990; Thompson 1991). These conditions may have conferred to Antarctic sponges and other benthic invertebrates, some particular biological and molecular strategies. For instance, the frequent production of asexually produced buds reported in representatives of Class Hexactinellida (*Rossella* and *Anoxycalyx*, Teixidó et al. 2006) suggests a high relevance of clonal reproduction in the Antarctic to preserve adapted genotypes to those stable conditions. Moreover the low variability of some mitochondrial and nuclear sequences in species *Rosella* spp. (Vargas et al. 2016) might also form part of mechanisms of DNA reparation directed to keep the suitable genotypes in these environments.

The study of the three populations *S. chupachups* using microsatellite markers showed a widespread asexual reproductive strategy, with ca. 25% of the individuals being clones. Most clones belonged to the same population but some of them were also found in two, ca. 2 Km apart populations, likely due to rafting resulting from currents, trawling operations from vessels and iceberg scouring.

Isolation and stability may have promoted in this sponge species, the asexual reproduction to perpetuate suitable genotypes well adapted to the Antarctic particular conditions.

Moreover, heterozygote excess (negative *F*IS) was found in any of the three populations of *S. chupachups*. This is an uncommon feature for sponge populations, which most often show homozygote excess (reviewed in Uriz & Turon 2012). Inbreeding in sponge populations has mainly been attributed to restricted dispersal and population structure (Carlon, 1999; Grosberg, 1987), bottleneck, genetic drift and to a decline in the number of individuals caused by several harmful conditions (Pérez-Portela, 2014).

Several causes for explaining the heterozygote excess in *S. chupachus* can be envisaged. It is unlikely due to outcrossing (mating among individuals from different populations), or to negative assortative mating in small populations (Storz et al. 2001), as connectivity is usually low among sponge populations (Mariani et al 2005) and avoidance of self and consanguineous mating has never been reported in sponges. Rather, it might be explained by mechanisms of heterozygote selection. Over-dominant selection of loci with heterozygotic fitness advantage may induce an excess of heterozygotes at other neutral loci closely linked to those under selection (Strobeck 1979; Nei 1987; Coulson et al. 1998). As heterozygosity cannot be lost through sexual recombination in clonal individuals, it would be maintained or even increased in the sponge populations as proposed for other organisms (Welch and Meselson, 2000; Stoeckels et al. 2006).

The three populations showed a low number of alleles, compared with sponge populations from other latitudes (e.g. Dailianis et al. 2011; González-Ramos et al. 2015). The low genetic diversity of this species suggests its fragility in front to environmental drastic changes, which may lead to mass mortality events. However, mechanisms of selection for heterozygotes would compensate the negative effect of clonal reproduction on genetic diversity. Similar genetic strategies might be also present in other Antarctic sponges with a high production of asexual bodies, such as the hexactinellid *Rosella* and *Anoxycalyx* (Teixidó et al. 2006), though genetic studies on these species are still missing.

Furthermore, the three populations of *S. chupachups* showed moderate to low genetic structure. The presence of private alleles in all of them confirms that they are poorly connected as it is commonly reported for sponge populations of other species at similar geographic distances (Duran et al. 2004a, b, c; Nichols

and Barnes, 2005; Calderon et al. 2007; Blanquer et al., 2009; López-Legentil and Pawlik, 2009; Blanquer and Uriz, 2010; Dailianis et al. 2011).

Two out of the three study populations were experiencing bottleneck, which may be associated to a founder effect. This agrees with previous observations of the species forming monospecific beds after iceberg scouring (Gutt 1996, 2000). The high rate of asexually produced individuals and the release of young individuals from sexual origin (Bergquist 1972; Sarà et al. 2002, as *S. borealis*) may facilitates the species colonization of new bare areas thanks to the higher growth rates and resistance of the already formed individuals released, with respect to the slow growth rates and fragility of recruits from larvae. Altogether these features explain the pioneer nature of *S. chupachups.*

CONCLUSIONS

Chapter 1

- 1) The Tetillidae phylogenetic reconstructions are mostly congruent with all the four markers used. The family phylogeny shows seven monophyletic well-supported clades, which correspond to genera: *Cinachyra*, *Acanthotetilla*, *Tetilla*, *Cinachyrella*, *Craniella*, *Antarctotetilla* gen. nov., and *Levantiniella* gen. nov. *Antarctotetilla* is recorded from Antarctic and Subantarctic waters while *Levantiniella i*s only known from Middle-east Mediterranean Sea.

- 2) The maximum parsimony phylogeny on phenotypic characters, plus the motifs of the 18S secondary structure (V4 region), recovered a similar tree than the molecular phylogenies except for the position of two *Craniella* sp., and *Amphitethya* cf. *microsigma*, which grouped either with *Fangophilina* sp. or *Cinachyrella*, respectively, in the molecular trees, and within *Cinachyra* or as a sister species to *Tetilla*, respectively, in the morphological tree.

- 3) We confirm that the two main morphological characters (cortex and porocalices) used to identify genera in Tetillidae do not contain information to resolve the phylogeny of our targeted sponges. Conversely, the combination of both molecular and maximum parsimony phylogenies proved that the cortex structure (i.e. with or without collagen, with or without spicules, and arranged in, one or two layers) and the shape (deep flask-shaped, hemispherical, and small, shallow) of porocalices, are diagnostic characters for genera.

- 4) Other Tetillidae as the three purported cf. *Fangophillina* spp., a misidentified *Craniella sagitta* from New Zealand, and several species of *Cinachyrella*, require both additional morphological re-examination and further phylogenetic analyses.

- 5) The mitochondrial and nuclear markers used could not discriminate species of the genera *Antarctotetilla* and *Cinachyra,* which are clearly differentiated morphologically. Low or no sequence variability of these markers within representatives of these genera may be explained either by a slow genetic evolutionary rate or by a recent radiation of these genera with phenotypic characters evolving faster than the genes studied.

Chapter 2

- 6) Two new Tetillidae genera: *Antarctotetilla* and *Levantiniella* are formally described. Their main diagnostic morphological features are pores grouped in small surface depressions and the presence of a slight cortical region (here named ‘’pseudocortex’’) in *Antarctotetilla*, and small surface cavities that resemble porocalices in *Levantiniella*.

- 7) After the revision of the syntype of *Tethya coactifera* Ledenfeld, 1907 and the holotype of *Tethya crassispicula* Ledenfeld, 1907 with the COI minibarcode sequence, we can confirm these two species belong to the Antarctic clade of Tetillidae. However, the minibarcode was not informative at genus or species levels. Only from a detailed morphological revision, we can conclude that *T. coactifera* belongs to *Antarctotetilla*, while *T. crassispicula* is a *Cinachyra*.

- 8) A new species of Tetillidae is described: *Antarctotetilla pilosa* nov. sp. The species diagnostic characters are a hair-like hispidation through the whole sponge surface and a third type of anatriaene with short and thick clades.

- 9) Up to now, five *Antarctotetilla* species were retrieved: A*. leptoderma*, *A.grandis*, *A. sagitta*, *A. coactifera* and *A. pilosa* nov sp.

Chapter 3

- 10) The population genetics of three populations of *Stylocordyla chupachups* using microsatellites markers showed that the 25% of the overall individuals analysed resulted from asexual reproduction, which represents a high asexual reproduction rate compared with that reported for non-Antarctic sponge species.

- 11) The three populations of *S. chupachups* analysed showed an excess of heterozygotes, which is unusual in sponge populations that show homozygote excess. This population genetic pattern can be explained by heterozygote selection.

- 12) *S. chupachups* show some characteristics of a pioneer species, as it forms monospecific beds on bare areas resulting from iceberg scouring. A founder effect was found in two of the study populations, which suggests that these populations may be the result of recent colonization events.

- 13) The low genetic diversity in *S. chupachups* in part resulting from a high rate of clonal reproduction suggests the fragility of this sponge species in the Antarctic ecosystems if any drastic perturbation occurs. However, compensatory genetic mechanisms, such an ancestral selection for heterozygotes, may be acting, and together with sexual reproduction, may preserve the genetic diversity.

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APPENDIX

File 1.1 Morphological data matrix (including secondary structure shapes of the 18S V4 variable region) used for the maximum parsimony phylogeny.

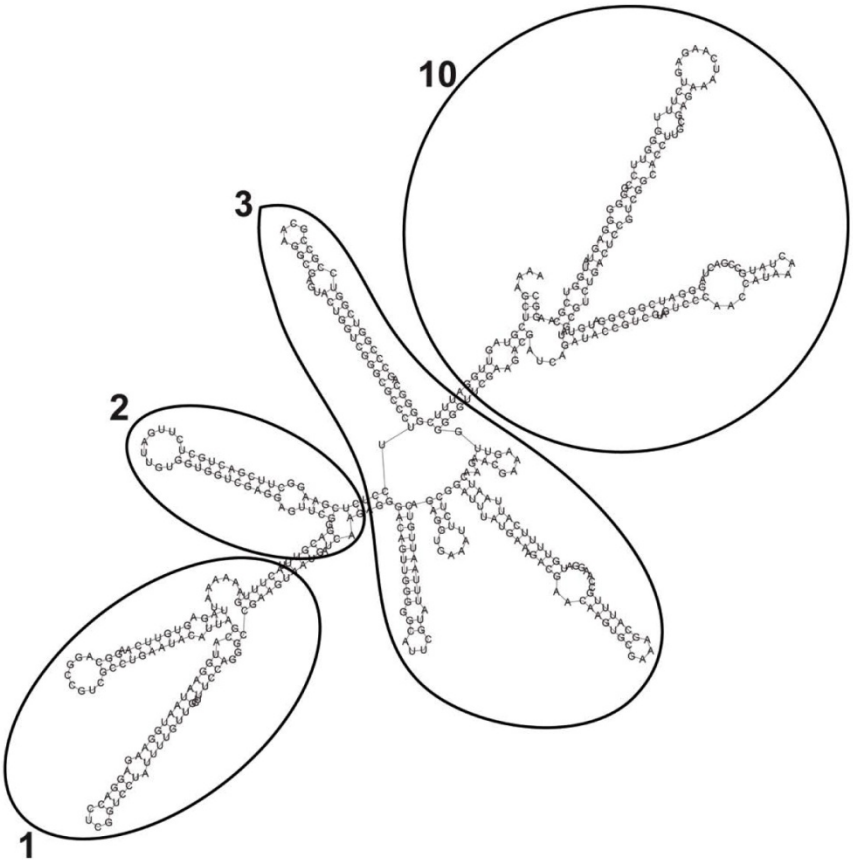
NAME	single oscule	multioscule	Big Hemisferical porocalices	small rounded porocalices	flask shaped porocalices	Pores grouped	Pores Sieve-like	Pores not Grouped
Acanthotetilla celebensis (de Voogd & van Soest, 2007)	?	?	0	1	0	0	0	0
Acanthotetilla walteri (Peixinho, Fernandez, Oliveira, Caires & Hajdu, 2007)	?	?	0	1	0	0	0	0
Acanthotetilla seychellensis (Thomas, 1973)	?	?	0	?	0	0	0	0
Cinachyra antartica (Current study)	?	?	0	0	1	0	0	0
Cinachyra barbata (Current study)	?	?	0	0	1	0	0	0
Tetillidae ANT 27211 (Current study)	0	1	0	0	0	1	0	0
Cinachyrella alloclada (Uliczka, 1929)	?	?	1	0	0	0	0	0
Cinachyrella paterifera (Wilson, 1925)	?	?	1	0	0	0	0	0
Cinachyrella australiensis (Carter, 1886)	?	?	1	0	0	0	0	0
Cinachyrella levantinensis (Vacelet, Bitar, Carteron, Zibrowius & Perez, 2007)	?	?	0	1	0	0	0	0
Cinachyrella schulzei (Keller, 1891)	?	?	1	0	0	0	0	0
Craniella cranium (Müller, 1776)	?	?	0	0	0	0	0	1
Craniella cf. *leptoderma* (Szitenberg, 2013)	1	0	0	0	0	1	0	0
Craniella sp. QMG 316342 (Szitenberg, 2013)	?	?	0	0	1	0	0	0
Craniella sp. QMG 316372 (Belinky, 2012)	?	?	0	0	1	0	0	0
Craniella sp. ZMBN 85240 (Cardenas, 2011)	?	?	0	0	0	0	0	1
Craniella sp. BIOICE 3659 (Szitenberg, 2010)	?	?	0	0	0	0	0	1
Craniella sp. QMG 318785 (Szitenberg, 2013)	?	?	0	0	0	0	0	1
Craniella zetlandica (Carter, 1872)	?	?	0	0	0	0	0	1
Cinachyrella kuekenthali (Uliczka, 1929)	?	?	1	0	0	0	0	0
Fangophilina sp. (Szitenberg, 2013)	?	?	0	0	0	0	0	1
Geodia cydonium (Jameson, 1811)	?	?	0	0	0	0	0	1
Geodia neptuni (Sollas, 1886)	?	?	0	0	0	0	0	1
Cinachyrella apion (Uliczka, 1929)	?	?	1	0	0	0	0	0
Paratetilla bacca (Selenka, 1867)	?	?	1	0	0	0	0	0
Tetilla leptoderma (Current study)	1	0	0	0	0	1	0	0
Tetilla grandis (Current study)	0	1	0	0	0	1	0	0
Tetilla japonica (Lampe, 1886)	?	?	0	0	0	0	0	1
Tetilla murycii (Peixinho, Pinheiro & Menegola, 2011)	?	?	0	0	0	0	0	1
Tetilla radiata (Selenka, 1879)	?	?	0	0	0	0	0	1
Tetilla sagitta (Cardenas, 2008)	0	1	0	0	0	1	1	0
Amphitetya microsigma (Lendenfeld, 1907)	?	?	0	0	0	0	0	1
Craniella sagitta (Szitenberg, 2013)	0	1	0	0	0	0	0	1

NAME	CORTEX only colagene	Double layered CORTEX	CORTEX colagene + oxeas	CORTEX colagene + sterraster	CORTEX palisade of megacanthoxeas	CORTEX amphitriaenes and sigmaspires	No CORTEX	Pseudocortex
Acanthotetilla celebensis (de Voogd & van Soest, 2007)	0	0	0	0	1	0	0	0
Acanthotetilla walteri (Peixinho, Fernandez, Oliveira, Caires & Hajdu, 2007)	0	0	0	0	1	0	0	0
Acanthotetilla seychellensis (Thomas, 1973)	0	0	0	0	1	0	0	0
Cinachyra antartica (Current study)	1	0	0	0	0	0	0	0
Cinachyra barbata (Current study)	0	0	1	0	0	0	0	0
Tetillidae ANT 27211 (Current study)	0	0	0	0	0	0	0	1
Cinachyrella alloclada (Uliczka, 1929)	0	0	0	0	0	0	1	0
Cinachyrella paterifera (Wilson, 1925)	0	0	0	0	0	0	1	0
Cinachyrella australiensis (Carter, 1886)	0	0	0	0	0	0	1	0
Cinachyrella levantinensis (Vacelet, Bitar, Carteron, Zibrowius & Perez, 2007)	0	0	0	0	0	0	1	0
Cinachyrella schulzei (Keller, 1891)	0	0	0	0	0	0	1	0
Craniella cranium (Müller, 1776)	0	1	0	0	0	0	0	0
Craniella cf. *leptoderma* (Szitenberg, 2013)	0	0	0	0	0	0	0	1
Craniella sp. QMG 316342 (Szitenberg, 2013)	0	0	1	0	0	0	0	0
Craniella sp. QMG 316372 (Belinky, 2012)	0	0	1	0	0	0	0	0
Craniella sp. ZMBN 85240 (Cardenas, 2011)	0	1	0	0	0	0	0	0
Craniella sp. BIOICE 3659 (Szitenberg, 2010)	0	1	0	0	0	0	0	0
Craniella sp. QMG 318785 (Szitenberg, 2013)	0	1	0	0	0	0	0	0
Craniella zetlandica (Carter, 1872)	0	1	0	0	0	0	0	0
Cinachyrella kuekenthali (Uliczka, 1929)	0	0	0	0	0	0	1	0
Fangophilina sp. (Szitenberg, 2013)	0	0	0	0	0	0	1	0
Geodia cydonium (Jameson, 1811)	0	0	0	1	0	0	0	0
Geodia neptuni (Sollas, 1886)	0	0	0	1	0	0	0	0
Cinachyrella apion (Uliczka, 1929)	0	0	0	0	0	0	1	0
Paratetilla bacca (Selenka, 1867)	0	0	0	0	0	0	1	0
Tetilla leptoderma (Current study)	0	0	0	0	0	0	0	1
Tetilla grandis (Current study)	0	0	0	0	0	0	0	1
Tetilla japonica (Lampe, 1886)	0	0	0	0	0	0	1	0
Tetilla murycii (Peixinho, Pinheiro & Menegola, 2011)	0	0	0	0	0	0	1	0
Tetilla radiata (Selenka, 1879)	0	0	0	0	0	0	1	0
Tetilla sagitta (Cardenas, 2008)	0	0	0	0	0	0	0	1
Amphitetya microsigma (Lendenfeld, 1907)	0	0	0	0	0	1	0	0
Craniella sagitta (Szitenberg, 2013)	0	0	0	0	0	0	0	1

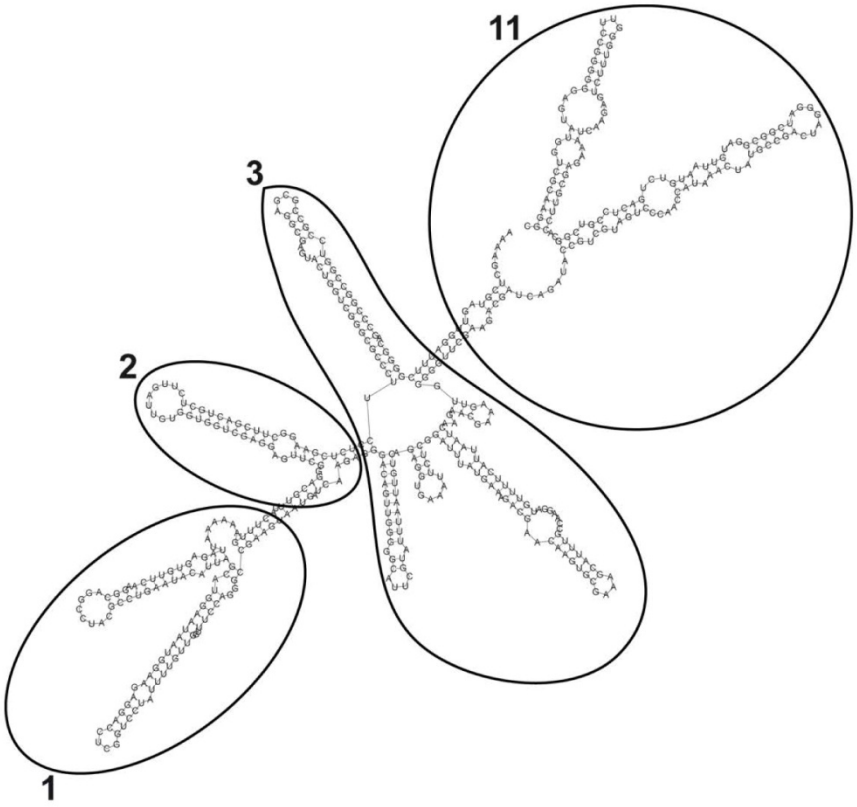
NAME	Corrugated Surface	Smooth surface	Hispid surface	Conulose surface	Hair-like	Short shafted triaenes	Megacanthoxeas	Sigmaspires	Aster	Amphitriaenes
Acanthotetilla celebensis (de Voogd & van Soest, 2007)	0	0	1	0	0	0	1	1	0	0
Acanthotetilla walteri (Peixinho, Fernandez, Oliveira, Caires & Hajdu, 2007)	0	0	1	0	0	0	1	1	0	0
Acanthotetilla seychellensis (Thomas, 1973)	0	0	1	0	0	0	1	1	0	0
Cinachyra antartica (Current study)	0	1	0	0	0	0	0	1	0	0
Cinachyra barbata (Current study)	0	0	1	0	0	0	0	1	0	0
Tetillidae ANT 27211 (Current study)	0	0	1	0	1	0	0	1	0	0
Cinachyrella alloclada (Uliczka, 1929)	0	0	1	0	0	0	0	1	0	0
Cinachyrella paterifera (Wilson, 1925)	0	1	1	0	0	0	0	1	0	0
Cinachyrella australiensis (Carter, 1886)	0	0	1	0	0	0	0	1	0	0
Cinachyrella levantinensis (Vacelet, Bitar, Carteron, Zibrowius & Perez, 2007)	0	0	1	0	0	0	0	1	0	0
Cinachyrella schulzei (Keller, 1891)	0	1	0	0	0	0	0	1	0	0
Craniella cranium (Müller, 1776)	1	0	0	0	0	0	0	1	0	0
Craniella cf. *leptoderma* (Szitenberg, 2013)	1	0	0	0	0	0	0	1	0	0
Craniella sp. QMG 316342 (Szitenberg, 2013)	0	0	1	0	0	0	0	1	0	0
Craniella sp. QMG 316372 (Belinky, 2012)	0	0	1	0	0	0	0	1	0	0
Craniella sp. ZMBN 85240 (Cardenas, 2011)	0	0	1	0	0	0	0	1	0	0
Craniella sp. BIOICE 3659 (Szitenberg, 2010)	0	0	1	1	0	0	0	1	0	0
Craniella sp. QMG 318785 (Szitenberg, 2013)	1	0	0	0	0	0	0	1	0	0
Craniella zetlandica (Carter, 1872)	1	0	0	0	0	0	0	1	0	0
Cinachyrella kuekenthali (Uliczka, 1929)	0	0	1	0	0	0	0	1	0	0
Fangophilina sp. (Szitenberg, 2013)	0	0	1	0	0	0	0	1	0	0
Geodia cydonium (Jameson, 1811)	0	0	1	0	0	0	0	0	1	0
Geodia neptuni (Sollas, 1886)	0	0	1	0	0	0	0	0	1	0
Cinachyrella apion (Uliczka, 1929)	0	0	1	0	0	0	0	1	0	0
Paratetilla bacca (Selenka, 1867)	0	0	1	0	0	1	0	1	0	0
Tetilla leptoderma (Current study)	1	0	0	0	0	0	0	1	0	0
Tetilla grandis (Current study)	0	1	0	0	0	0	0	1	0	0
Tetilla japonica (Lampe, 1886)	0	1	0	0	0	0	0	1	0	0
Tetilla murycii (Peixinho, Pinheiro & Menegola, 2011)	0	1	0	0	0	0	0	0	0	0
Tetilla radiata (Selenka, 1879)	0	1	0	0	0	0	0	0	0	0
Tetilla sagitta (Cardenas, 2008)	1	0	0	0	0	0	0	1	0	0
Amphitetya microsigma (Lendenfeld, 1907)	0	1	0	1	0	0	0	1	0	1
Craniella sagitta (Szitenberg, 2013)	0	0	1	0	0	0	0	1	0	0

NAME	ssr1	ssr2	ssr3	ssr4	ssr5	ssr6	ssr7	ssr8	ssr9	ssr10	ssr11	ssr12	ssr13
Acanthotetilla celebensis (de Voogd & van Soest, 2007)	1	0	0	0	0	1	1	0	0	0	0	0	0
Acanthotetilla walteri (Peixinho, Fernandez, Oliveira, Caires & Hajdu, 2007)	1	0	0	0	0	1	1	0	0	0	0	0	0
Acanthotetilla seychellensis (Thomas, 1973)	1	0	0	0	0	1	1	0	0	0	0	0	0
Cinachyra antartica (Current study)	1	1	1	0	0	0	0	0	0	1	0	0	0
Cinachyra barbata (Current study)	1	1	1	0	0	0	0	0	0	1	0	0	0
Tetillidae ANT 27211 (Current study)	1	1	1	0	0	0	0	0	0	1	0	0	0
Cinachyrella alloclada (Uliczka, 1929)	1	0	0	0	0	1	1	0	0	0	0	0	0
Cinachyrella paterifera (Wilson, 1925)	1	0	0	0	0	1	1	0	0	0	0	0	0
Cinachyrella australiensis (Carter, 1886)	1	0	0	0	0	1	1	0	0	0	0	0	0
Cinachyrella levantinensis (Vacelet, Bitar, Carteron, Zibrowius & Perez, 2007)	1	0	0	0	0	1	1	0	0	0	0	0	0
Cinachyrella schulzei (Keller, 1891)	1	0	0	0	0	1	1	0	0	0	0	0	0
Craniella cranium (Müller, 1776)	0	0	0	1	1	1	0	0	0	0	0	0	0
Craniella cf. *leptoderma* (Szitenberg, 2013)	1	1	1	0	0	0	0	0	0	1	0	0	0
Craniella sp. QMG 316342 (Szitenberg, 2013)	1	1	1	0	0	0	0	0	0	0	1	0	0
Craniella sp. QMG 316372 (Belinky, 2012)	1	1	1	0	0	0	0	0	0	0	1	0	0
Craniella sp. ZMBN 85240 (Cardenas, 2011)	0	0	0	1	1	1	0	0	0	0	0	0	0
Craniella sp. BIOICE 3659 (Szitenberg, 2010)	0	0	0	1	1	1	0	0	0	0	0	0	0
Craniella sp. QMG 318785 (Szitenberg, 2013)	0	0	0	1	1	1	0	0	0	0	0	0	0
Craniella zetlandica (Carter, 1872)	0	0	0	1	1	1	0	0	0	0	0	0	0
Cinachyrella kuekenthali (Uliczka, 1929)	1	0	0	0	0	1	1	0	0	0	0	0	0
Fangophilina sp. (Szitenberg, 2013)	1	1	0	1	1	0	0	0	0	0	0	0	0
Geodia cydonium (Jameson, 1811)	1	0	0	0	0	0	0	1	1	0	0	0	0
Geodia neptuni (Sollas, 1886)	1	0	0	0	0	0	0	1	1	0	0	0	0
Cinachyrella apion (Uliczka, 1929)	1	0	0	0	0	1	1	0	0	0	0	0	0
Paratetilla bacca (Selenka, 1867)	1	0	0	0	0	1	1	0	0	0	0	0	0
Tetilla leptoderma (Current study)	1	1	1	0	0	0	0	0	0	1	0	0	0
Tetilla grandis (Current study)	1	1	1	0	0	0	0	0	0	1	0	0	0
Tetilla japonica (Lampe, 1886)	0	0	0	0	0	0	0	0	0	0	0	1	1
Tetilla murycii (Peixinho, Pinheiro & Menegola, 2011)	0	0	0	0	0	0	0	0	0	0	0	1	1
Tetilla radiata (Selenka, 1879)	0	0	0	0	0	0	0	0	0	0	0	1	1
Tetilla sagitta (Cardenas, 2008)	1	1	1	0	0	0	0	0	0	1	0	0	0
Amphitetya microsigma (Lendenfeld, 1907)	?	?	?	?	?	?	?	?	?	?	?	?	?
Craniella sagitta (Szitenberg, 2013)	1	1	1	0	0	0	0	0	0	1	0	0	0

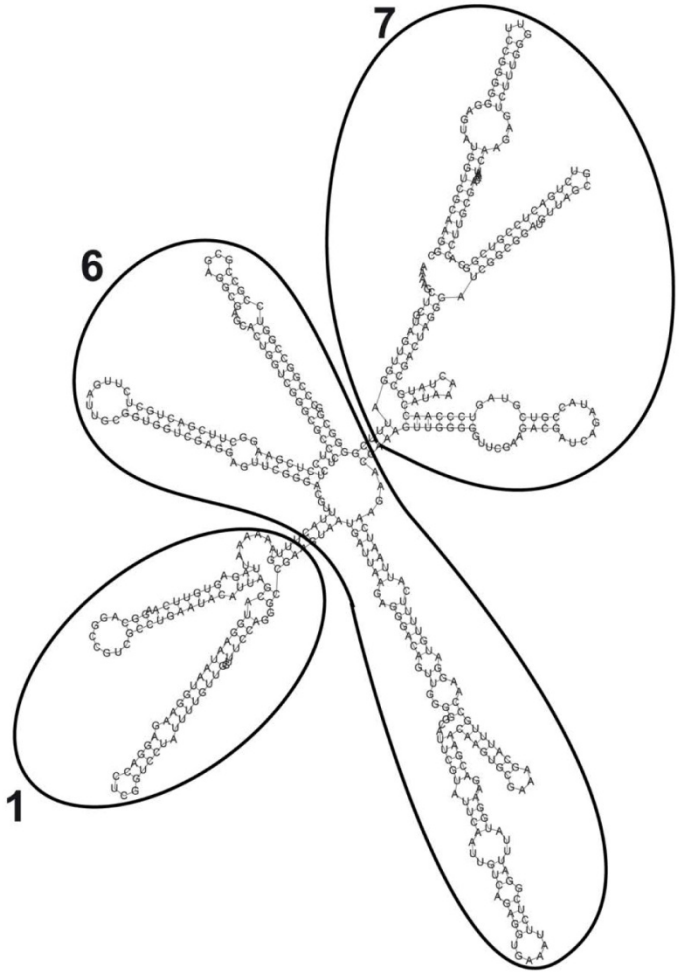
File 1.2 The different parts of the predicted secondary structures (V4 region of 18S) are encircled and numbered.



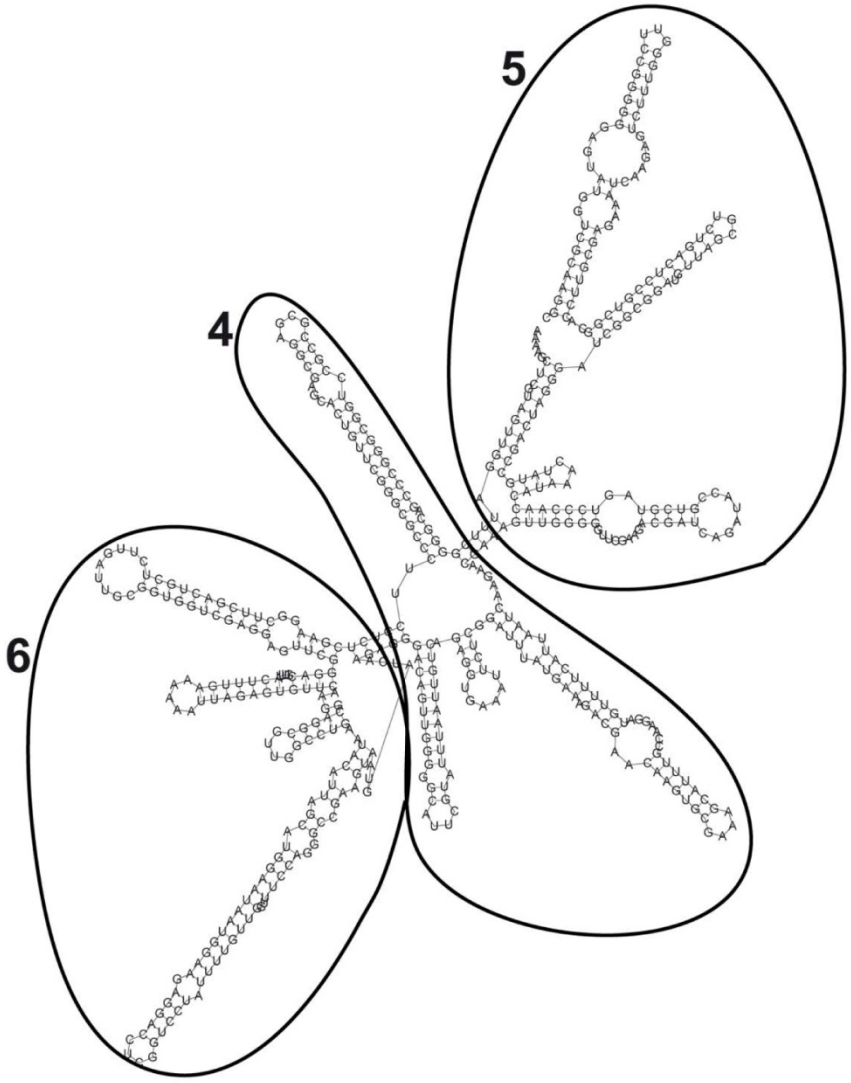
Cinachyra - *Antarctotetilla* - Tetillidae sp.1, sp.2, sp.3.



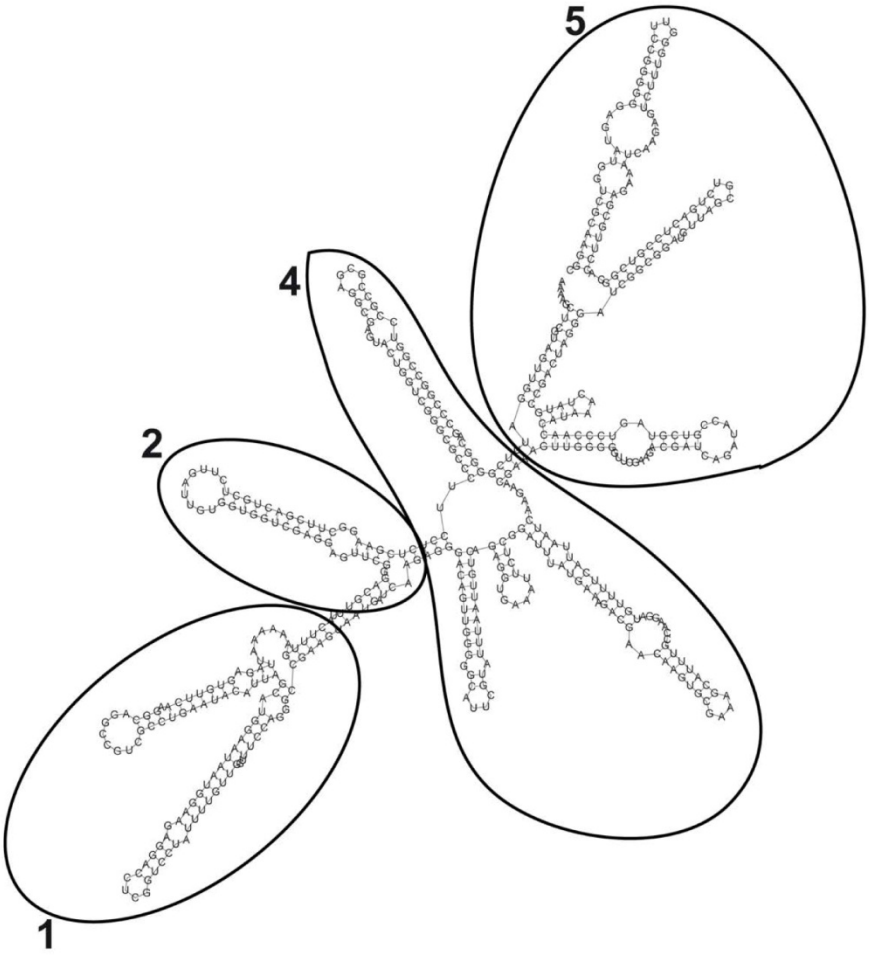
Cinachyra sp. QMG 316342, QMG 316372.



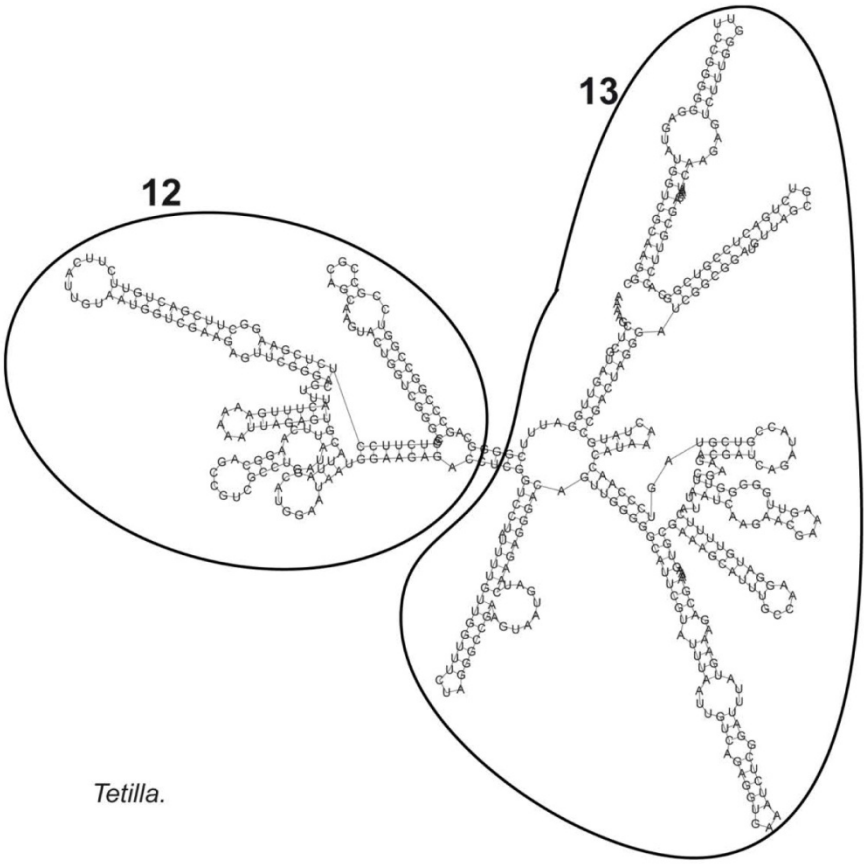
Cinachyrella - Paratetilla - Acanthotetilla - Levantinella.



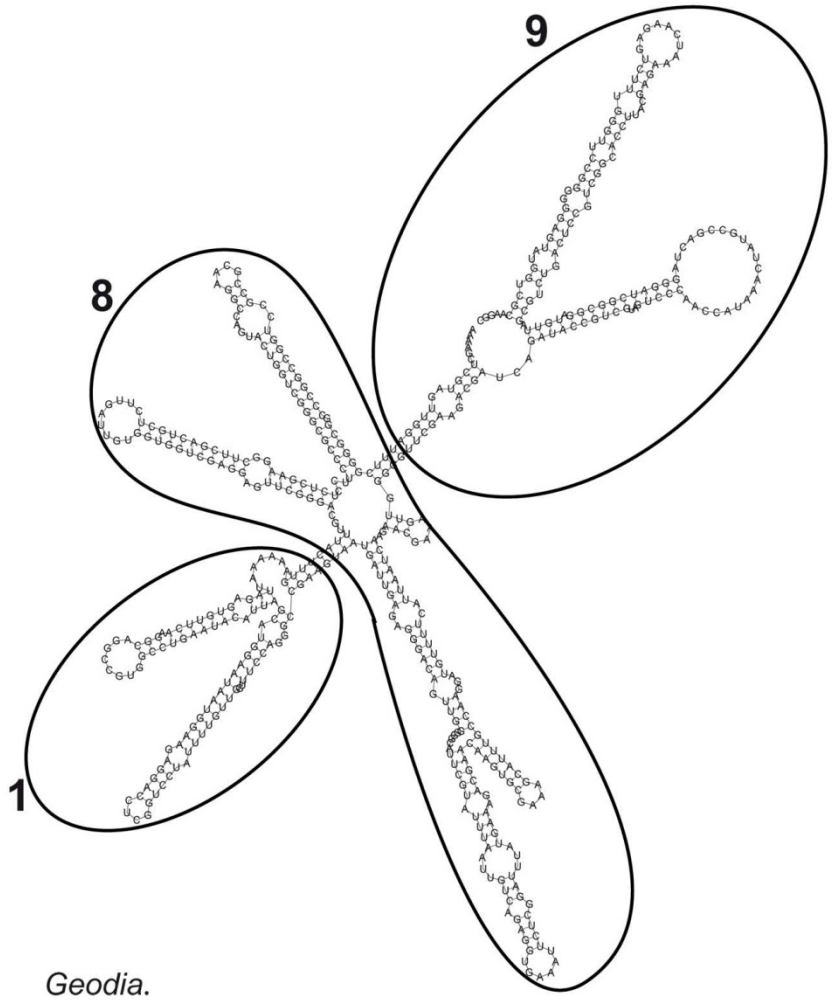
Craniella.



Fangophilina sp.



Tetilla.



Geodia.

File 2.1 Revised sequence of *A. pilosa* (ex Tetillidae sp.3) and two COI minibarcode sequences of type species (*T. coactifera* and *T. crassispicula*).

>*Antarctotetilla_pilosa_* cytochrome oxidase subunit I (COI, M1-M6)

CCGGATTAATGTTGGGTGACGACCATTTATACAATGTTATGGTCACGGCCCACGGTCTTATAATGGTCTTTTTCGTAGTTATGCCGGTTATKATAGGTGKGTTCGGTAATTGAAT
GGTCSMCSTTTACATYGGKGCWCMGRATAYGGCTTWTSCAAGATTAAACAATRTTAGTTTKTGAGTTTTACCCCCCTCATTAATACTAMTACTAGGCTCTGCTTTTGTTGAACA
AGGGGTTGGGACAGGATGGACCCKTTATCCGCCATTATCAAGTATACAGGCTCATTCYGGGGGGMTCMGTYGATGYGGCAATTTTTWGTCTTCATTTGGCTGGGATCTCTTCAA
TTTTAGGGGCAATGAATYTTATAASTACTATCTTTAATATGCGGGCACCTGGGATTACCATGGATAGATTGCCTCTATTTGTTTGATCTATTTTAATAACAACTTATTTATTATTAT
TAGCTTTGCCAGTATTGGCTGGTGCCATAACTATGCTTTTAACAGATAGAAATTTTAATACAACGTTCTTCGATCCCG

>*Tethya_coactifera*_type_ cytochrome oxidase subunit I (COI, Minibarcode)

ATACTTATTATTTGGTGTTTTTTCGGGTATGATAGGAACTGGATTTAGCTTGCTTATTAGATTAGAACTATCCGCTCCCGGATTAATGTTGGGTGACGACCAT
TTATACAATGTTATGGTCACG

>*Tethya_crassispicula*_type_ cytochrome oxidase subunit I (COI, Minibarcode)

WACCTTATACTTATTATTTGGTGTTTTTTCGGGTATGATAGGAACTGGATTTAGCTTGCTTATTAGATTAGAACTATCCGCTCCCGGATTAATGTTGGGTGA
CGACCATTTATACAATGTTATGGTCACG

PLOS ONE

RESEARCH ARTICLE

Phylogenetic Reassessment of Antarctic Tetillidae (Demospongiae, Tetractinellida) Reveals New Genera and Genetic Similarity among Morphologically Distinct Species

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Abstract

Species of Tetillidae are distributed worldwide. However, some genera are unresolved and only a few genera and species of this family have been described from the Antarctic. The incorporation of 25 new COI and 18S sequences of Antarctic Tetillidae to those used recently for assessing the genera phylogeny, has allowed us to improve the resolution of some poorly resolved nodes and to confirm the monophyly of previously identified clades. Classical genera such as *Craniella* recovered their traditional diagnosis by moving the Antarctic *Tetilla* from *Craniella*, where they were placed in the previous family phylogeny, to *Antarctotetilla* gen. nov. The morphological re-examination of specimens used in the previous phylogeny and their comparison to the type material revealed misidentifications. The proposed monotypic new genus *Levantinella* had uncertain phylogenetic relationships depending on the gene partition used. Two more clades would require the inclusion of additional species to be formally established as new genera. The parsimony tree based on morphological characters and the secondary structure of the 18S (V4 region) almost completely matched the COI M1-M6 and the COI+18S concatenated phylogenies. Morphological synapomorphies have been identified for the genera proposed. New 15 28S (D3-D5) and 11 COI I3-M11 partitions were exclusively sequenced for the Antarctic species subset. Remarkably, species within the Antarctic genera *Cinachyra* (*C. barbata* and *C. antarctica*) and *Antarctotetilla* (*A. leptoderma*, *A. grandis*, *and A. sagitta*), which are clearly distinguishable morphologically, were not genetically differentiated with any of the markers assayed. Thus, as it has been reported for other Antarctic sponges, both the mitochondrial and nuclear partitions used did not differentiate species that were well characterized morphologically. Antarctic Tetillidae offers a rare example of genetically cryptic (with the traditional markers used for sponges), morphologically distinct species.

KT124366 KT124322 KT124348 KT124359 KT124349 KT124360 KT124345 KT124326 KT124339 KT124316 KT124315 KT124327 KT124350 KT124334 KT124312 KT124352 KT124342 KT124333 KT124320 KT124332 KT124369 KT124371 KT124337 KT124370

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Introduction

Sponges dominate some benthic communities in the Antarctic, in terms of both biomass [1], [2] and diversity [3]. The Antarctic clockwise circumpolar current [4] and the low water temperatures contribute to the biogeographic isolation of the Antarctic continental shelf, which partly explains the high degree of sponge endemism in the area [5], [6], [7]. Taxonomic affinities between Antarctic sponges and those of the Magellanic region (South America) and the Falkland Islands have also been reported [8], [5], [9] but the studies are still incomplete and subject to debate [10], [7]. Most of the currently known Antarctic sponge species were discovered during the oceanographic campaigns of the twentieth century [11], [12], [13], [14], [8], [15], [16], [17], [18], [19], [20], [21], [22], [23]. However, recent findings of many new species [24], [7], [25], [26], [27], [28], [29], [30], [31], [32] suggest that the sponge biodiversity of this area has not been fully explored yet and that more species remain to be discovered.

While collecting sponges during the Antarctic Polarstern ANT-XXVII/3 expedition in 2011, we realized the difficulty to identify the fairly common, well known, large conspicuous species belonging to the family Tetillidae Sollas, 1886. The World Porifera Database [33] currently lists only four valid Tetillidae species from the Antarctic—*Cinachyra barbata* (Sollas, 1886), *Cinachyra antarctica* (Carter, 1872), *Tetilla leptoderma* (Sollas, 1886) and *Craniella sagitta* (Lendenfeld, 1907). Furthermore, intra-specific variations of the mitochondrial cytochrome c oxidase subunit 1 (COI), from Antarctic and New Zealand Tetillidae, also suggest that the diversity of this group is underestimated [34]. We also noticed that there is no consensus in the literature regarding the allocation of some Antarctic species to the genus *Craniella* or *Tetilla* [35], [36], [34].

The family Tetillidae (Demospongiae, Tetractinellida, Spirophorina) contains 156 species distributed worldwide [33]. Many of them inhabit sedimentary bottoms to which they anchor by means of long spicule bundles, which represent a suitable substrate for many other hard-bottom invertebrates [37]. Their representatives are characterized by a globular habit, a radiate skeleton composed chiefly of the following spicules: megascleres are protriaenes, oxeas, and sometimes ortho/anatriaenes or calthrops, which often protrude the ectosomal layer outward; microscleres are characteristic sigmaspires and occasionally raphides [36]. To this day, the Tetillidae have no clear morphological synapomorphy, as triaenes are shared with all Tetractinellida, and sigmaspires are found in most Spirophorina families. The Tetillidae appears monophyletic with COI [38], but polyphyletic in 18S and 28S (C1-D2) phylogenies [39], [40], [38].

Using the COI of 14 Tetillidae species, Szitenberg et al. [41] suggested that most Tetillidae genera were not monophyletic. Later, Szitenberg et al. [34], using this time a set of three molecular markers (COI, 28S and 18S) on 28 Tetillidae species belonging to eight genera, obtained five main clades (with COI and 28S): (i) *Acanthotetilla* (ii) *Cinachyrella*, *Paratetilla*, and *Amphitethya*, (iii) *Cinachyrella levantinensis*, (iv) tropical-temperate *Tetilla*, and (v) *Craniella*, *Cinachyra*, and *Fangophilina*. Results were similar with 18S, except that *Acanthotetilla* sequences were lacking from the NCBI genbank. One of the main issues raised by this study concerned the *Craniella/Cinachyra/Fangophilina* clade, which included all the Antarctic species. Results suggested the polyphyly of the genus *Craniella* distributed in three clades: (i) *Craniella* cf. *leptoderma* (Antarctic, New Zealand), (ii) *Craniella sagitta* (Antarctic, New Zealand) and (iii) a *Craniella* clade with boreo-arctic Atlantic species mixed with New Zealand/Australian species. Based on this polyphyly, Szitenberg et al. [34] propose to reallocate the Antarctic *Tetilla*, *Fangophilina*, and *Cinachyra* to the genus *Craniella*, despite the absence of morphological support for such a proposal.

The goal of the current study was to investigate the relationships between the Antarctic and tropical/temperate Tetillidae to revise the taxonomy and phylogeny of the family and to assess

the purported endemism of its Antarctic genera. We improved the sampling of previous phylogenies by incorporating additional Antarctic and Sub-Antarctic specimens from geographically distant localities. We used four gene partitions (mitochondrial and nuclear) to conduct molecular phylogenetic analyses. The morphology of new specimens, type species, and some specimens previously sequenced [34] was examined. Finally, we also performed a maximum parsimony phylogenetic analysis based on morphological characters and the secondary structure of the V4 region of the 18S rDNA.

Our findings confirm the monophyly of the Antarctic genera and allow us to erect two new Tetillidae genera: *Antarctotetilla* gen. nov., restricted to Antarctic and sub-Antarctic waters, and *Levantinella* gen. nov. so far limited to eastern Mediterranean. This study also resurrects a sub-Antarctic species and reveals four potential new species. The genetic homogeneity of the markers used among morphologically distinct Antarctic Tetillidae species, contrasts with the habitual finding of genetically distinct, morphologically cryptic species. A restricted geographical distribution of some Tetillidae genera has become evident.

Material and Methods

The collection of sponge samples was conducted in strict accordance with Spanish and European regulations under the rules of the Spanish National Research Council with the approval of the Directorate of Research of the Spanish Government. The study was found exempt from ethics approval by the ethics commission of the University of Barcelona since, according to article 3.1 of the European Union directive (2010/63/UE) from the 22/9/2010, no approval is needed for sponge sacrification, as they are the most primitive Animals and lack any nervous system Moreover, the collected sponges are not listed in CITES."

Sampled sponges

The majority of the samples were collected in Antarctic and sub-Antarctic regions during the Polarstern ANT-XXVII/3 expedition from Punta Arenas, Chile (February 8, 2011) to Cape Town, South Africa (18 April 2011) with Agassiz (AGT) and bottom trawl (BT) gears. During this expedition, Tetillidae were collected in South Georgia, South Orkneys Islands, and Newmayer in the Antarctic continent. A Remote Operated Vehicle (ROV) was deployed during the Polarstern cruise to gather samples between 300 and 450 meters and to photograph underwater living specimens. Given the large size (up to 30 cm in diameter) of most Antarctic Tetillidae, once the individuals were photographed, a fragment ca. 3 cm^3 in size was preserved in absolute ethanol, which was changed three times before packing at -20°C for transportation and storage at the CEAB (Centre d'Estudis Avançats de Blanes, Spain). Other Tetillidae from this study were collected during the Collaborative East Antarctic Marine Census (CEAMARC) Dec. 2007- Jan. 2008 in Adélie Land, Antarctica [42], [43]. The CEAMARC specimens were dredged between 170 and 1700 m; the complete specimens were bulk-fixed with ethanol (80%) in 60L metallic drums. A few samples also came from a fishery-independent biomass survey "POissons de KERguelen" (POKER II) conducted in 2010 on the Kerguelen Plateau. The complete specimens were collected using a Grande Ouverture Verticale (GOV) trawl, frozen on board and bulk-fixed in ethanol 80% at the Muséum National d'Histoire Naturelle (MNHN), Paris, France. Specimens from these two expeditions are housed at the MNHN and stored in the 'Zoothèque' at a constant 18°C temperature. Three additional samples were collected between Lavoisier and the Antarctic Peninsula between 847 and 960 m (R/V LM Gould, 2010) and were obtained from Bill Baker (University of South Florida). The samples used in this study, voucher numbers, Genbank accession numbers, and collecting localities are provided in Table 1.

Table 1. List of species used in the study, with collection reference number, accession number of the sequences stored in the Genbank, revised species name, and geographical origin.

		Genbank accession numbers					
SPECIES	**Voucher number**	**COI M1-M6**	**18S**	**COI I3-M11**	**28S (D3-D5)**	**Revised species name**	**Collection sites**
Tetilla leptoderma	ANT 27111	**KT124318**	**KT124341**	**KT124328**	**KT124362**	*Antarctotetilla leptoderma*	Sub Antarctic (South Georgia)
Tetilla leptoderma	ANT 27112	**KT124319**	**KT124343**	**KT124329**	**KT124365**	*Antarctotetilla leptoderma*	Antarctica (Newmayer)
Tetilla grandis	ANT 27123	**KT124324**	**KT124344**	**KT124330**	**KT124363**	*Antarctotetilla grandis*	Antarctica (Newmayer)
Tetilla grandis	ANT 27124	**KT124325**	**KT124346**	**KT124331**	**KT124364**	*Antarctotetilla grandis*	Antarctica (Newmayer)
Cinachyra barbata	ANT 27212	**KT124314**	**KT124356**	**KT124336**	_		Sub Antarctic (South Georgia)
Tetillidae	ANT 27211	**KT124313**	**KT124355**	_	**KT124361**	Tetillidae sp. 3	Sub Antarctic (South Orkneys)
Cinachyra antarctica	ANT 27204	**KT124317**	_	_	**KT124367**		Antarctica (Newmayer)
Tetilla leptoderma	ANT 27107	_	**KT124351**	_	**KT124358**	*Antarctotetilla leptoderma*	Antarctica (Newmayer)
Tetilla leptoderma	ANT 27108	**KT124323**	**KT124347**	_	_	*Antarctotetilla leptoderma*	Sub Antarctic (Souh Orkneys)
Tetilla leptoderma	ANT 27109	_	**KT124354**	_	_	*Antarctotetilla leptoderma*	Antarctica (Newmayer)
Cinachyra antartica	ANT 27223	_	**KT124353**	**KT124335**	**KT124368**		Antarctica (Newmayer)
Cinachyra barbata	ANT 27205	**KT124321**	**KT124340**	_	**KT124366**		Antarctica (Newmayer)
Tetilla leptoderma	ANT 27105	**KT124322**	**KT124348**	_	**KT124359**	*Antarctotetilla leptoderma*	Antarctica (Newmayer)
Tetilla leptoderma	ANT 27106	_	**KT124349**	_	**KT124360**	*Antarctotetilla leptoderma*	Antarctica (Newmayer)
Tetilla grandis	MNHN-Poker II-Chalut 32 sp.4	**KT124326**	**KT124345**	_	_	*Antarctotetilla grandis*	Sub Antarctic (Kerguelen)
Cinachyra antarctica	MC 7485	**KT124316**	**KT124339**	_	_		Between Lavoisier and Antarctica
CInachyra antarctica	MC 7486	**KT124315**	_	_	_		Between Lavoisier and Antarctica
Tetilla sagitta	MNHN-IP 2009 359	**KT124327**	_	**KT124334**	_	*Antarctotetilla sagitta*	Antarctica (Adelie Land)
Cinachyra barbata	MNHN-IP 2009 506a	**KT124312**	**KT124350**	_	_		Antarctica (Adelie Land)
Tetilla sagitta	MNHN-IP 2009 351	_	**KT124352**	**KT124333**	**KT124369**	*Antarctotetilla sagitta*	Antarctica (Adelie Land)
Cinachyra barbata	MNHN-IP 2009 387	_	**KT124342**	_	_		Antarctica (Adelie Land)
Tetilla sagitta	MNHN-IP 2009 31	**KT124320**	_	**KT124332**	**KT124370**	*Antarctotetilla sagitta*	Antarctica (Adelie Land)
Cinachyra barbata	NIWA 28877	JX177864	JX177977	_	_	*Cinachyra* cf. *barbata*	Antarctica (Oates land)
Cinachyra antarctica	NIWA 28951	JX177868	_	_	_		Antarctica (Oates land)
Cinachyra antarctica	NIWA 28957	JX177867	_	_	_		Antarctica (Oates land)
Cinachyra antarctica	QMG 311149	JX177914	_	_	_	*Cinachyra* sp.	Antarctica, Ross island (Mcmurdo base)
Craniella sagitta	NIWA 25206	JX177917	JX177981	_	_	Tetillidae sp.2	New Zealand (Chatham rise)
Craniella sagitta	NIWA 28491	JX177915	_	_	_	Tetillidae sp.2	New Zealand (Chatham rise)
Craniella sagitta	NIWA 28929	JX177863	_	_	_	Tetillidae sp.1	Antarctica (Oates land)
Craniella cf. *leptoderma*	NIWA 28910	JX177865	JX177982	_	_	*Antarctotetilla* cf. *grandis*	Antarctica (Oates land)
Craniella cf. *leptoderma*	NIWA 36097	JX177866	_	_	_	*Antarctotetilla grandis*	Antarctica (Ross island)
Craniella cf. *leptoderma*	NIWA 52077	JX177916	_	_	_	*Antarctotetilla leptoderma*	New Zealand (Chatham rise)

(*Continued*)

Table 1. (*Continued*)

		Genbank accession numbers					
SPECIES	**Voucher number**	**COI M1-M6**	**18S**	**COI I3-M11**	**28S (D3-D5)**	**Revised species name**	**Collection sites**
Craniella cf. *leptoderma*	NIWA 28496	JX177897	_	_	_	*Antarctotetilla leptoderma*	New Zealand (Chatham rise)
Craniella cf. *leptoderma*	NIWA 28524	JX177895	JX177976	_	_	*Antarctotetilla leptoderma*	New Zealand (Chatham rise)
Craniella cf. *leptoderma*	NIWA 28507	JX177896	JX177975	_	_	*Antarctotetilla leptoderma*	New Zealand (Chatham rise)
Craniella sp.	QMG 316342	HM032747	JX177983	**KT124337**	**KT124371**	*Cinachyra* sp.	Australia (South Norfolk ridge)
Craniella zetlandica	PC 252	KC122679	_	_	_		Røst reef, Norway
Craniella zetlandica	VM 14754	_	JX177986	_	_		Iceland
Craniella neocaledoniae	NIWA 28591	_	JX177984	_	_		New Zealand
Craniella sp.	QMG 318785	HM032752	JX177985	_	_		Australia (South Norfolk ridge)
Craniella sp.	BIOICE 3659	HM032750	_	_	_		Iceland
Craniella sp.	QMG 316372	HM032748	HE591469	**KT124338**	**KT124372**	*Cinachyra* sp.	Australia (South Norfolk ridge)
Craniella sp.	ZMBN 85240	HM592668	_	_	_	*Craniella* cf. *cranium*	Norway
Craniella cranium	ZMBN 85239	HM592669	_	_	_	*Craniella* aff. *zetlandica*	Norway
Fangophilina sp.	NIWA 28601	JX177919	JX177979	_	_	cf. *Fangophillina*	New Zealand (Challenger Plateau)
Fangophilina sp.	NIWA 28586	JX177918	JX177978	_	_	cf. *Fangophillina*	New Zealand (Challenger Plateau)
Fangophilina sp.	NIWA 28617	JX177912	JX177980	_	_	cf. *Fangophillina*	New Zealand (Challenger Plateau)
Paratetilla sp.	QMG 314224	HM032744	_	_	_		Australia (Curacoa Island)
Cinachyrella schulzei	QMG 320143	HM032746	_	_	_	*Cinachyrella* cf. *tenuiviolacea*	Australia (Keppel Islands)
Cinachyrella schulzei	QMG 320636	HM032745	JX177971	_	_	*Cinachyrella* cf. *tenuiviolacea*	Australia (Melanie Patches)
Cinachyrella apion		AJ843895	_	_	_		Bermuda
Cinachyrella sp.	TAU 25622	_	JX177962	_	_		Tanzania
Cinachyrella sp.	TAU 25621	HM032740	JX177964	_	_		Tanzania
Cinachyrella sp.	QMG 320270	HM032741	JX177963	_	_		Australia (Wellington point, Moreton Bay)
Craniella cf. *leptoderma*	QMG 315031	HM032749	JX177974	_	_	*Antarctotetilla* cf. *sagitta*	Antartica (Casey Antartic Research Base)
Cinachyrella australiensis	QMG 321405	HM032743	_	_	_		Australia (Sunshine Coast)
Cinachyrella australiensis	QMG 320216	JX177902	JX177966	_	_		Australia (Keppel Islands)
Cinachyrella australiensis	QMG 320656	_	JX177968	_	_		Australia (Munro Reef, Coral Sea)
Cinachyrella australiensis	QMG 320656	_	JX177967	_	_		Australia
Cinachyrella apion	ZMBN 81789	HM592667	_	_	_		USA
Cinachyrella apion	SBP-B25	EF519601	_	_	_		Caribean Sea
Cinachyrella apion		FJ711645	_	_	_		Panama
Cinachyrella apion		_	AJ627186	_	_		Bermuda

(*Continued*)

Table 1. (*Continued*)

		Genbank accession numbers					
SPECIES	**Voucher number**	**COI M1-M6**	**18S**	**COI I3-M11**	**28S (D3-D5)**	**Revised species name**	**Collection sites**
Cinachyrella cf. *paterifera*	0M9H2022-P	_	KC902343	_	_		Australia
Cinachyrella sp.	USNM 1204826	_	KC901899	_	_		Panama
Cinachyrella sp.	USNM 1204829	_	KC902189	_	_		Panama (Bocas del Toro)
Cinachyrella alloclada	DH S271	JX177913	JX177965	_	_		Panama
Cinachyrella alloclada	USNM 1133831	_	KC902108	_	_		Panama
Cinachyrella alloclada	0M9G1250-W	_	KC902264	_	_		USA
Cinachyrella alloclada	TAU 25623	HM032738	_	_	_		Bahamas
Tetilla radiata	MNRJ 576	HM032742	_	_	_		Brazil (Rio De janeiro)
Tetilla murycii	UFBA 2586POR	JX177898	_	_	_		Brazil (Camamu Bay)
Cinachyrella levantinensis	TAU 25529	JX177906	JX177970	_	_		Lebanese Coast
Cinachyrella levantinensis	TAU 25568	JX177904	JX177969	_	_	*Levantinella levantinensis*	Lebanese Coasts
Cinachyrella levantinensis	MHNM 16194	JX177905	HM629803	_	_	*Levantinella levantinensis*	Lebanese Coast
Cinachyrella levantinensis	DH S124	JX177903	_	_	_	*Levantinella levantinensis*	Lebanese Coast
Cinachyrella levantinensis	TAU 25456	_	HM629802	_	_	*Levantinella levantinensis*	Lebanese Coast
Tetilla japonica		_	TTL18SR	_	_		Japan
Tetilla japonica	TAU 25619	JX177901	_	_	_		Japan
Cinachyrella sp.	SP.11	_	AY734439	_	_		Australia?
Cinachyrella sp.	SP.22	_	AY734437	_	_		Australia?
Cinachyrella sp.	SP.24	_	AY734438	_	_		Australia?
Cinachyrella kuekenthali	SBP-K75	EF519603	_	_	_		Caribean Sea
Cinachyrella kuekenthali	SBP-B79	EF519602	_	_	_		Caribean Sea
Cinachyrella kuekenthali		FJ711646	_	_	_		Panama
Cinachyrella kuekenthali		NC010198	_	_	_		USA
Cinachyrella kuekenthali		EU237479	_	_	_		USA
Paratetilla bacca	TAU 25620	JX177900	_	_	_		Thailand
Paratetilla bacca	LB 622	JX177894	_	_	_		Indonesia
Paratetilla bacca	LB 671	JX177893	JX177972	_	_		Indonesia
Paratetilla bacca	0M9H2290-H	_	KC902195	_	_		Australia
Amphitethya cf. *microsigma*	SAM S1189	JX177910	_	_	_	*Amphitethya microsigma*	South Australia?
Acanthotetilla celebensis	RMNH POR 2877	JX177893	_	_	_		Indonesia
Acanthotetilla walteri	UFBA 2021	JX177907	_	_	_		Brazil
Acanthotetilla seychellensis	0CDN 8107-V	_	KC902033	_	_		American Samoa
Cinachyrella kuekenthali	USNM 1133786	_	KC902290	_	_		Panama

(*Continued*)

Table 1. (*Continued*)

		Genbank accession numbers					
SPECIES	**Voucher number**	**COI M1-M6**	**18S**	**COI I3-M11**	**28S (D3-D5)**	**Revised species name**	**Collection sites**
Cinachyrella kuekenthali		_	EU702414	_	_		USA
Cinachyra sp.	0CDN 8726-T	_	KC902124	_	_		Guam
Craniella sp.	0CDN 5142-X	_	KC902265	_	_		Philippines
Geodia cydonium		_	AY348878	_	_		Mediterranean Sea
Geodia cydonium	ZMA POR 21652	HM592738	_	_	_		Portugal
Geodia neptuni		_	AY737635	_	_		Caribean Sea
Thenea levis	ZMBN 85230	HM592717	_	_	_		Norway
Theonella swinhoei	ZMA POR 16637	HM592745	_	_	_		Egypt

Reference numbers of individuals sequenced *de novo* in the current study are indicated in bold. Abbreviations: BIOICE, The inter-Nordic BIO-Iceland project; DH, LB, personal collections of Dorothée Huchon and Lisa Becking; MC, National Museums, Northern Ireland, Holywood; MHNM, Muséum d'Histoire Naturelle Palais Longchamp, Marseille, France;MNHM, Muséum National d'Histoire Naturelle, Paris, France; MNRJ–Museu Nacional do Rio de Janeiro, Brazil; NIWA, National Institute of Water & Atmospheric Research, New Zealand; PC, personal collection, University of Bergen, Norway; QMG, Queensland Museum, Australia; RMNH, Rijksmuseum van Natuurlijke Historie, Leiden, Nederland; SAM, South Australian Museum, Australia; SBP, Sponge Barcoding Project (http://www.palaeontologie.geo.uni-muenchen.de/SBP/); TAU, Steinhardt National Collection of Natural History, Zoological Museum at Tel Aviv University, Israel; UFBA, Universidade Federal da Bahia, Brazil; USNM, United States National Museum, U.S.A.; VM, Museum of Natural History and Archaeology, a part of the University of Science and Technology, Trondheim, Norway; ZMA, Zoölogisch Museum van de Universiteit van Amsterdam, Holland; ZMBN, Zoologisk Museum, Bergen, Norway; 0CDN, 0M9G, Smithsonian Institution/National Museum of Natural History, U.S.A.

doi:10.1371/journal.pone.0160718.t001

Additional specimens of particular interest to obtain a more comprehensible sampling for our taxonomic study and to verify previous identifications were obtained on loan from several institutions: paratype of *Fangophilina submersa* Schmidt, 1880 (MSZ.PO160, Musée Zoologique de Strasbourg, France); paratype of *Craniella quirimure* Peixinho, Cosme, Hajdu, 2005 (MNRJ 8417, Museu nacional/UFRG, Brazil); paratype of *Tetilla radiata* Selenka, 1879 (MNRJ 576, Museu nacional/UFRG, Brazil); *Tetilla muricyi* Fernandez, Peixinho, Pinheiro, Menegola, 2011 (UFBA 2569, Museu de História Natural da Bahia, Brazil); *Craniella* sp. (QMG 316342, and QMG 316372, Queensland Museum, Brisbane, Australia); nine individuals of *Cinachyrella levantinensis* Vacelet, Bitar, Carteron, Zibrowius, Pérez, 2007 from Lebanon (06/07/2003-1 and 31/07/2003-2, Station Marine d'Endoume, Marseille) and seven specimens collected across the shore of Ma'agan Michael, Israel (courtesy of Jean Vacelet); *Craniella sagitta* Lendenfeld, 1907 (syn. *Tethya sagitta*) (NIWA 28491 and NIWA 28929 National Institute of Water & Atmospheric Research, New Zealand); a small piece of the syntype of *Tethya sagitta* Lendenfeld, 1907 (ZMB Por 3504, Museum für Naturkunde Leibniz, Germany); *Fangophilina* sp. (NIWA 28601 National Institute of Water & Atmospheric Research, New Zealand). Moreover, several specimens from Szitenberg et al. [34] were re-examined from photographs or specimens (see Results).

Selected outgroups for the phylogenetic analyses, which mainly aimed at establishing relationships among genera, belonged to the Astrophorina (families Geodiidae and Theneidae) since previous molecular phylogenies of Demospongiae based on mitochondrial [44], [38] and nuclear [45], [46] genes placed Astrophorina either as a sister clade of the Tetillidae (COI), or sister to some Tetillidae (18S, 28S).

DNA extraction, amplification and sequencing

Genomic DNA was extracted according to the manufacturer's protocol for the DNeasy Blood & Tissue kit (Qiagen). Two mitochondrial markers were sequenced, both from COI: the

M1-M6 partition, using primers LCO1490 and HCO2198 [47] and the I3-M11 partition, using primers PorCOI2 fwd. and PorCOI2 rev. [48]. Two nuclear markers were also sequenced: 18S, using primers 1F and 1795R, [49] and the D3-D5 partition of 28S, using primers Por28S-830F and Por28S-1520R [50]. Different amplification protocols were performed for each marker: 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); COI I3-M11 partition (95°C, 3 min, [94°C, 30 s, 57°C, 45 s, 72°C, 90 s] x 35–40 cycles, 72°C, 10 min); 18S (94°C, 5 min, [94°C, 1 min, 50–55°C, 1 min, 72°C, 1 min] x35-40 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. Amplifications of the COI I3-M11 partition were performed in a 50 μL volume reaction, containing 34,45 μL H_20, 5 μL buffer (INVITROGEN), 0,75 μL MgCl (INVITROGEN), 2,4 μL DMSO (dimethyl sulfoxide), 2 μL BSA, 2 dNTP (Sigma), 1 μL primers forward/reverse, 0,4 Taq (INVITROGEN) and 1 μL of genomic DNA. Amplifications of 18S rRNA 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. Purified PCR products were sequenced in both directions using Applied Biosystems 3730xl DNA analyzers (Macrogen, South Korea).

Sequence alignment and phylogenetic reconstructions

Once the poriferan origin of the obtained sequences was verified using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), sequences were aligned using Clustal W v.1.81 [51]. In cases where the forward and reverse reads do not match, we used BioEdit v.7.2.5 [52] and kept either the best quality of the two reads or introduced an IUPAC ambiguity code into the consensus sequence.

JModelTest 0.1.1 [53] was used to determine the best-fitting evolutionary model for each dataset. The model GTR+I+G was used for the mitochondrial and nuclear genes under the Akaike information criterium. Phylogenetic trees were constructed under Bayesian Inference (BI) and Maximum Likelihood (ML) criteria. BI analyses were performed with MrBayes 3.2.1 [54]. 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 PhyMLv3.0 program [55], [56]. We assessed the robustness of the tree clades in PhyML by a nonparametric bootstrap resampling with 1000 replicates.

Incongruence Length Difference (ILD) test (PAUP 4.0b10) was run [57] to verify sequence homogeneity or incongruence between the 18S and COI markers. The ILD test indicated no significant conflict ($p = 0.93$) between the two markers so a concatenated 18S-COI dataset was constructed with the species for which we had sequences for both markers.

18S rRNA secondary structure and morphological analysis

RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) [58], [59] was used to determine the predicted secondary structure of the 18S V4 variable region for all species

following Voigt et al. [60]. We used the default setting for all parameters except for the folding temperature. As the specimens came from different localities, we fixed the folding temperatures according to that of the specimen locality. However, the specimens belonging to *Geodia* spp., which were used as outgroups, lived in locations with contrasting temperatures. Only in this case, we used the default setting of 37°C [61]. The program automatically chooses the secondary structures with the lowest free energy (dG in kcal/mol) [60]. Following Gazave et al. [62], we encoded the different parts of the predicted secondary structures as elements and treated them as binary characters (presence/absence) in the morphological matrix. As the V4 of 18S secondary structure motifs were conserved across genera, according to the species sequenced, we assumed that the few species for which the 18S sequence was not available, shared the secondary structure motifs of the genus. The morphological/secondary structure matrix, consisting of 26 morphological characters and 13 18S secondary structure motifs, is made available in S1 File. A phylogenetic-tree was built with the morphological matrix under the maximum parsimony (MP) criterion using PAUP 4.0b10 [57] using a heuristic search and the branch swapping method with the tree bisection and reconnection (TBR) algorithm and ACCTRAN character-state optimization.

Results

Mitochondrial COI

The COI (M1-M6 partition) dataset comprised 70 sequences (16 new) of 537 nucleotides (nt.) (158 nt. variable, of which 148 nt. were parsimony informative). Phylogenetic trees from ML and BI analyses retrieved congruent topologies, although some clades were differently supported (Fig 1). The Antarctic/New Zealand Tetillidae clustered in a well-supported group (93/1), which split in three well-defined clades henceforth called clades 1, 2 and 3.

Clade 1 (80/0.95, ML bootstrap supporting values /BI posterior probability) contained all the species of *Cinachyra* sequenced plus an unidentified species named Tetillidae ANT 27211.

Clade 2 (88/0.95) clustered individuals of *Tetilla grandis*, *T. sagitta*, and *T. leptoderma*, including those individuals called *Craniella leptoderma* in previous phylogenies. The position of three *Craniella sagitta* sequences (NIWA 25206, 28491, and 28929) was unresolved. These specimens were placed clearly apart from our *Tetilla sagitta* specimens from Antarctica, which morphologically conformed to the type species. Those three sequences belonged to two haplotypes with a difference of 8 nt.: NIWA 28491 and NIWA 25206 specimens from New Zealand *versus* NIWA 28929 from Antarctica.

Clade 3 (-/0.91) included the sequences of *Fangophilina* sp. (NIWA 28601, NIWA 28586, and NIWA 28617) and *Craniella* sp. (QMG 316342 and 316372), and was retrieved only in BI analyses.

Clade 4 (81/1) was a sister group of Antarctic sponges and included non-Antarctic/New Zealand species of *Craniella*.

Clade 5 (100/1) contained the non-Antarctic *Tetilla* (i.e. from tropical seas).

Clade 6 (98/1) included sequences of *Cinachyrella levantinensis* from the eastern Mediterranean, and was clearly apart from the rest of the *Cinachyrella* species.

Clade 7 (84/0.99) consisted of *Cinachyrella* species from tropical and subtropical waters and was divided in two well-supported sub-clades (posterior probability 0.80 and 0.84): the first included *C. australiensis*, *C. kuekenthali*, *Amphitethya* cf. *microsigma*, and *C. apion*, while the second included *C. alloclada*, *Cinachyrella* sp., *C. schulzei*, and *Paratetilla bacca*. The latter two species clustered together (100/1).

Clade 8 (100/1), included two species of *Acanthotetilla*, and appeared as the sister group of the remaining Tetillidae.

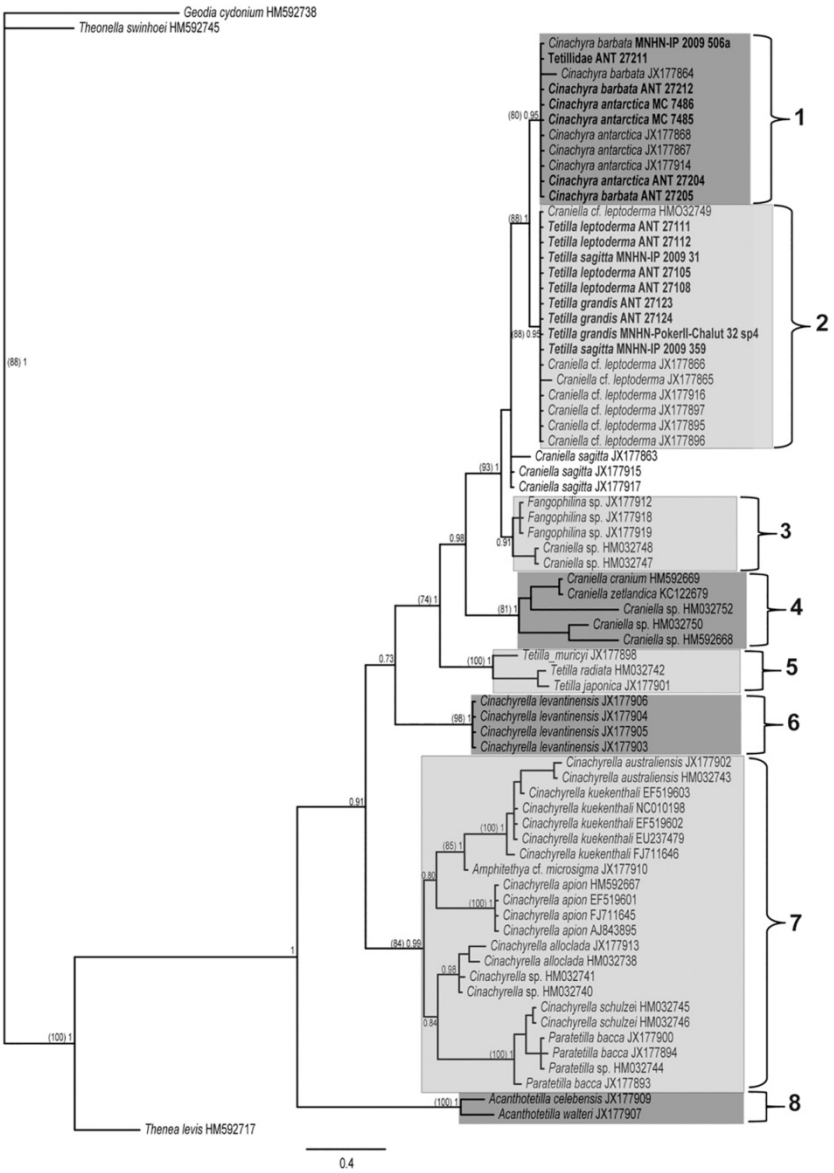

Fig 1. COI M1-M6, BI phylogeny of Tetillidae, which was congruent with ML tree. Species names are followed by their accession numbers (sequences downloaded from Genbank) or the specimen reference. Individuals sequenced in this study are in bold. Only supporting values higher than 70% (ML bootstrap, between parentheses on the left) or 0.75 (BI posterior probability) are represented on the tree nodes.

doi:10.1371/journal.pone.0160718.g001

Almost no intra-species variation was found for the M1-M6 partition for the Antarctic genera, with the notable exception of two individuals: *Cinachyra barbata* (JX177864), which differed in 3 nt. from the other *Cinachyra* sequences, and *Craniella* cf. *leptoderma* (JX177865), which differed in 2 nt. from the other *Tetilla*/*Craniella* sequences (Fig 1).

We obtained 11 new sequences of the COI I3-M11 partition, 614 nt. long (11 nt. variable and parsimony informative). This partition (Fig 2), although it has been considered more variable than the M1-M6 partition [63], failed to reveal any difference among the Antarctic species of *Cinachyra* or *Tetilla*/*Craniella*.

Nuclear 18S rRNA and 28rDNA

The full-length 18S dataset comprised 48 sequences (19 new) of 1483 nt. (83 nt. variable, of which 60 nt. were parsimony informative). ML and BI analyses gave congruent topologies (Fig 3). These

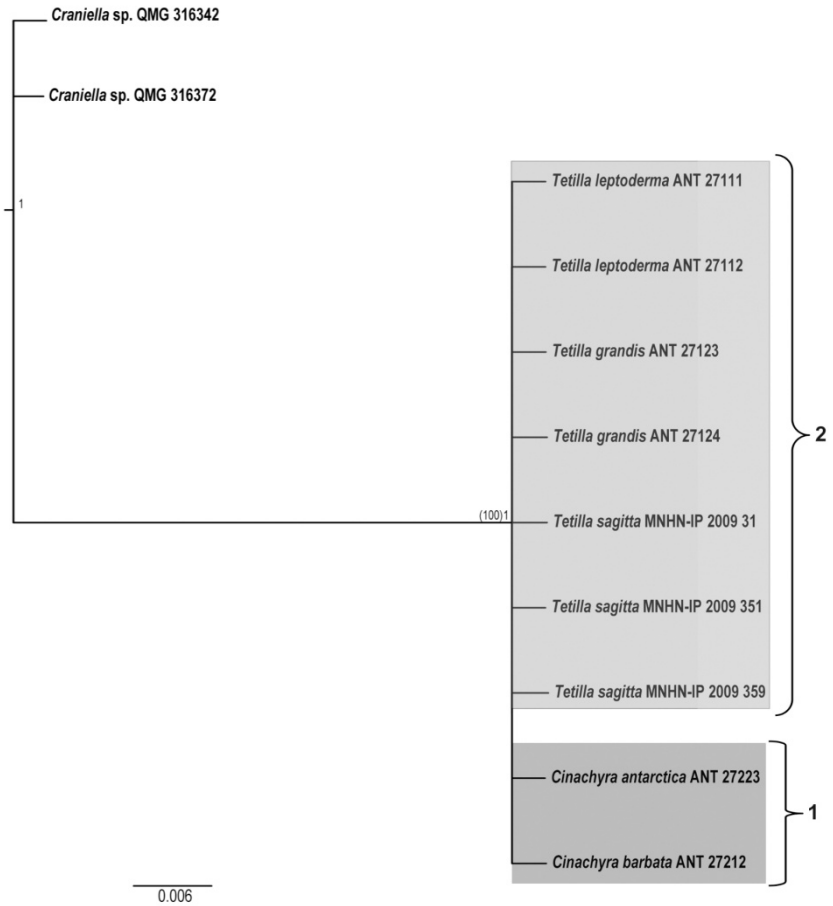

Fig 2. COI I3-M11 BI phylogeny of the Antarctic individuals of Tetillidae, which was congruent with ML tree, showing no clear separation between *Cinachyra* and *Antarctotetilla*. Bootstrapping and posterior probability (ML and BI, respectively) values are represented on the node of the only resulting clade. Individuals sequenced in the current study are indicated in bold.

doi:10.1371/journal.pone.0160718.g002

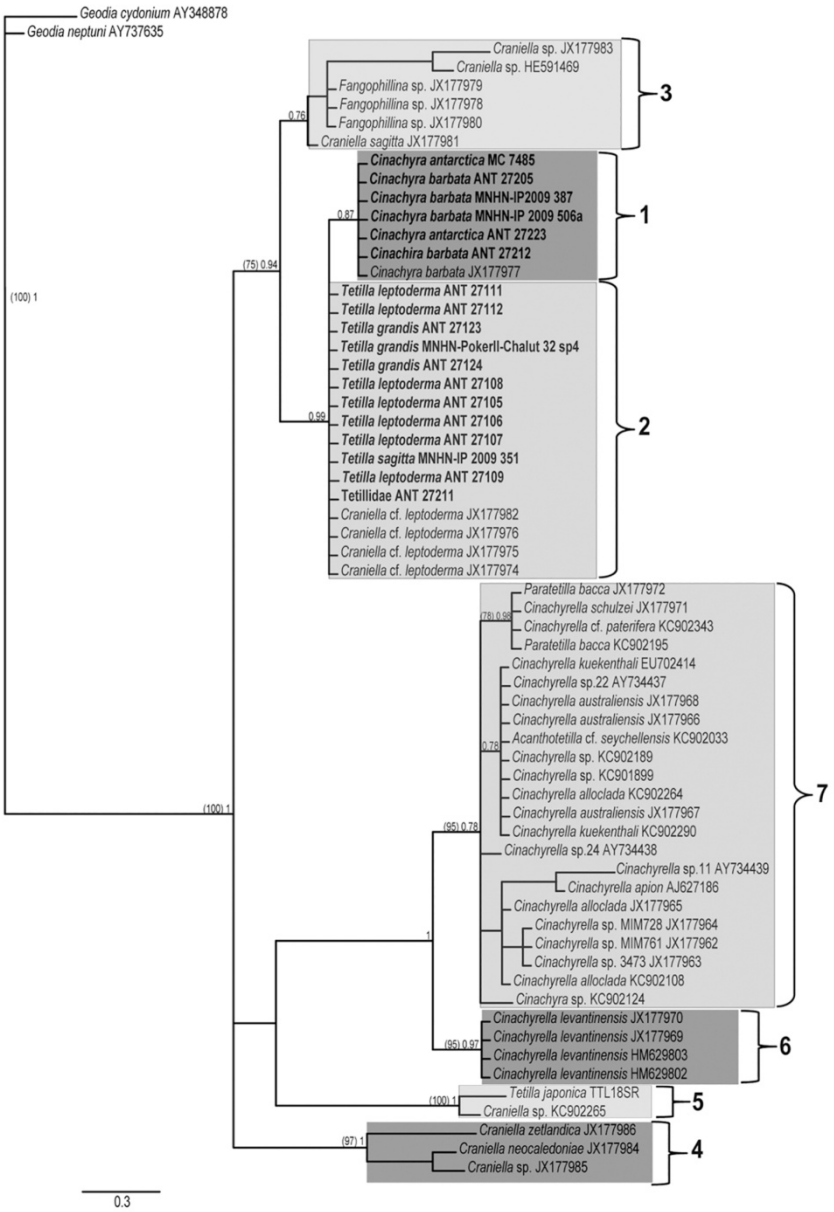

Fig 3. 18S rRNA BI phylogeny of Tetillidae, which was congruent with ML tree. Species names are followed by the accession numbers (sequences downloaded from Genbank) or the specimen reference. Individuals sequenced in this study are in bold). Only supporting values higher than 70% (ML bootstrap, between parentheses on the left) or 0.75 (BI posterior probability) are represented on the tree nodes.

doi:10.1371/journal.pone.0160718.g003

trees recovered the same clades as the COI tree, except for the absence of clade 8, since no sequences of *Acanthotetilla* were available for this marker, and clade 2. Like in the COI analyses, 18S failed to discriminate among species of the Antarctic *Tetilla* (including species named *Craniella* in previous phylogenies) or *Cinachyra*. As in the COI phylogeny, the Antarctic/New Zealand Tetillidae clustered in a clade (75/0.94) comprising *Cinachyra* spp., *Tetilla* spp, *Craniella sagitta* (NIWA 25206), *Fangophilina* spp., and *Craniella* sp. (QMG 316342 and 316372) representatives.

Clade 1 (-/0.99) contained the species that split in clades 1 and 2 in COI phylogeny. The specimen Tetillidae ANT27211 groups this time with *Tetilla* spp. and not with *Cinachyra* spp., as in the COI phylogeny.

Clade 3 (-/0.76) comprises *Fangophilina* sp. plus *Craniella* sp. (QMG 316342 and 316372) and *Craniella sagitta* (NIWA 25206) as in the COI phylogeny but without statistical support.

Clade 4 (97/1) included the same *Craniella* species as in the COI phylogeny plus *C. neocaledoniae*, a species absent from the COI sampling.

Clade 5 (100/1) encompassed non-antarctic *Tetilla* (i.e. from tropical seas).

Clade 6 (95/0.97) was formed exclusively by *C. levantinensis* sequences. This clade was sister to clade 7 whereas it was sister to clade 1–5 in the COI tree.

Clade 7 (95/0.78), as in the COI tree, clustered all *Cinachyrella* species plus *Paratetilla bacca*.

The 28S rRNA gene (D3-D5 partition) comprised 15 new sequences of 650 nt. (11 nt. variables, of which 10 nt. were parsimony informative). Phylogenetic trees were consistent in ML and BI analyses (Fig 4). Species within any of these two genera (*Cinachyra* and *Tetilla*/*Craniella*) were not discriminated.

Concatenated COI and 18S rRNA

The dataset for the concatenated mitochondrial and nuclear partitions (COI M1-M6 partition and 18S) comprised 39 sequences of 2019 nt. The resulting phylogenetic trees were consistent in ML and BI analyses (Fig 5) and were for the most part similar to the COI tree (i.e. clades 1, 3, 4, 5, and 7), except for clade 6, which was shared only with the 18S tree (Fig 3). On the other hand, contrarily to 18S phylogeny, clade 2 was well supported (87/0.92). Clade 3 was similar to both COI and 18S phylogenies. The supporting values of the clades slightly varied in some cases with respect to those of the previous phylogenies.

Morphological identifications and re-examination of specimens

To understand our phylogenetic results, which were fairly congruent with both nuclear and mitochondrial genes, we examined the morphology of our specimens, several holotypes and also some specimens previously sequenced by Szitenberg et al. [34]. The resulting decisions from our examinations are detailed below and summarized in Table 1 and S2 File.

Clade 1 comprised individuals that belonged to the *Cinachyra* genus. Most of these individuals were morphologically similar except for the specimen of *C. barbata* (NIWA 28877), and that of *Cinachyra antarctica* (QMG 311149). The former differed from the other *C. barbata* in 3 nt, and pictures of the specimen (courtesy of M. Kelly) show porocalices spread on the sponge body instead of being concentrated on the lateral zone: we decided to name it *Cinachyra* cf. *barbata* until the specimen could be studied. Underwater pictures (courtesy of J. Hooper) of *Cinachyra antarctica* (QMG 311149) show a hairy surface covered with sediments with high palisades of spicules just around the openings: we tentatively renamed it as *Cinachyra* sp.

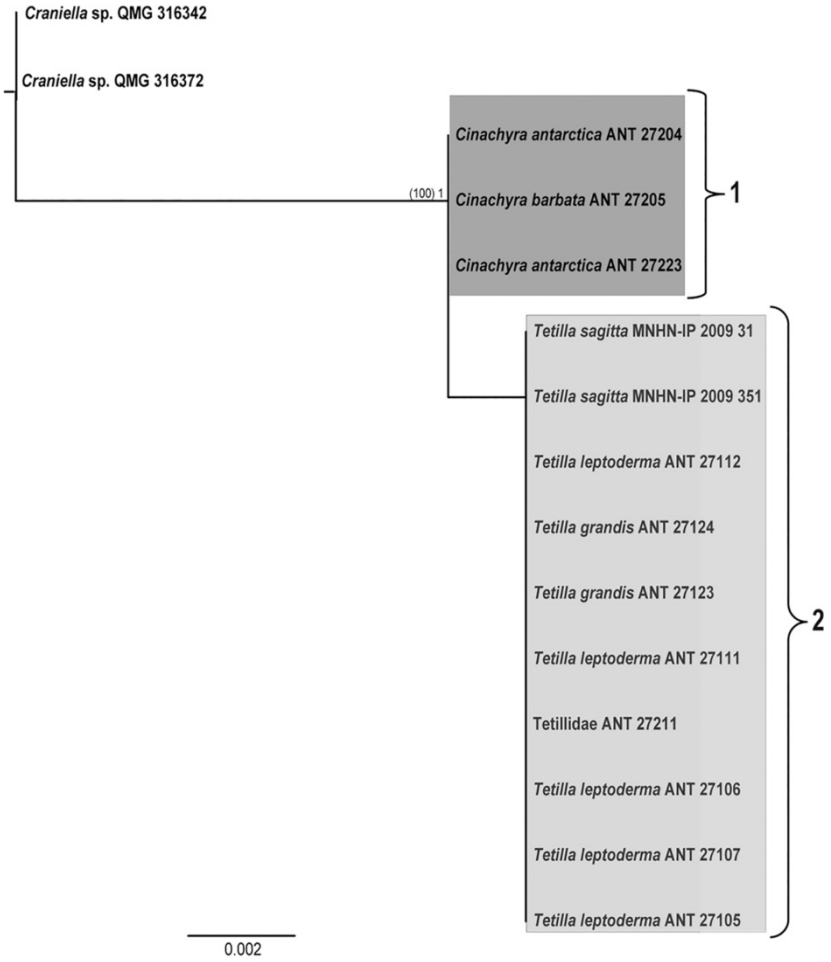

Fig 4. 28S (D3-D5) BI and ML phylogeny of the Antarctic individuals of Tetillidae, which was congruent with ML tree, showing no species differences within the genera *Cinachyra* and *Antarctotetilla*. Species names are followed by the accession numbers (sequences downloaded from Genbank) or the specimen reference. Individuals sequenced in this study are in bold. Only supporting values higher than 70% (ML bootstrap, between parentheses on the left) or 0.75 (BI posterior probability) are represented on the tree nodes.

doi:10.1371/journal.pone.0160718.g004

Clade 2 included *Tetilla*/*Craniella* specimens from Antarctica/New Zealand. These specimens belonged to three species (*leptoderma*, *sagitta* and *grandis*) that had been formally placed either in the genus *Tetilla* or the genus *Craniella* by previous authors. However, these species did not have the characteristic conspicuous double-layered cortex of *Craniella* [36]. Instead, they all had a loose arrangement of cortical oxeas perpendicular to the surface, which we will henceforth call 'pseudocortex' (Figs 6F and 6H and 7B and 7D). Therefore these species cannot belong to *Tetilla* either, which lacks a cortical specialization. Moreover, all these species have

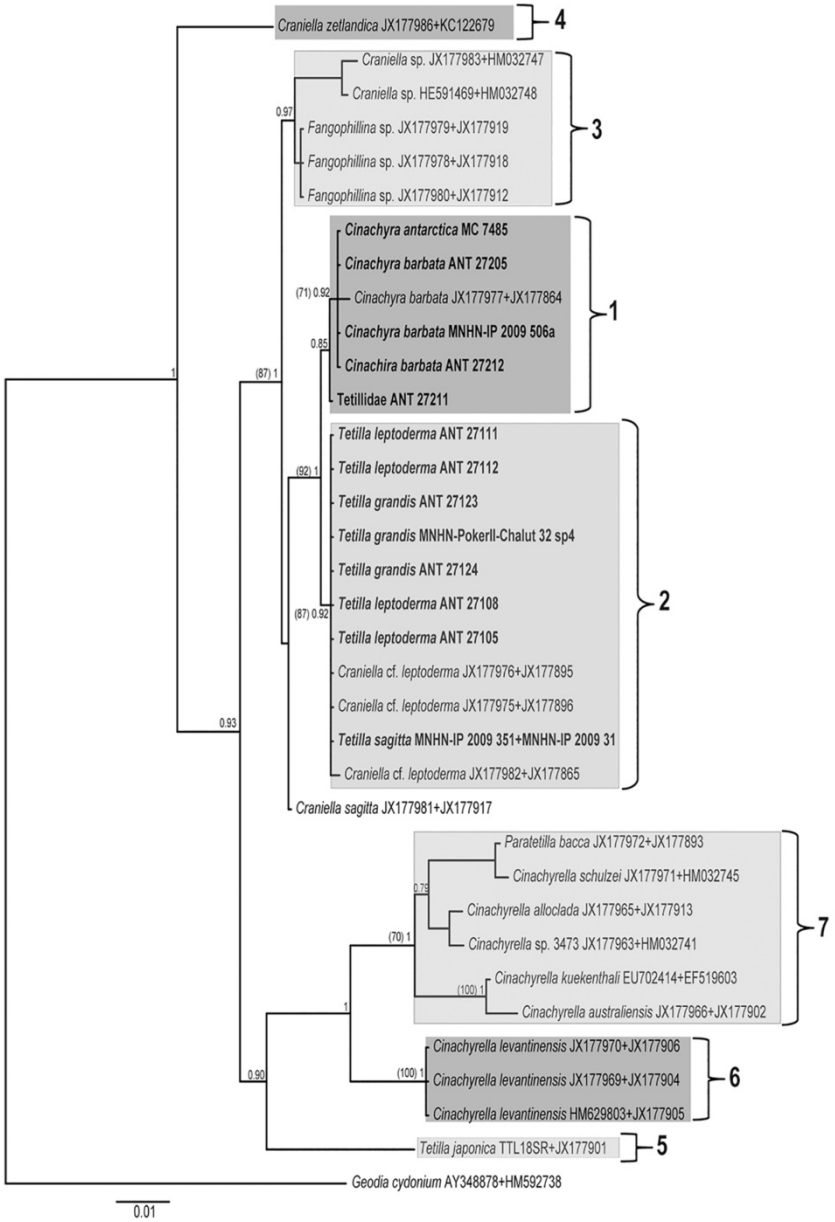

Fig 5. 18S rRNA–COI M1-M6 concatenate BI phylogeny of Tetillidae, which was congruent with ML tree. Species names are followed by the accession numbers (sequences downloaded from Genebank) or the specimen reference. Individuals sequenced in this study are in bold. Only supporting values higher than 70% (ML bootstrap, between parentheses on the left) or 0.75 (BI posterior probability) are represented on the tree nodes.

doi:10.1371/journal.pone.0160718.g005

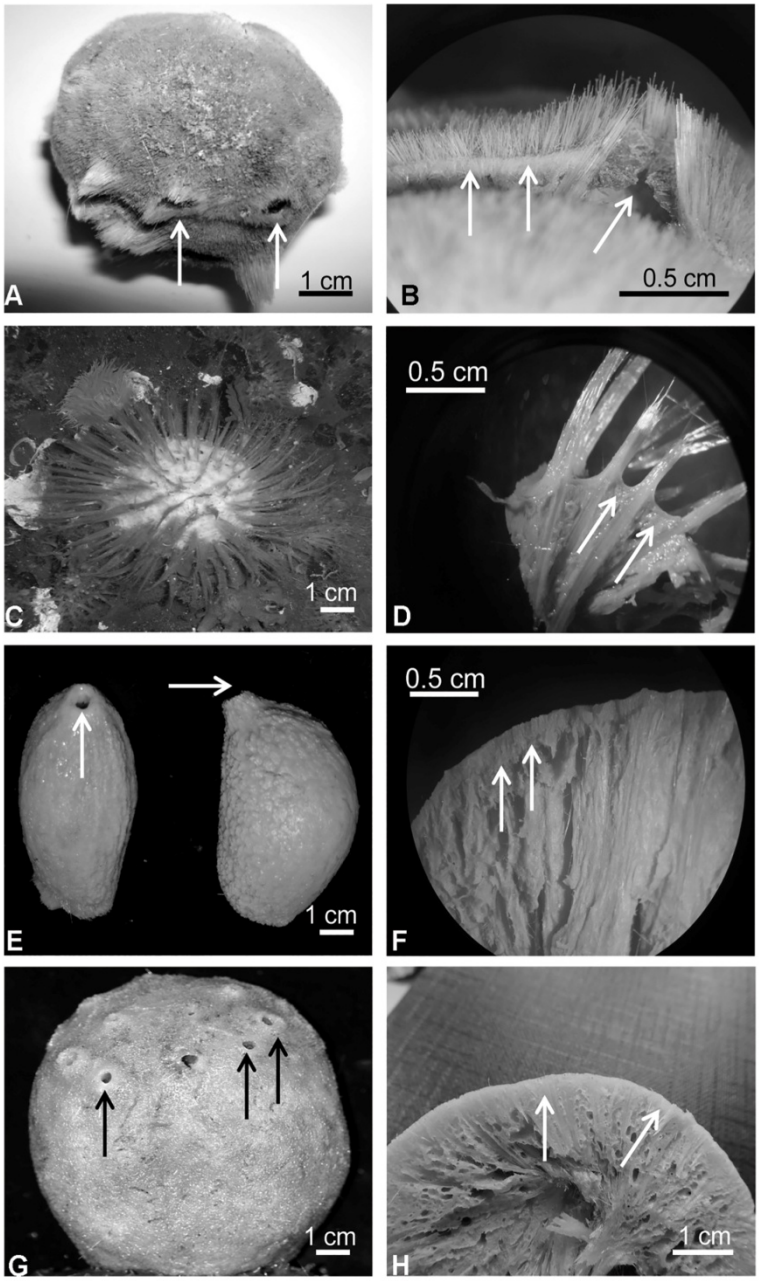
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Fig 6. Pictures of the species of Tetillidae studied. A) *Cinachyra barbata* from Newmayer (Antarctica) arrows point to the porocalices. B) Transversal section of *C. barbata*: arrows point to the cortex and one porocalyx. C) Underwater picture of *Cinachyra antarctica* from McMurdo, (Antarctica). D) Transversal section of *C. antarctica*: arrows point to the collagenous cortex. E) *Antarctotetilla leptoderma* from South Georgia: arrows point to the unique osculum on top. F) Transversal section of *A. leptoderma*: arrows point to the dense ectosomal layer (pseudocortex). G) *Antarctotetilla grandis* from Newmayer, Antarctica: arrows point to the multiple oscula. H) Transversal section of *A. grandis*: arrows point to the slightly marked ectosomal layer. All the pictures are by the authors.

doi:10.1371/journal.pone.0160718.g006

pores clustered in small, depressed areas and their oscula, single or multiple, are usually larger than in typical *Craniella*. Finally, they never harbored direct developing embryos as observed in both typical *Craniella* and some *Tetilla*. All these characteristics prompted us to erect a new genus for these three species: *Antarctotetilla* gen. nov. (see definition below). We will henceforth call these species *Antarctotetilla leptoderma*, *Antarctotetilla sagitta* and *Antarctotetilla grandis*. The latter had been synonymized with *A. leptoderma* [14], [22] but it is clearly different from *A. leptoderma* as it has several small oscula and a spherical body while *A. leptoderma* is slightly elongate/ovoid body with a sole large oscule on top. Therefore we propose to officially resurrect this species so far only recorded from the Antarctic and Sub-Antarctic.

Five out of the eight specimens of *Craniella* cf. *leptoderma* from Szitenberg et al. [34] were confirmed morphologically to be *A. leptoderma*. Pictures of NIWA 28910 and NIWA 36097 showed that these specimens had a smooth surface and multiple small oscula, which matched the morphology of *Antarctotetilla grandis*. Finally, underwater pictures of QMG 315031 showed that the specimen had at least two oscula, which differs from the large single oscule on top, constantly present in *A. leptoderma*. QMG 315031 was therefore tentatively re-identified as *Antarctotetilla* cf. *sagitta*.

The three specimens of *Craniella sagitta* (NIWA 28491, NIWA 25206, and NIWA 28929 (Table 1) were examined (see pictures of NIWA 28491 in Fig 7F) and compared with the syntype of *Tethya sagitta* (ZMB Por 3504). These three specimens possessed a pseudocortex but lacked the main diagnostic characters of the species, such as pores grouped in sieve-like areas and oscula on the top of smooth flattened zones (Fig 7C and 7E) [16]. Thus, they cannot be identified with *A. sagitta* but we are unsure to which species or even genera they should belong. We could distinguish two morphotypes that corresponded to two haplotypes (differing in 8 nt.), which suggests that they represent two different species, here named Tetillidae sp. 1 and 2. Interestingly, these specimens were originally identified as two different species based on external morphology (S2 File).

Clade 3 included two misidentified genera: *Craniella* sp. (QMG 316342 and QMG 316372) and *Fangophilina* sp. (NIWA 28601, NIWA 28586, and NIWA 28617). The individuals called *Craniella* sp. from the Norfolk Ridge (Fig 8A), had porocalices and a spiculous cortex, similar to that of *Cinachyra*. They are therefore tentatively identified as *Cinachyra* sp. As for *Fangophilina* sp., its morphology was different from the species type of *Fangophilina*, *F. submersa* (Fig 7H). However, the small size of these specimens (ca. 1cm in diameter) prevented us to verify whether the only visible orifice was a true porocalyx or an oscule and thus they have been provisionally named cf. *Fangophilina* sp.

Clade 4 included *Craniella* species from the Boreo-Arctic Atlantic and the Norfolk ridge. We confirm that they possessed the typical double-layered cortex of *Craniella* (Fig 9D), and were thus considered true *Craniella*. *Craniella cranium* Müller, 1776 (ZMBN 85239) and *Craniella* sp. (ZMBN 85240) from Korsfjord-Norway [64] were reexamined. ZMBN 85239 was re-identified as *Craniella* aff. *zetlandica* since it only differed from *C. zetlandica* Carter, 1872 in the presence of sigmaspires. ZMBN 85240 was re-identified as *Craniella* cf. *cranium* because it closely resembled *Craniella pilosa* Montagu, 1818, a synonym of *C. cranium* (Fig 8B and 8C).

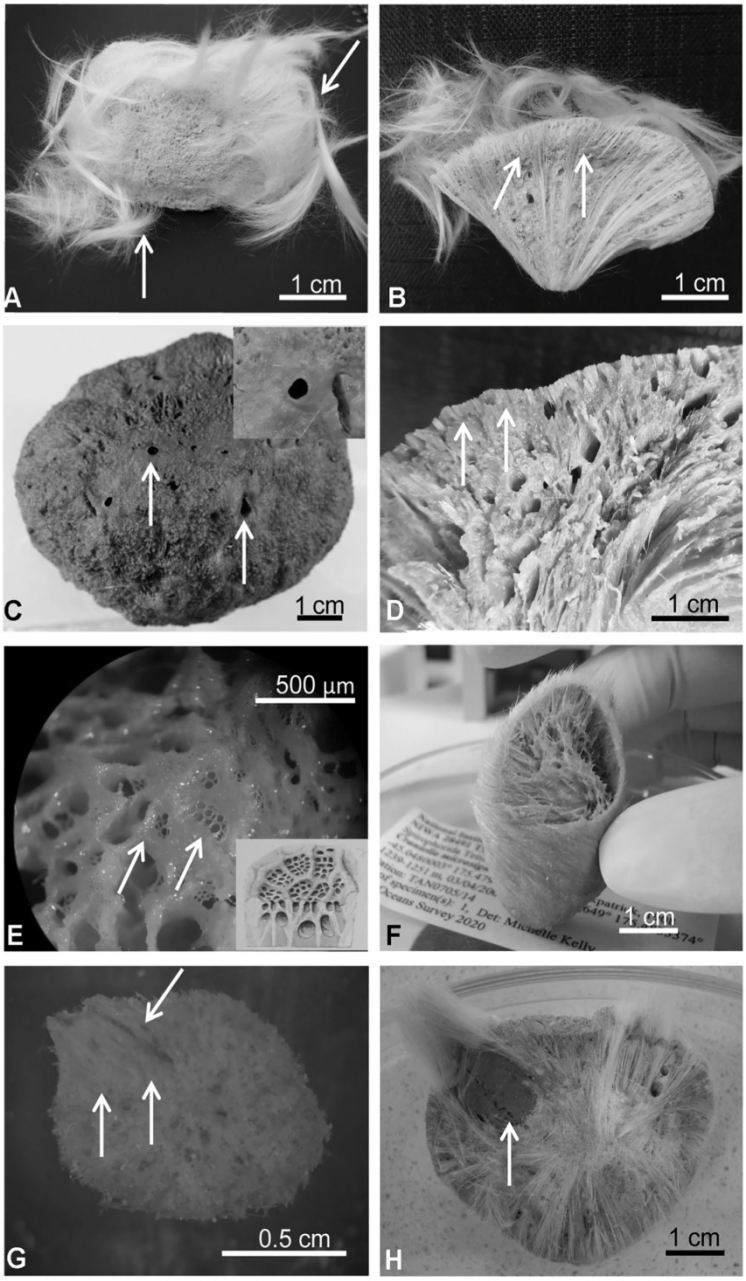
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Fig 7. Pictures of the species of Tetillidae studied. A) Tetillidae ANT 27211 from Newmayer, Antarctica: arrows point to the hair-like hispidation pattern formed by bundles of fusiform oxeas, protriaenes and sometimes anatriaenes. B) Transversal section of Tetillidae ANT 27211: arrows point to the ectosomal layer. C) *Antarctotetilla sagitta* from Adélie Land: arrows point to the oscula; inset: detail of the even surface around the oscula. D) Transversal section of *A. sagitta*: arrows point to the ectosomal layer. E) Surface of *A. sagitta*: arrows point to the pores clustered in sieve-like areas; inset: *T. sagitta* pores in sieves by Kirkpatrick (1908). F) *Craniella sagitta* NIWA 28491 from New Zealand. G) cf. *Fangophilina* sp. NIWA 28601 from New Zealand: arrows point to the osculum. H) Lectotype of *Fangophilina submersa* MZSPO 160 from Northern Gulf of Mexico: arrow points to the porocalyx. All the pictures are by the authors except figures G, which were courtesy of Sadie Mills.

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However, we keep the 'cf.' for now, until a world-wide revision of *C. cranium* can be made, this species having a long and complicated taxonomic history.

Clade 5 contained *Tetilla* species, which are characterized by the absence of a true cortical structure. Re-examination of a picture of the type species, *T. euplocamus* (Fig 8D), and the types of *T. muricyi* Fernandez, Peixinho, Pinheiro, and Menegola, 2011 (Fig 8E and 8F) and *T. radiata* Selenka, 1879, under the stereomicroscope seems to confirm previous studies of *Tetilla radiata* Santos and Hajdu, 2003 (Figs 5 and 6), and *T. muricy* Fernandez et al. 2011 (Fig 4C and 4D), which showed the absence of a cortical specialization in these *Tetilla*. However, a loose layer of para-tangential large oxeas below the surface may be present in some cases, as it has been reported for *T. rodriguezi* Fernandez et al. 2011 (Fig 6C).

Clade 6 only contained *Cinachyrella levantinesis*. We re-examined nine specimens of *C. levantinensis* trying to understand why they did not group with any of the other *Cinachyrella* species in the molecular trees. *C. levantinensis* lacks cortex as *Cinachyrella* (Figs 8H and 9E). Its surface is mostly covered with a dense layer of sand, which was retained by the protruding spicules (Fig 8G) as in some *Cinachyrella* (Fig 9F). However, while *Cinachyrella* species have typical large porocalices, *C. levantinensis* has numerous small rounded depressions, difficult to assign to porocalices with certainty. As stated by Vacelet et al. [65], these depressions were not visible in specimens heavily covered by sand. These depressions are sometimes concentrated in sand free lateral areas, whereas usually porocalices are evenly distributed in typical *Cinachyrella*. All these characteristics prompted us to officially create *Levantinella* gen. nov. (see definition below) to welcome this species.

Clade 7 contained only specimens of *Cinachyrella*. Some individuals of dubious identification were revised (S2 File). *Amphitethya* cf. *microsigma* (SAM-S1189) from Szitenberg et al. [34] showed an external morphology and spicules corresponding to the original description of *A. microsigma* Lendenfeld, 1907. Furthermore the individual SAM-S1189 was collected in southern Australia, not far from the type locality of *A. microsigma*. We therefore confirmed the species identification. Using the online SpongeMaps (http://www.spongemaps.org), we also examined underwater pictures of *Cinachyrella schulzei* Keller, 1891 (QMG 320143, QMG 320636) from Szitenberg et al. [41, 34]. These specimens are reddish pink (QMG 320636) to pale pink (QMG 320143), and completely devoid of sand, in contrast to the typical yellowish-grey color of *C. schulzei*, which is often covered with sand [66]. These specimens may instead be conspecific with *Cinachyrella tenuiviolacea* Pulitzer-Finali, 1982, which has a typical pink color and is fairly common in Australian shallow waters and were here provisionally referred to as *C.* cf. *tenuiviolacea*.

Maximum parsimony phylogeny on phenotypic characters

The MP analysis included 33 OTUs, one for each species, 26 morphological characters, and 13 motifs of the V4 18S secondary structure (S3 File), which were treated as binary elements (S1 File). The astrophorin *Geodia cydonium* and *Geodia neptuni* were used as outgroups. The MP

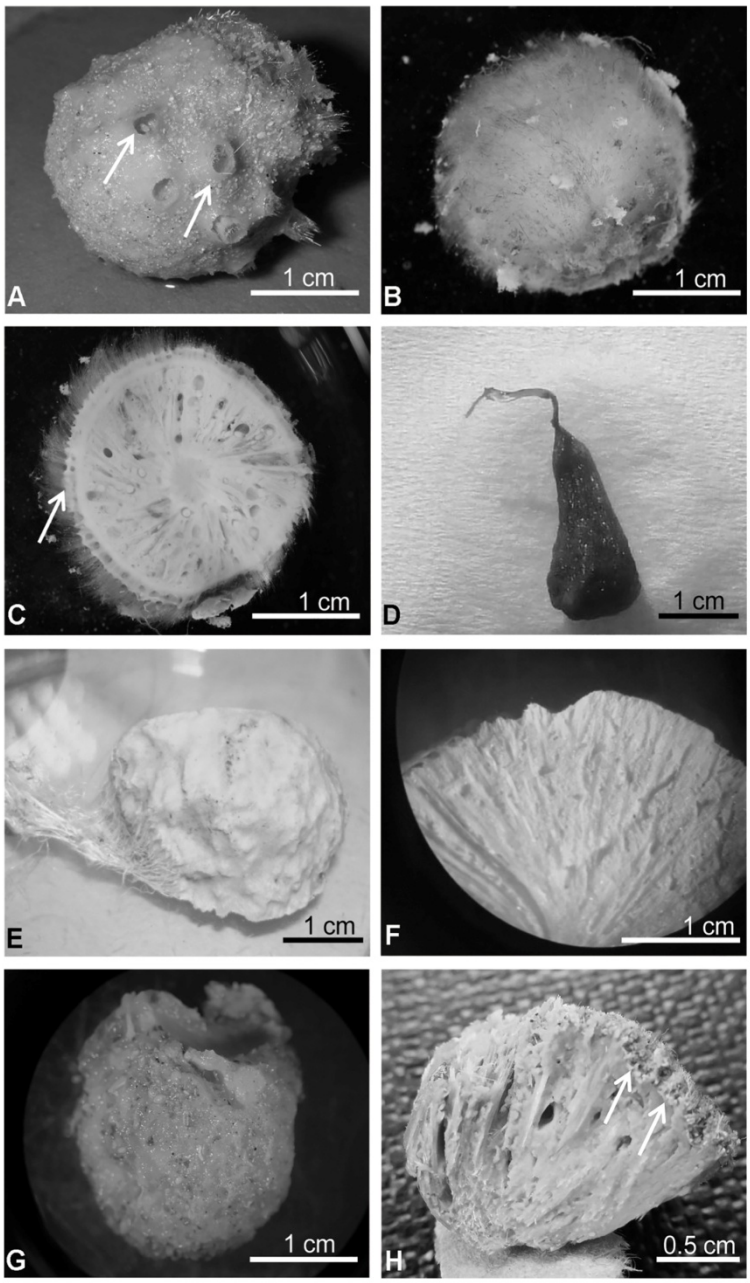
A
1 cm
B
1 cm
C
1 cm
D
1 cm
E
1 cm
F
1 cm
G
1 cm
H
0.5 cm

Fig 8. Pictures of the species of Tetillidae studied. A) *Craniella* sp. QMG 316342 from Australia: arrows point to the porocalices. B) *Craniella* cf. *cranium* ZMBN 85240 from Norway. C) Transversal section of *Craniella* cf. *cranium* ZMBN 85240: arrow points to the double-layered cortex. D) Holotype of *Tetilla euplocamos* MZSPO 206 from Brazil. E) Paratype of *Tetilla muricyi* UFBA 2569 from Brazil. F) Transversal section of the paratype of *T. muricyi* UFBA 2569. G) *Levantinella levantinensis* from Lebanon. H) Transversal section of *L. levantinensis*: arrows point to the dense ectosomal region formed by sediment accumulation. All the pictures are by the authors except figures A, and D, which were courtesy of John Hooper, and Marie Meister, respectively.

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analysis retrieved 6 most parsimonious trees of 48 steps (CI = 0.684; RI = 0.887). Character states are shown at the nodes of the phylogram corresponding to tree number 1 (Fig 10). Clade support resulting from the majority-rule consensus tree is also shown on the phylogram (Fig 10).

The Tetillidae appeared divided in two strongly supported clades (100% bootstrap value), here called clade A and B. Clade A was characterized by pores grouped in several ways and was formed by two well supported sub-clades: A1 embraced two groups with the same V4 secondary structure, A1a formed by *Acanthotetilla*, with acanthoxeas, small porocalices, and a cortical region made of a palisade of acanthoxeas. The clade A1b was formed by the genus *Cinachyrella* and *Levantinella* gen. nov. The latter species is characterized by absence of a well-formed cortex, small, little conspicuous porocalice-like structures, and a dense incorporation of sand to its surface. The genus *Cinachyrella* included *Paratetilla bacca* despite the presence of triaenes in the former, and was characterized by the absence of a defined cortex and the presence of true hemispherical large porocalices. No morphological characters could differentiate the two subgroups of *Cinachyrella* found in the molecular phylogenies (i.e. the *C. australiensis*/*C. apion* group versus the *C. alloclada*/*C.* cf. *tenuiviolacea* group).

A2 corresponded to the Antarctic clade, characterized by sharing the same V4 secondary structure. A2a included the *Cinachyra* genus, with cortex and flask-shaped porocalices as synapomorphies, and two *Cinachyra* sp. (QMG 316342 and QMG 316372), which also have cortex and porocalices but show a slightly different secondary structure. Clade A2b, with pseudocortex as a synapomorphy (Fig 9A, 9B and 9C), was subdivided in 2 groups: A2ba and A2bb. A2ba included *Antarctotetilla* gen. nov. with pores clustered in shallow small depressions as a synapomorphy, and Tetillidae sp.3 (ANT 27211, Figs 7A and 9B) totally covered by long hair-like spicules, without porocalices, and with pores clustered in shallow small depressions. A2bb comprised Tetillidae sp. 1 and sp. 2 individuals (NIWA 28491, 25206 and 28929).

Clade B included species that had spread pores as a synapomorphy and was also formed by two well-supported sub-clades:

B1 included two groups. Group B1a was formed by species of *Craniella* with a two-layered cortex made of collagen plus spicules, and a hispid-conulose surface, and a specific V4 secondary structure. Group B1b included cf. *Fangophilina sp*, with a particular V4 secondary structure region and a hispid surface (Fig 7G).

B2 was divided in two groups: B2a formed by the genus *Tetilla* (tropical species) with a particular V4 secondary structure and B2b containing *Amphitethya microsigma*.

Species of the genera *Cinachyra* and *Antarctotetilla* gen. nov. were clearly differentiated, despite the fact that they shared the same V4 secondary structure: *Cinachyra antarctica* has a collagen cortex as an autopomorphy (Figs 6C and 6D and 9H) while *C. barbata* and the two *Cinachyra* sp. (QMG 316342 and QMG 316372) have a spicule-collagenous cortex (Figs 6A and 6B and 9G).

All molecular analyses suggested that the latter species was related to cf. *Fangophilina* sp. and not to *Cinachyra*. Within *Antarctotetilla* gen. nov., *A. leptoderma* and *A. sagitta* have a corrugate surface (Figs 6E and 7C), but while the former has a single large apical osculum, *A.*

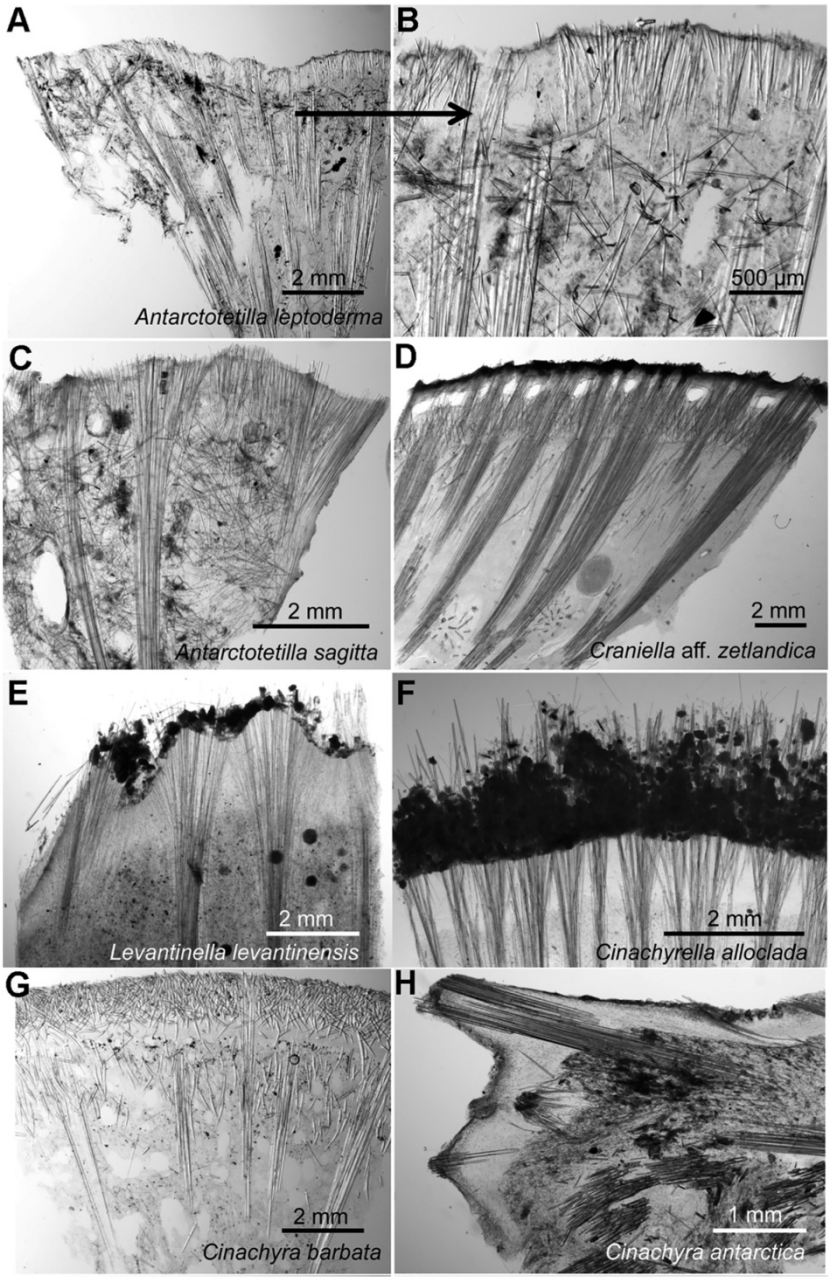
A
2 mm
Antarctotetilla leptoderma
B
500 µm
C
2 mm
Antarctotetilla sagitta
D
2 mm
Craniella aff. zetlandica
E
2 mm
Levantinella levantinensis
F
2 mm
Cinachyrella alloclada
G
2 mm
Cinachyra barbata
H
1 mm
Cinachyra antarctica

Fig 9. Thick sections of Tetillidae species showing differences in the ectosome or cortex structures. A) *Antarctotetilla leptoderma* from Adélie Land, Antarctica, MNHN IP-2009-544a. B) *A. leptoderma*, close-up of C. C) *Antarctotetilla sagitta* from Adélie Land, Antarctica, MNHN IP-2009-31. D) *Craniella* aff. *zetlandica* from Korsfjord, Norway, ZMBN 85239. E) *Levantinella levantinensis* from Israel, PC 705. F) *Cinachyrella alloclada* from Bocas del Toro, Panama, ZMBN 81788. G) *Cinachyra barbata* from Adélie Land, Antarctica, MNHN IP-2009-387. H) *Cinachyra antarctica* from Adélie Land, Antarctica, MNHN IP-2009-328.

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sagitta shows several small oscules (Fig 7C). On the other hand, *A. grandis* shows an even surface, spread small oscula, and pores clustered in small depressions (Fig 6G), while *A. sagitta* is characterized by their pores in sieves covering sub-ectosomal spaces (Fig 7C and 7E).

Formal diagnoses of the currently proposed genera

Family Tetillidae

Tetilla Schmidt, 1868

Type species: *Tetilla euplocamos* Schmidt, 1868.

Diagnosis: Tetillidae without porocalices, without cortical specialization, without auxiliary megascleres [36].

Cinachyrella Wilson, 1925

Type species: *Tetilla hirsuta* Dendy, 1889.

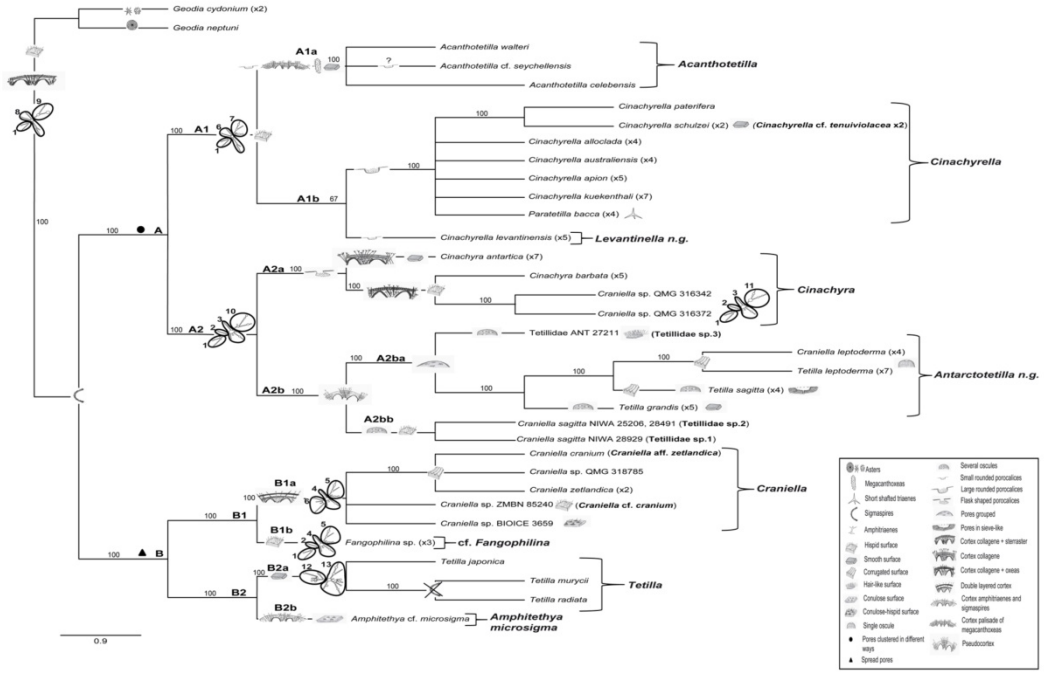

Fig 10. A) Phylogram of one of the most parsimonious trees on morphological characters plus the several zones identified for the V4 region of 18S secondary structure–SSRs–(numbered and encircled). Characters that represent either synapomorphies or apomorphies are depicted in the tree. The supporting bootstrap values of clades resulting from the Majority-rule consensus tree are also indicated. **B)** legends to character drawings on the tree.

doi:10.1371/journal.pone.0160718.g010

Diagnosis: Tetillidae usually with hemispherical porocalices (except in some stalked species), without spiculous cortical specialization; modified from [36].

Remarks: *Amphithetya* and *Paratetilla* are within the *Cinachyrella* clade in our pylogenies. The several well-supported subgroups within the *Cinachyrella* clade might correspond to subgeneres. However, a more in deep study of *Cinachyrella* by including additional species would be necessary to formally propose those subgeneres.

Levantinella gen. nov.

Type species: *Levantinella levantinensis* (Vacelet et al., 2007) by monotypy.

Diagnosis: Tetillidae with small porocalices formed by rounded shallow depressions, without cortex, without auxiliary megascleres.

Craniella Schmidt, 1870

Type species: *Craniella tethyoides* Schmidt, 1870.

Diagnosis: Globular sponges without porocalices and with a distinct, two-layered cortex (visible to the naked eye). The outer cortex layer often with sub-dermal cavities and the inner layer made of collagen and a tight arrangement of cortical oxeas. Presence of direct-developing embryos within the sponge tissue; modified from [36].

Cinachyra Sollas, 1886

Type species: *Cinachyra barbata* Sollas, 1886.

Diagnosis: Tetillidae with a collagenous cortex, sometimes reinforced by auxiliary oxeas, with flask-shaped porocalices; modified from [36].

Remarks: Most described *Cinachyra* have been transferred to *Cinachyrella* (see World Porifera Dadabase)

Antarctotetilla gen. nov.

Synonymy: *Tetilla sensu* Sollas, 1886 (part.), *Craniella sensu* Kirkpatrick, 1908 (part.).

Type species: *Tetilla leptoderma* Sollas, 1886 by designation.

Diagnosis: Tetillidae with single or multiple oscule(s) on top, pores clustered in shallow depressions (no porocalices) and a pseudocortex (very slight cortical differentiation) made of perpendicular oxeas, loosely arranged.

Remarks: Our molecular and morphological results suggest that that three species should be assigned to this genus: *A. leptoderma*, *A. sagitta*, and *A. grandis*. These species are only known (up to now) from the Antarctic, New Zealand, Kerguelen Islands and the Magellanic region. Moreover, *Craniella coactifera* Lendenfeld, 1907 shows strong similarities with *A. grandis* and is likely a synonym of this species. We reallocate *C. coactifera to Antarctotetilla* gen. nov. and keep this species valid until its type can be compared with the type of *A. grandis*.

Discussion

Molecular markers

The molecular markers used in this study were informative enough to resolve the relationships of Tetillidae genera but did not resolve Antarctic species. Phylogenetic trees inferred with the COI M1-M6 partition (also known as the barcoding Folmer fragment) gave a better resolution of the genera, in part because of the higher number of individuals sequenced for this marker and those already available in Genbank. However, although the COI M1-M6 partition differentiated all the species of temperate and tropical genera of Tetillidae included in this study it failed to separate species within the Antarctic genera *Cinachyra* and *Antarctotetilla* gen. nov., despite clear morphological differences. Although uncommon, strictly identical COI M1-M6 sequences for different sponge species have previously been found in other demosponge groups [67], [68], [69], [70] and also in Antarctic hexactinellid sponges: only two COI haplotypes were found among the Antarctic *Rosella* species [71], which had been recognized by Barthel and Tendal [72].

Similarly, the 18S, 28S (D3–D5) and COI I3-M11 partitions did not succeed in discriminating all Antarctic species. This was to be expected from the slow evolving 18S or even from the faster evolving 28S (D3-D5), which has rather been used for inter-species relationships. However, this was rather surprising for the COI I3-M11 partition, which is considered more variable than the Folmer partition [63], and has been used in population genetics and phylogeography studies of demosponges [73], [48], [74].

This may be an indication of contrasting evolutionary rates between sponge groups [75], [69]. Thus, our results suggest either a particularly slow genetic evolutionary rate of the markers 28S (D3-D5) and COI or a recent radiation with phenotypic characters evolving faster than the genes studied. Further studies on other Demospongiae families and/or more variable markers are required to shed light on the evolutionary processes that affect Antarctic sponges, which are poorly studied so far.

Phylogeny and taxonomic actions

Insufficient knowledge on the morphological characters of Antarctic/New Zealand Tetillidae along with misidentifications biased the interpretation of previous phylogenetic studies [34]. The re-examination of some of these specimens as well as holotypes (S2 File) proved essential to understand previous puzzling results such as the polyphyly of *Craniella* [34]. Overall, our results improve and clarify the Tetillidae phylogeny. All COI and 18S trees, as well as the COI +18S trees were mostly congruent, except for the positions of Tetillidae sp.3 (ANT 27211), Tetillidae sp.1 ("*Craniella sagitta*" NIWA 28929), Tetillidae sp.2 ("*Craniella sagitta*" NIWA 25206 and 28491) and *Levantinella levantinensis*.

The MP trees allowed us to identify phenotypic synapomorphies for the proposed genera. The MP phylogeny on phenotypic characters, plus the motifs of the 18S secondary structure (V4 region), differed from the molecular phylogenies only in the position of the two *Cinachyra* sp. (QMG 316342 and QMG 316372, previously wrongly identified as *Craniella* sp.): they form a strongly supported clade with cf. *Fangophilina* sp. (NIWA 28601, 28586, 28617) in molecular trees, whereas they group with *Cinachyra* in the MP tree (because of their porocalices and spiculous cortex).

According to Szitenberg et al. [34], the presence of cortex or/and porocalices, which have been traditionally used to differentiate Tetilllidae genera [35], [36], do not represent synapomorphies according to our molecular analyses. Instead, the types of cortex (with spicules, without spicules, with one or two layers) and/or of porocalices (e.g. deep flask-shaped, hemispherical porocalices, and small, shallow cavities) are the derived characters shared within each genus.

In the current study, we see the Tetillidae basically divided in seven well-supported clades, instead of the five recovered in Szitenberg et al. [34]. Most of these clades correspond to genera: *Antarctotetilla* gen. nov., *Cinachyra*, *Acanthotetilla*, *Tetilla*, *Cinachyrella*, *Craniella*, and *Levantinella* gen. nov.

The new genus *Antarctotetilla* contains exclusively those Tetillidae without cortex, without porocalices, and with grouped ostia. The presence of a pseudocortex (not visible with the naked eye) instead of a clearly conspicuous cortex in those species may explain why they have been assigned to the genus *Tetilla* by previous authors [76], [77], [19], [22]. However, the type species of *Tetilla* (*T. euplocamos* Schmidt, 1868) and other tropical representatives that we have examined (e.g. *T. radiata*, *T. muricyi*), do not have any kind of obvious cortical specialization.

After moving the traditional Antarctic "*Tetilla*" (*Craniella* in Szitenberg et al. [34]) to *Antarctotetilla* gen. nov., the genus *Tetilla* recovers its classical diagnosis [35] by including those

Tetillidae without cortex and without porocalices. Similarly, by moving the two misidentified *Craniella* sp. (QMG 316342 and QMG 316372) to *Cinachyra* sp., *Craniella senso stricto* [35], [36] was recovered as a monophyletic genus, with a characteristic two-layered cortex as synapomorphy.

Szitenberg et al. [34] proposed the inclusion of *Fangophilina* and *Cinachyra* within the genus *Craniella*, as either junior synonyms or sub-genera. However, this proposal was based on a series of misidentifications: QMG 316342 and QMG 316372 had been wrongly identified as *Craniella* sp. (now *Cinachyra* sp.), NIWA 28929, NIWA 28491 and NIWA 25206 had been wrongly identified as *Craniella sagitta* (here renamed Tetillidae sp. 1 and sp. 2). Conversely four confirmed *A. sagitta* specimens (ANT IP 31, IP 351, IP 359 and *A.* cf. *sagitta* QMG 315031) joined the *Antarctotetilla* gen. nov. clade in both molecular and morphological phylogenies.

We missed true *Fangophilina* species in our molecular analyses, since, as stated above, the three cf. *Fangophilina* sp. did not completely match the diagnostic morphological characters of the genus. The type species *F. submersa* was reported to have two opposite porocalyces: one with an inhalant function and the other exhalant [78], [36]. The revision of the type material showed that only the cavity containing the ostia is a true porocalyx since the other one corresponded to a deep cloacal osculum. Morphological and genetic investigations on further individuals of *F. submersa*, which is known just by a single specimen, are necessary to resolve the phylogenetic position and morphological variation of this genus, which has been considered dubious [36].

Our phylogenetic trees retrieved the type species of *Amphitethya* (*A. microsigma*) and *Paratetilla* (*P. bacca*) within the large *Cinachyrella* clade, as in previous phylogenies [34]. The position of *Paratetilla* within *Cinachyrella* is also recovered in the morphological tree as species of both genera have similar external morphology. Although the type species of *Cinachyrella*, *C. hirsuta* Dendy, 1889, is not included in our sampling, we are confident that it would group in the large *Cinachyrella* clade, based on its morphology [36]. We would therefore have enough arguments to synonymize *Amphitethya* Lendenfeld, 1907 and *Cinachyrella* Wilson, 1925 with *Paratetilla* Dendy, 1905, the oldest genus name. However, reallocating the so far described 40 species of *Cinachyrella* [33] to *Paratetilla* without previous reexamination would not be the most conservative option since it would hide the morphological difference we currently recognize (calthrop-like triaenes) to identify *Paratetilla*. Instead, we prefer to wait for further molecular phylogeny studies on this group to take taxonomic action. Since *Cinachyrella* species are distributed in several clades, we believe a future revision of this group with a wider sampling might indicate where to place *C. hirsuta*.

The presence of *Amphitethya* within the *Cinachyrella* clade of our molecular trees was unexpected, due to its stalked morphology and the absence of porocalices, which, conversely, placed *A. microsigma* as a sister species of *Tetilla* in the morphological tree. However, we note that the characteristic amphitriaenes of *Amphitethya* have also been occasionally observed in *Paratetilla* species: *P. aruensis* [79], and *P. merguiensis* [80].

In our 18S phylogeny, a few sequences from Redmond et al. [39] had suspicious phylogenetic affinities: *Acanthotetilla* cf. *seychellensis* (KC902033), and *Cinachyra* sp. (KC902124) clustered with *Cinachyrella* while *Craniella* sp. (KC902265) clustered with *Tetilla*. We suspect these are misidentifications but we could not re-examine the corresponding specimens.

Szitenberg et al. (S3 File) [34] suggested erecting *Levantinella* gen. nov. for the species *C. levantinensis*, which substantially diverged from the rest of the *Cinachyrella* species in their study. However, no formal definition was proposed thus making *Levantinella* a *nomen nudum*. We agree with these authors in that this species belongs to a new genus and we formally propose to make *Levantinella* gen. nov. available with *C. levantinensis* as type species by monotypy.

The phylogenetic affinities of *Levantinella*, however, differed depending on the gene partition used: it appeared as a sister group of the *Tetilla/Craniella/Fangophilina/Antarctotetilla/Cinachyra* clade with COI while it was a sister group to the other *Cinachyrella* with 18S.

The family Tetillidae appeared paraphyletic or polyphyletic in previous 18S phylogenies [39], [38] and 28S phylogenies [81]: the *Craniella/Cinachyra/Antarctotetilla* gen. nov. clade was sister to the Astrophorina. However, these results may be due to a sampling bias, a possibility further suggested by a wide COI phylogeny that recovers a monophyletic Tetillidae [38]. A more thorough worldwide study of representatives of this family is needed to further test its monophyly.

Geographical distribution of Tetillidae genera

The geographical location of the studied Tetillidae suggests a temperature related distribution of some genera (Fig 11). *Cinachyrella* shows a tropical–subtropical distribution, while *Craniella* species mainly inhabit temperate-cold seas. The genera *Cinachyra* and *Antarctotetilla* appear confined to the Antarctic and Sub-Antarctic regions, contributing to the reported Antarctic sponge endemism [82], [5], which underlines the importance of the Polar Front in isolating the Southern Ocean fauna. Other *Cinachyra* species, which have been reported out of the Antarctic, may have been incorrectly attributed to this genus. For example, *Cinachyra helena* Rodriguez and Muricy, 2007 from Brazil does not belong to *Cinachyra* since its purported porocalyx

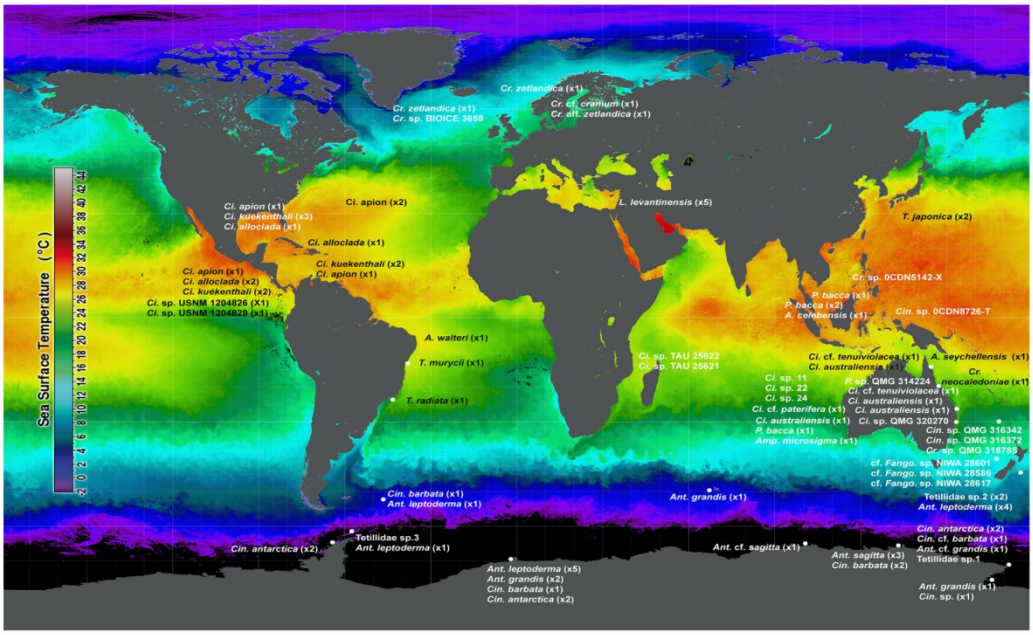

Fig 11. Distribution of the Tetillidae species analysed in this study overlying a temperature map in South hemisphere winter (NASA Goddard Space Flight Center, Ocean Ecology Laboratory, Ocean Biology Processing Group; (2014): Sea-viewing AQUA MODIS Sea Surface Temperature, August 2013. NASA OB.DAAC. http://oceancolor.gsfc.nasa.gov/cgi/l3. Accessed on: 2015/04/29). White points represent exact sampling locations. Cr. = *Craniella*; Ci. = *Cinachyrella*; L. = *Levantinella; Fango*. = *Fangophilina*; A. = *Acanthotetilla*; T. = *Tetilla*; Amp. = *Amphitethya*; P = *Paratetilla*; Cin. = *Cinachyra*; Ant. = *Antarctotetilla*.

doi:10.1371/journal.pone.0160718.g011

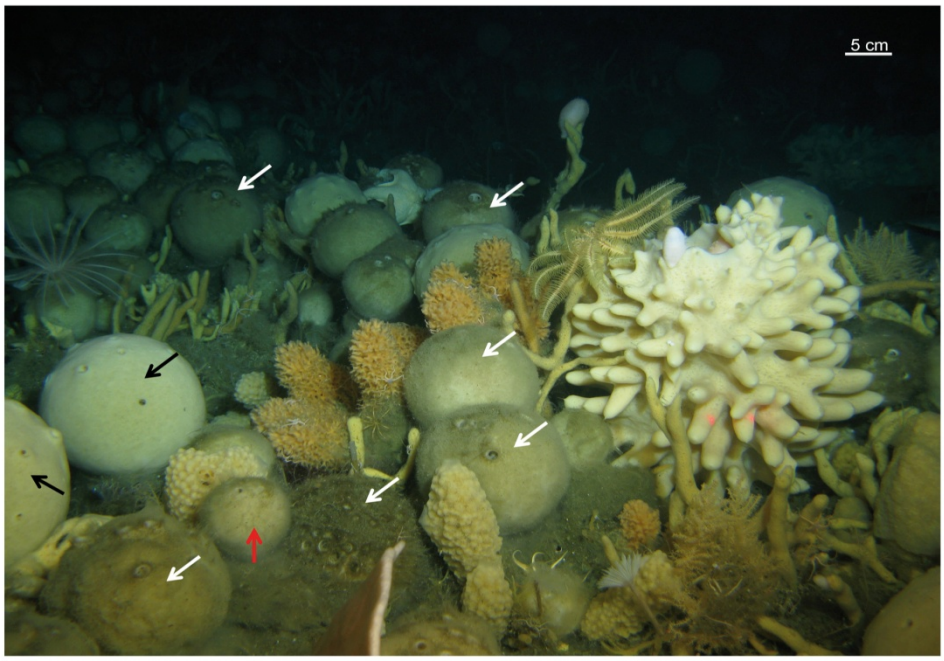

Fig 12. Tetillidae grounds on the Antarctic bottoms. Black arrows point to *Antarctotetilla grandis*. White arrows indicate *Cynachyra* spp. Red arrow points to Tetillidae sp3.

doi:10.1371/journal.pone.0160718.g012

rather looks like a cloacal oscula with macro-orifices inside the depression [83]. Moreover, its two-layered cortex [83] suggests that it is a *Craniella*. We therefore propose to reallocate this species to the genus *Craniella*. Other described *Cinachyra* have been moved to *Cinachyrella* posteriorly (see WPD). Current representatives of the genus *Tetilla* are mainly living in arctic-temperate-warm seas.

In relation to depth, *Cinachyrella* is distributed in shallow-waters (<30 m depth) with few exceptions that can be found up to 100 m depth (e.g. *Cinachyrella kuekenthali*, *Amphitethya microsigma*) while other genera such as *Craniella* or *Fangophilina* are nearly restricted to the deep-sea. *Antarctotetilla* and *Cinachyra* are eurybathic inhabiting from 30 to 600 m of depth, but they are particularly abundant between 200 and 300 m of depth where they may dominate in sponge grounds (Fig 12).

Sponge molecular phylogenies have greatly contributed to emend the traditional sponge systematics, in particular for demosponges [50], [84]. The sponge phylogenetic tree resolution continuously improves as new genetic markers, and more importantly, additional taxa are included in the datasets. However, a careful morphological identification of the individuals sequenced and included in the molecular phylogenies is required for a precise interpretation of the molecular results. In other words, molecular phylogenies should consistently be associated with thorough morphological studies of the specimens sequenced, as we have tried to do with the Tetillidae species.

Supporting Information

S1 File. Morphological data matrix (including secondary structure shapes of the 18S V4 variable region) used for the maximum parsimony phylogeny.
(PDF)

S2 File. Original and revised identifications of Tetillidae voucher specimens from previous studies (Cárdenas et al. 2011, Szitenberg et al. 2010, Szitenberg et al. 2013) after morphological re-examination. Localities, depths, and Genbank accession numbers of the corresponding sequences are also listed. QMG, Queensland Museum, Brisbane, Australia; NIWA, National Institute of Water & Atmospheric Research, New Zealand; SAM, South Australia Museum; ZMBN, Zoological Museum in Bergen, Norway. In bold, specimens re-examined in this study. Specimens with (*) were only seen on pictures.
(PDF)

S3 File. The different parts of the predicted secondary structures (V4 region of 18S) are encircled and numbered.
(PDF)

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Conceived and designed the experiments: MJU.

Performed the experiments: MC GA.

Analyzed the data: MC GA.

Contributed reagents/materials/analysis tools: MJU PC.

Wrote the paper: MJU MC PC GA.

Sampling: MJU PC. Laboratory work: MC GA. Phylogenetic reconstructions: MC. Data interpretation: MC MJU PC.

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Rio de Janeiro, April 25th, 2018

Dear Iosune,

I am pleased to inform of the acceptance of the manuscript by Mirco Carella and M. J. Uriz, entitled **Description of two new genera (*Antarctotetilla, Levantiniella*) and a new species of Tetillidae**, for publication in *Zootaxa*. The manuscript is currently passing through a detailed linguistic editorial revision, and I expect it to be uploaded to *Zootaxa*'s desktop printing facility in New Zealand, in no longer than two weeks time. Then, it usually takes about four weeks to be published, depending of speedy review of proofs.

Thank you for publishing with *Zootaxa*.

Cordially,

Eduardo C.M. Hajdu
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Prof. Dr. Eduardo C.M. Hajd
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