Phylogeny, Taxonomy and Evolution of the Astrophorida (Porifera, Demospongiae)

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“La mer est salée parce qu’il y a des morues dedans. Et si elle ne déborde pas, c’est parce que la Providence, dans sa sagesse, y a placé aussi des éponges”

”The sea is salted because there are cods inside. And if it does not overflow, it is because providence, in its wisdom, also placed sponges there.”

Alphonse Allais (French writer, 1854-1905)

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Once upon a time, in early autumn 2005, Christoffer, without knowing me at all, welcomed me into his lab. One day, he handed me a sponge molecular paper casually suggesting that I could work on sponge phylogenetics. Why not? I replied. The next day I was meeting Hans Tore who presented the Norwegian *Geodia* species to me. In August 2006, I was starting a Ph.D. on the Astrophorida. So I owe a great debt to my supervisors Christoffer and Hans Tore who very early trusted and believed in me, who always favorably welcomed my ideas and projects and whose door was always open. It has been so nice working with you and in your lab. Takk så myke! Tusen takk!

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While finishing this Ph.D., I think of my French great-grandfather, André Vuillet (1883-1914), an entomologist who never finished a promising thesis 'Doctorat ès-Sciences' since he got killed during the 1st World War. I also think of his daughter (my grandmother) Claudine Hardy-Vuillet (1909-2004) who, although she had to stop her biologist career early, deeply loved science. She liked to hear my stories and I think she would have been interested and proud of this work. I cannot help to think that these persons were partially responsible, directly or indirectly, for my very early interest in "this view of life".

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Bergen, March 2010

Paco

A mi abuelita Mama Leonor, al otro lado del Atlántico.

ABSTRACT

The Astrophorida (Porifera, Demospongiae) currently represent ca 660 extant species worldwide. In tropical and parts of warm temperate waters they are common at quite shallow depths, while in boreal/antiboreal and Arctic waters they are usually deep-water species. They have a very diverse external morphology (massive to thin encrusting, subspherical-, fan-, cup- or irregularly-shaped) and display a wide array of external colors. They can be several meters large to a few millimeters thick. However, they all share the same spicule combination: small aster-shaped spicules (microscleres) associated with large four-rayed spicules (megascleres) called triaenes. This unique shared derived character (synapomorphy) is not found in any other Porifera groups. According to the last major morphological revision of the Astrophorida, five families are included in this order: Ancorinidae, Calthropellidae, Geodiidae, Pachastrellidae, and Thrombidae. To date, molecular phylogenetic studies including Astrophorida species are scarce and offer limited sampling. Phylogenetic relationships within this order are therefore for the most part unknown, hypotheses based on morphology largely untested and the spicule evolutionary processes poorly studied. This thesis presents five papers investigating the 1) taxonomy, 2) phylogeny and 3) evolution of the Astrophorida.

1) The first aim of this thesis was to build a molecular phylogeny on solid taxonomical grounds. The three first papers are integrative taxonomical and nomenclatural studies on Atlantic Astrophorida species, notably from the Caribbean coast of Panama and from Norway. In the course of these studies, three species were synonymized, two species were resurrected and two were new to science. This thesis also proposes a list of the North-East Atlantic/Mediterranean Sea Astrophorida species here considered valid.

2) The second aim of this thesis was to investigate the phylogenetic relationships within the Astrophorida with molecular data. The two following papers are molecular phylogeny analyses using a cytochrome *c* oxidase subunit I (COI) gene partial sequence and the 5' end terminal part of the 28S rDNA, first considering the Geodiidae alone, then the Astrophorida. Sampling included all five families of this order, three 'lithistid' families of Astrophorida affinities as well as two putative Astrophorida (*Alectona* and *Neamphius*) still classified today in the Alectonidae, Hadromerida. The COI and 28S (C1-D2) datasets were concatenated in a single matrix containing a total of 152 taxa (29 genera, 2 sub-

genera, 89 species) and 1,527 characters. The resulting tree showed that i) the Astrophorida was monophyletic, ii) the sub-orders Euastrophorida and Streptosclerophorida were both found polyphyletic, iii) the Calthropellidae were monophyletic (and found to be a subfamily of the Geodiidae), iv) the Geodiidae, the Ancorinidae and the Pachastrellidae appeared polyphyletic and had to be redefined, v) a new subfamily of the Geodiidae was revealed (Caminellinae subfam. nov.) and finally vi) some genera were found to be polyphyletic (*Ecionemia, Erylus, Poecillastra*, *Penares, Rhabdastrella*, *Stelletta* and *Vulcanella*). Based on these results, a revised classification of the Astrophorida is proposed, along with a key to the families, sub-families and *incertae sedis*. The use of a phylogenetic classification of the Astrophorida (following the principles of phylogenetic nomenclature and the rules of the *PhyloCode*) was also explored.

3) The third aim of this thesis was to investigate the evolution of Astrophorida sponge spicules, particularly diverse in this order. In the two last papers, spicule categories were mapped on the molecular phylogenetic trees. The main result was that spicule homoplasy is more common than what we expected: convergent evolution and secondary losses have happened many times, in all the clades, for megascleres and microscleres. The implications of these results are discussed with respect to the function of spicules, their evolution and the taxonomy of sponges.

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1. INTRODUCTION

1.1. General introduction to the phylum Porifera

Ancestors of the extant sponges (phylum Porifera Grant, 1836) are considered to be the first animals to appear on the planet. This has been confirmed many times by a majority of molecular studies that find the Porifera at the base of the Metazoa tree (Medina *et al.* 2001; Lavrov *et al.* 2005; Peterson & Butterfield 2005; Jiménez-Guri *et al.* 2007; Park *et al.* 2007). According to the fossil record, siliceous sponges are present just before the Cambrian (~580 Mya) (Li *et al.* 1998; Huang *et al.* 2008) and are already well diversified in the Lower Cambrian (Xiao *et al.* 2005). Meanwhile, sponge specific biomarkers (carbonate rock texture and hydrocarbon remains of C_{30} sterols) place the origin of sponges in the Early Neoproterozoic, at least 100 Ma before the Cambrian, at ~635 Mya (Love *et al.* 2009) or ~779 Mya (Neuweiler *et al.* 2009). Molecular clock analyses are consistent with the biomarker record and strongly suggest that sponges with siliceous spicules were present during the Precambrian, but were not fossilized (Savolainen *et al.* 2005; Sperling *et al.* 2010). Sperling *et al.* (2010) speculate that this "spicule gap" could result from a higher solubility of biogenic silica due to clay-poor Precambrian sediments.

Porifera encompass ca 8,500 described species, and estimations of undescribed species usually double that number (van Soest 2007). Porifera are present worldwide, in all aquatic habitats, including freshwater environments, tropical reefs, Arctic/Antarctic regions and the deep-sea. Porifera are distributed in four classes: Hexactinellida Schmidt, 1870, Demospongiae Sollas, 1885, Homoscleromorpha Lévi, 1973 and Calcarea Bowerbank, 1864. Compared to most other phyla, phylogenetic relationships among Porifera are largely unresolved. More than fifty years after Lévi (1957) considered Porifera to be the last major group of Metazoa in which the orders were still not clearly defined, we can unfortunately notice this is still the case. The main reasons for this are i) the paucity of characters used in sponge classification and ii) the homoplasy richness of this group. Sponge taxonomy is primarily based on spicule morphology and spicule arrangement within the sponge body (Hooper & van Soest 2002b). Other characters such as texture, form and coloration are less reliable as they are frequently influenced by environmental factors (Bell & Barnes 2000; McDonald *et al.* 2002; Meroz-Fine *et al.* 2005). But even spicule size, shape and type can sometimes be influenced by environmental conditions (cf. 1.4.3.). Therefore, sponge

systematics have given rise to numerous debates, most of which result from a lack of suitable variable morphological characters to distinguish sponges at the species level and higher.

In this context, molecular data was highly welcomed because it provided new and independent evidence to test morphological hypotheses. The first application of molecular systematics to sponges dates back to the 1980s, using allozyme divergence to discriminate between conspecific sponge populations. In the 1990s, molecular studies comparing sequences of ribosomal RNA have been used to reappraise the phylogenetic relationships among sponge genera, families and orders, mainly using the 18S small subunit and 28S large subunit rRNA genes. Since then, the use of a genetic approach has been a valuable contribution to the study of many long-standing problems in sponge taxonomy (see Boury-Esnault & Solé-Cava 2004 for a review) one of them being the status of the phylum Porifera. Indeed, these last 10 years the question regarding the "natural" existence of this group has been regularly debated and tested. Early phylogenetic studies generally used ribosomal and/or nuclear housekeeping genes (e.g. aldolase (ALD), catalase (CAT), elongation factor 1-alpha (EF1a)) and sampled few sponges species: they suggested the Porifera were not monophyletic (Adams *et al.* 1999; Peterson & Addis 2000; Medina *et al.* 2001). Similar results were obtained with a wider sampling (Borchiellini *et al.* 2001; Borchiellini *et al.* 2004b; Sperling *et al.* 2007; Sperling *et al.* 2009). Later studies including Expressed Sequence Tags (EST) and complete mitochondrial genomes, with limited sampling, were finding the Porifera monophyletic, albeit with contradictory supports and sister-groups (Jiménez-Guri *et al.* 2007; Dunn *et al.* 2008; Lavrov *et al.* 2008; Philippe *et al.* 2009; Schierwater *et al.* 2009). A few ribosomal gene studies (with limited sampling) also found the Porifera monophyletic (Dohrmann *et al.* 2008; Lavrov *et al.* 2008). Although the issue is not settled, most studies nonetheless agree that the Silicea Gray 1867 (Hexactinellida + Demospongiae) are monophyletic (Adams *et al.* 1999; Borchiellini *et al.* 2001; Medina *et al.* 2001; Dohrmann *et al.* 2008; Philippe *et al.* 2009; Sperling *et al.* 2010). Meanwhile, Homoscleromorpha and Calcarea are either sister-groups within the Porifera or paraphyletic and closer to the Eumetazoa. Also, based on molecular results, the 13 extant orders of Demospongiae (85% of all living sponges) are currently distributed in four clades: G1/Keratosa, G2/Myxospongia, G3/Haplosclerida and G4/Democlavia (Borchiellini *et al.* 2004b; Sperling *et al.* 2009) (Fig. 1).

The overall goal of this thesis was to provide new insights on the evolutionary relationships within the order Astrophorida Sollas, 1888, using molecular and morphological data. This introduction will be divided in three parts. After a short presentation of the

Astrophorida, I will present what was known of their taxonomy and phylogeny before the beginning of this study, including a brief overview of molecular markers in sponge phylogenetic studies. Finally, I will introduce a few facts about Astrophorida morphology and spiculogenesis, both of which will be helpful for the following discussion on spicule evolution.

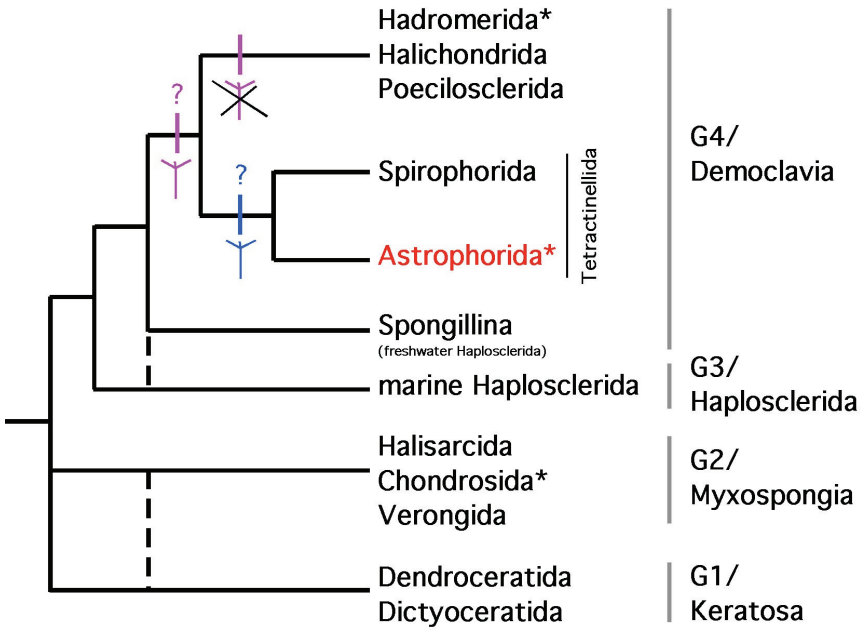

Figure 1. Current molecular phylogenetic relationships of the Demospongiae. Dashed lines indicate uncertain branches. * indicates the presence of microsclere asters. Two hypotheses for the gain of triaenes are illustrated: the hypothesis suggested by the fossil record and the hypothesis suggested by molecular clock analyses.

1.2. General presentation of the Astrophorida

The Astrophorida is geographically and bathymetrically widely distributed around the world, and represent around 660 extant species worldwide (van Soest *et al*. 2010; this study). Astrophorida species have colonized hard- as well as soft-bottoms from various depths. In tropical and parts of warm temperate waters Astrophorida species are common at quite shallow depths, while in boreal/antiboreal and Arctic waters they are usually deep-water species (they are poorly known in the Antarctic). In gravely hard-bottom habitats on the outer shelf and upper slope, Astrophorida can dominate ecosystems in terms of abundance and

biomass (Maldonado & Young 1996; Klitgaard & Tendal 2004). They have also been shown to dominate in some paleoenvironments (Pisera *et al.* 2006). Astrophorida species have a very diverse external morphology: massive to thin encrusting, subspherical-, fan-, cup- or irregularly-shaped. They display a wide array of external colors: white, purple, green, yellow, orange, black etc. They can be several meters large (e.g. *Stryphnus fortis* (Vosmaer), pers. obs.) to a few millimeters thick (e.g. some *Jaspis*). However, Astrophorida are all characterized by a clear morphological synapomorphy: the simultaneous presence of aster-shaped microscleres and tetractinal megascleres. According to the fossil record, Astrophorida may represent one of the oldest orders of demosponges. Well-preserved typical triaenes (ortho- and plagiotriaenes) and euasters (oxyasters and sterrasters) are common in Early and Middle Cambrian Australian terrains (van Kempen 1990; Reitner & Mehl 1995; Mehl 1998). They even abound in Mesozoic spicule assemblages (van Kempen 1990). In rare cases, the whole sponge can be fossilized, as in the fossil *Geodia avicula* from the Miocene (Brimaud & Vachard 1986). On the contrary, Astrophorida lithistids are easily fossilized because their spicules are tightly holding together (Brimaud & Vachard 1986; Lévi 1991; Pisera 1999). Surprisingly, molecular clock estimates suggest that Astrophorida and Spirophorida may have diverged only 380 Ma ago (late Devonian) (Sperling *et al.* 2010). Either i) molecular clock estimates are too shallow or ii) the triaenes and euasters found from the Early Cambrian are not homologous to the ones of extant Astrophorida or iii) triaenes and euasters are plesiomorphic and have appeared in the ancestor of the G4/Democlavia, which might have originated in the Cambrian (according to the molecular clock); in that case, triaenes would have been lost in the ancestor(s) of the rest of the Democlavia: Hadromerida, Poecilosclerida and Halichondrida (Fig. 1).

Sexual reproduction is poorly studied and documented in the Astrophorida. With the exception of the armored planktonic larva (= hoplitomella larva) of *Alectona* and *Thoosa* (Topsent 1920; Vacelet 1999) no larvae are known (Maldonado & Bergquist 2002). All Astrophorida, except for *Alectona* and *Thoosa*, are considered to be oviparous. Few Astrophorida species have actually been shown to be gonochoric and oviparous: *Erylus discophorus* (Schmidt) (Scalera Liaci & Sciscioli 1969; 1970), *Geodia barretti* Bowerbank (Spetland *et al.* 2007), *Geodia cydonium* (Jameson) (Mercurio *et al.* 2007). *Stelletta grubei* (Schmidt) (Scalera Liaci & Sciscioli 1969; Sciscioli *et al.* 1991) and shallow-water Theonellidae of the Red Sea (Ilan *et al.* 2004). Furthermore, I have observed oogenesis in *Rhabdastrella cordata* Wiedenmeyer (#S1026) from South Australia. Spermatogenesis has been observed in *Thenea muricata* (Bowerbank) and *Yucatania sphaerocladoides* (Hartman &

Hubbard) (Sollas 1882b; Babiç 1915; Hartman & Hubbard 1999). Sperm release has been rarely documented except for a *Geodia* sp. in Jamaica (Reiswig 1970) and *Geodia barretti* in an aquarium at the University of Bergen (pers. obs.). Oocyte release has to my knowledge never been observed in this group. Conversely, asexual reproduction is common and fairly well documented in the Astrophorida: *Geodia hentscheli* Cárdenas *et al.* (Burton 1949), *Geodia cydonium* (pers. obs.), *Geodia phlegraei* (Sollas) (Greenland specimens, pers. obs.), *Thenea muricata* and *Thenea valdiviae* von Lendenfeld (Steenstrup & Tendal 1982).

1.3. Taxonomy and phylogeny of the Astrophorida

1.3.1. Historical review of the Astrophorida taxonomy

Demospongiae with triaenes (Astrophorida and Spirophorida) are grouped in the Tetractinellida Marshall, 1876 (Fig. 1). The Astrophorida was originally a suborder of the Choristida Sollas, 1885 which united all the Tetractinellida with aster microscleres, except for the lithistids. The fact that today's definition of the Astrophorida has not changed reflects the stability and phylogenetic relevance of the morphological characters used to define it. Lévi (1973) later made of this group an order and modified some of its contents, notably excluding the Placospongiidae and including the Thrombidae and the Calthropellidae. According to their last major revision in the *Systema Porifera*, five families are included in this order: Ancorinidae Schmidt 1870, Calthropellidae Lendenfeld 1907, Geodiidae Gray 1867, Pachastrellidae Carter 1875, and Thrombidae Sollas, 1888 (Hooper & van Soest 2002a). Thirty-eight genera and two subgenera are currently distributed in those families. In an effort to incorporate lithistids, the sub-orders Euastrophorida Reid, 1963 (Astrophorida with euasters) and Streptosclerophorida Dendy, 1924 (Astrophorida with streptasters) were erected. Lithistids with streptasters were then included in the Streptosclerophorida (Reid 1963; Lévi 1991).

Classification of the Astrophorida according to the *Systema Porifera*:

(Maldonado 2002; van Soest & Hooper 2002; Uriz 2002a; b; c)

Order Astrophorida Sollas, 1888

- Family Thrombidae Sollas, 1888
  - *Thrombus* Sollas, 1886
- Family Pachastrellidae Carter, 1875
  - *Acanthotriaena* Vacelet *et al.*, 1976
  - *Ancorella* von Lendenfeld, 1907
  - *Brachiaster* Wilson, 1925
  - *Characella* Sollas, 1886
  - *Cladothenea* Koltun, 1964
  - *Dercitus* Gray, 1867
  - *Pachastrella* Schmidt, 1868
  - *Poecillastra* Sollas, 1888
  - *Stoeba* Sollas, 1888
  - *Thenea* Gray, 1867
  - *Triptolemma* de Laubenfels, 1955
  - *Vulcanella* Sollas, 1886
    - *Vulcanella (Vulcanella)* Sollas, 1886
    - *Vulcanella (Annulastrella)* Maldonado, 2002
- Family Geodiidae Gray, 1867
  - *Caminus* Schmidt, 1862
  - *Erylus* Gray, 1867
  - *Geodia* Lamarck, 1815
  - *Isops* Sollas, 1880
  - *Pachymatisma* Bowerbank *in* Johnston, 1842
  - *Sidonops* Sollas, 1889
- Family Calthropellidae von Lendenfeld, 1907
  - *Calthropella* Sollas, 1888
  - *Chelotropella* von Lendenfeld, 1907
  - *Pachastrissa* von Lendenfeld, 1903
  - *Pachataxa* de Laubenfels, 1936
- Family Ancorinidae Schmidt, 1870
  - *Ancorina* Schmidt, 1862
  - *Asteropus* Sollas, 1888
  - *Cryptosyringa* Vacelet, 1979
  - *Disyringa* Sollas, 1888
  - *Ecionemia* Bowerbank, 1864
  - *Holoxea* Topsent, 1892
  - *Jaspis Gray, 1867*
  - *Melophlus* Thiele, 1899
  - *Penares* Gray, 1867
  - *Psammastra* Sollas, 1886
  - *Rhabdastrella* Thiele, 1903
  - *Stelletta* Schmidt, 1862
  - *Stryphnus* Sollas, 1886
  - *Tethyopsis* Stewart, 1870
  - *Tribrachium* Weltner, 1882
- *Lamellomorpha* Bergquist, 1968 *incertae sedis*

1.3.2. Molecular phylogenetics and the Astrophorida

The Astrophorida are part of the G4/Democlavia clade (Fig. 1). It is one of the few sponge orders to have been consistently, and with strong support, shown to be monophyletic (Chombard *et al.* 1998; Borchiellini *et al.* 2004b; Nichols 2005; Erpenbeck *et al.* 2007a). All molecular phylogenetic studies place them in a strongly supported sister-order relationship with the Spirophorida Bergquist and Hogg, 1969 (Chombard *et al.* 1998; Borchiellini *et al.* 2004b; Nichols 2005; Lavrov *et al.* 2008; Voigt *et al.* 2008; Sperling *et al.* 2009).

The first molecular phylogenetic study to focus on the Tetractinellida used the 5' end terminal part of the 28S rRNA gene (Chombard *et al.* 1998). The most-parsimonious tree they obtained (Fig. 2) notably suggests that i) the sub-orders Euastrophorida and Streptosclerophorida are monophyletic and paraphyletic respectively (if *Stryphnus* is considered a Streptosclerophorida), ii) some lithistids belong to the Astrophorida, iii) *Penares helleri* (Schmidt) (an Ancorinidae) should be reallocated to the Geodiidae (it has presumably secondarely lost its sterrasters) and iv) *Poecillastra* (a Pachastrellidae) is a more basal Astrophorida than the Geodiidae, Ancorinidae and lithistids. Later, Chombard (1998) added two additional sequences to her analyses: one from a Geodiidae (*Geodia cydonium*), the other from an Ancorinidae (*Stelletta dorsigera* Schmidt). Her results prompted her to propose to resurrect the Geodiidae subfamilies: Erylinae Sollas, 1888 and Geodinae Sollas, 1888. At the same time she suggested that the Geodiidae could be polyphyletic, although taxonomists had never previously challenged the monophyly of the family before.

Because they possess triaenes and asters, many lithistid families are also known and/or suspected to belong to the Astrophorida (Sollas 1888; Topsent 1928; Reid 1970; Lévi 1991) but have until now and in spite of molecular evidence (Kelly-Borges & Pomponi 1994; Chombard *et al.* 1998; McInerney *et al.* 1999) been kept apart in the classification (Hooper & van Soest 2002b). Other enigmatic taxa such as the excavating sponges *Alectona* and *Neamphius* (both belonging to the Alectonidae) have also been suggested to be derived Astrophorida species, based on morphological (Sollas 1888), molecular (Borchiellini *et al.* 2004a) and larval data (Topsent 1920; Vacelet 1999).

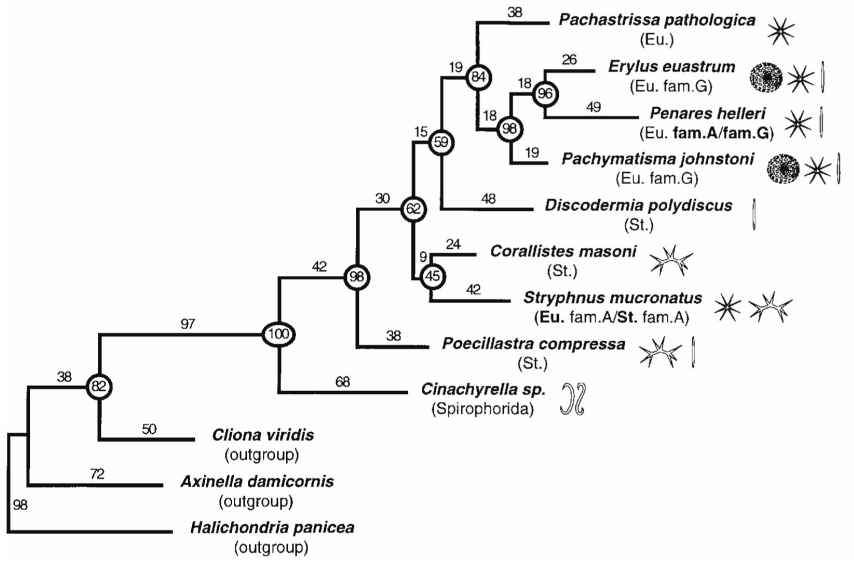

Figure 2. Most-parsimonious tree resulting from a parsimony analysis of 28S rDNA sequences (C1-D2). Length of branches are shown above each branch and circled numbers indicate the bootstrap proportions (1000 replicates). Microsclere composition of ingroup species are indicated as well as their previous and final assignments (in bold when modified by the present work). Eu. = Euastrophorida; St. = Streptosclerophorida; fam. A = family Ancorinidae; fam. G = family Geodiidae (Chombard *et al.* 1998; Figure 5).

1.3.3. Molecular markers in sponge phylogenetics

Early metazoans such as Porifera and Cnidaria (especially Anthozoa) have a slow-evolving mitochondrial DNA when compared to other phyla (Shearer *et al.* 2002; Duran *et al.* 2004; Lavrov *et al.* 2005; Wörheide 2006; Huang *et al.* 2008). Therefore, the cytochrome *c* oxidase subunit 1 (COI) has proven to be a good phylogenetic marker for higher-level sponge phylogenies (Nichols 2005; Erpenbeck *et al.* 2007a). Although the COI Folmer fragment has also been successfully used for inter-species and population studies (Duran & Rützler 2006; Blanquer & Uriz 2007; Heim *et al.* 2007b; Reveillaud *et al.* in press), it has in some cases appeared to be too conserved (Schröder *et al.* 2003; Addis & Peterson 2005; Heim *et al.* 2006; Huang *et al.* 2008), which raises issues when it comes to the barcoding of sponges (Box 1).

The 28S rDNA (C1-D2) partition has been used early in sponge molecular phylogenies (Lafay *et al.* 1992) and proven to be adequate in resolving poriferan intra-ordinal relationships (Borchiellini *et al.* 2004b). It is relatively unsaturated and suited to resolve Astrophorida relationships (Chombard 1998; Chombard *et al.* 1998). But the D1 domain

alone is not informative enough to resolve the inter-family relationships within the Astrophorida (Borchiellini *et al.* 2004a). The D2 domain is more variable and appropriate to investigate inter-species relationships (Usher *et al.* 2004; Barucca *et al.* 2007; Blanquer & Uriz 2007; Wörheide *et al.* 2008). Other studies have been using the 28S (D3-D5) partition, but it has proven more suited for higher-level phylogenies (McInerney *et al.* 1999; Alvarez *et al.* 2000; Erpenbeck *et al.* 2004; Erpenbeck *et al.* 2005b; Nichols 2005; Erpenbeck *et al.* 2007b) than for inter-species studies (Reveillaud *et al.* in press). Of course, for higher-level phylogenies, the 18S rDNA gene has been widely used (Adams *et al.* 1999; Borchiellini *et al.* 2004b; Dohrmann *et al.* 2006; Redmond *et al.* 2007; Redmond & McCormack 2008; Voigt *et al.* 2008) and it also has in some rare cases been used for inter-species studies (Blanquer & Uriz 2007). The 16S and 12S rDNA gene have been rarely used and they both seem quite conserved, more so than COI (Heim *et al.* 2007a; Dohrmann *et al.* 2008).

Chombard (1998) suggested that internal-transcribed-spacer 2 (ITS2) might be a good molecular marker to discriminate Geodiidae species. ITS1 and ITS2 rDNA sequences have been shown to have an appropriate rate of evolution for studies at the species level (e.g. Hoshino *et al.* 2008; Valderrama *et al.* 2009 and references therein). But, as in many other phyla, ITS divergent paralogues can be found (Lôbo-Hajdu *et al.* 2004; Wörheide *et al.* 2004; Alvarez *et al.* 2007). ITS phylogenetic results must therefore be treated with care, especially for analyses at the population-level (Wörheide *et al.* 2004; Nichols & Barnes 2005).

Other markers have been used, but to at a lesser extent: a series of nuclear housekeeping genes such as elongation factor 1-alpha (EF1a), aldolase (ALD), catalase (CAT) or triose-phosphate isomerase (TPI) (Erpenbeck *et al.* 2005a; Erpenbeck *et al.* 2006a; Sperling *et al.* 2007); nuclear introns such as the ATP synthetase beta subunit-gene (*ATPSb*-iII) intron (Bentlage & Wörheide 2007; Wörheide *et al.* 2008; Reveillaud *et al.* in press); mitochondrial markers such as NADH dehydrogenase subunit 5 (nad5) (Hoshino *et al.* 2008), cytochrome *c* oxidase subunit 3 (CO3) (Park *et al.* 2007) and the ATP synthase subunit 8 (Atp8) (Xavier *et al.* in press).

Box 1: Barcoding of sponges

DNA barcoding is an identification method that compares short specific DNA sequences from unidentified specimens to sequences of previously identified voucher specimens (Hebert *et al.* 2003). There are currently two separate tasks to which DNA barcodes can be applied: i) species identification and ii) new species discovery (DeSalle *et al.* 2005). For the first task, one needs species-specific sequences. For the second task, one needs a molecular marker able to delimitate species. The mitochondrial cytochrome *c* oxidase subunit 1 (COI) so commonly used in phylogenetics was a marker of choice for the 'Barcoding of Life' initiative (Hebert *et al.* 2003). It was thought the ca. 700 base pairs 5' partition of COI, also known as the Folmer fragment (Folmer *et al.* 1994) could facilitate the correct determination of specimens including polymorphic or cryptic species (Moritz & Cicero 2004; Schander & Willassen 2005). DNA identification would be of paramount importance for sponges, a group with limited morphological diagnostic features. As of now, sponge COI seems to evolve more slowly than in other phyla (with the exception of the Cnidarians) up to the point that there is a substantial overlap between intra- and closest interspecific variation (= no 'barcoding gap') (Huang *et al*. 2008) and that two different sponge species cannot be discriminated (Schröder *et al.* 2003; Addis & Peterson 2005; Heim *et al.* 2006). The former is mainly a problem for the DNA identification of new sponge species; the latter is a problem for identification of known species. The potential insufficient resolution of COI at the species level has prompted Erpenbeck *et al.* (2006b) to propose a more variable second partition, further downstream in the COI sequence, which has proven to be suited for inter- and intra-specific studies (López-Legentil & Pawlik 2009). It is also clear that barcoding of species should not rely on a single marker but on a multiple marker strategy from different genomes (Savolainen *et al.* 2005; Wörheide *et al.* 2007) so new markers are now on trial in sponges (e.g. 28S, ITS, nad5, CO3). In order to start tagging sponge specimens with DNA sequences and thus initiate a DNA species database, the Sponge Barcoding Project (SBP) was initiated during the 7th International Sponge Symposium in Buzios (Brazil) in 2006: www.spongebarcoding.org/ (Wörheide *et al.* 2007). Morphological description of the sequenced specimens on the SBP is of paramount importance insofar as proper sponge identifications can be challenging and ambiguous. For a discussion on DNA barcoding in sponges see Solé-Cava & Wörheide (2007).

1.4. The morphology of Astrophorida

1.4.1. Spicules

In Demospongiae, siliceous spicules are traditionally distributed into two categories on the basis of their size: megascleres and microscleres. A combination of triaenes and asters is the synapomorphy of the Astrophorida. The diversity of microscleres is especially high in the G4/Demosclavia clade and the Astrophorida has a much more diverse and abundant spicule repertoire in comparison with other sponge orders (e.g. Halichondrida, Haplosclerida). For example, *Geodia barretti* has up to ten different spicule types: two categories of oxeas, triaenes, anatriaenes, protriaenes, mesoprotriaenes and four types of asters. With the development of the scanning electron microscope (SEM) at the end of the '60s, spicule morphology revealed new microstructures (e.g. surface ornamentation, pattern of ramifications), which have been used to resolve spicule homology issues, species discrimination and sponge classification. This tool is now essential in any morphological work on sponges. The Astrophorida is a promising model group to study spicule evolution since they offer such a wide variety of spicules that can be traced through evolution and whose homologies can be tested through phylogenetic reconstruction. This is a much harder task in orders like Haplosclerida (McCormack *et al.* 2002; Redmond *et al.* 2007) or Halichondrida (Erpenbeck *et al.* 2005b; Erpenbeck *et al.* 2006a) offering mostly monaxonic spicules.

1.4.2. Skeleton organization

Astrophorida usually have a radial arrangement of their megascleres, more obvious in the peripheral region. A more or less thick layer of microscleres can form a conspicuous cortex; this is especially true for the Ancorinidae and the Geodiidae. Pachastrellidae, Calthropellidae and Thrombidae do not have a conspicuous cortex although they usually have a thin layer of microscleres at their surface, the term ectosome is then preferred. When long or short-shafted triaenes are present they generally have their cladomes placed under the ectosome or cortex and their rhabdomes perpendicular to it. In species with pseudo-calthrops (= very short-shafted triaenes, as in *Poecillastra compressa* (Bowerbank)) triaenes are more rare and irregularly positioned. When calthrops or mesotriaenes are present, they are usually

very abundant and present throughout the whole sponge, there is no radial arrangement then. Apart from the cortex/ectosome, microscleres can be present in the choanosome, especially around the canals and openings (oscules, pores). In addition to spicules, many species also have thick layers of collagen in their cortex. The Tetractinellida are considered to have secondarily lost spongin (a subtype of collagen, typical of sponges) (Borchiellini *et al.* 2004b) although it has been found in low quantities in *Stelletta grubei* (Simpson *et al.* 1985b), so it may be present in other Astrophorida as well.

1.4.3. Morphogenesis of spicules

Silicon (Si) is the second most abundant element (27%) in the lithosphere after oxygen. This makes silica (SiO_2) the most abundant mineral in the Earth's crust. 92% of the extant sponges have siliceous spicules (Boury-Esnault 2008). Spiculogenesis takes place in a silica under-saturated environment so it is an active process. This process is partially controlled genetically since each species has a characteristic repertoire of spicules and spicule arrangement. It is also partially controlled by physiological processes such as reproduction (Bavestrello *et al.* 1996; Frøhlich & Barthel 1997; Mercurio *et al.* 2000) or nutrition (Frøhlich & Barthel 1997) and environmental parameters (Uriz *et al.* 2003) such as silica concentrations (Stone 1970; Elvin 1971; Pé 1973; Yourassowsky & Rasmont 1984; Frøhlich & Barthel 1997; Maldonado *et al.* 1999; Mercurio *et al.* 2000), water temperature (Stone 1970; Elvin 1971; Bavestrello *et al.* 1993; Mercurio *et al.* 2000) and wave force (Palumbi 1986).

Megascleres and microscleres seem to be produced in specific sclerocytes which require a certain level of silicon concentration to work (Maldonado *et al.* 1999; Uriz *et al.* 2003). Biosilicification takes places around a proteic axial filament (triangular-shaped in the Astrophorida, at least in the megascleres (Simpson *et al.* 1985a; Müller *et al.* 2007)) surrounded by an intracellular membrane called the silicalemma. The axial filament is rich in silicateins (*silica* prot*eins*), assumed to be the key enzymes responsible for the synthesis of the spicules (Shimizu *et al.* 1998; Cha *et al.* 1999). Silicateins have been found in the Silicea sponges (Shimizu *et al.* 1998; Krasko *et al.* 2000; Müller *et al.* 2008). Axial filaments have been observed in megascleres and microscleres of Astrophorida species (Simpson *et al.* 1985a). Axial filaments may impose the overall geometry of the spicules while further spicule ornamentation (spines and swellings) might be controlled by other factors present in the silicalemma (Schönberg 2001; Pisera 2003; Uriz *et al.* 2003). Silicatein has actually been

detected on the surface of spicules, suggesting that they can grow by apposition extracellularly (Müller *et al.* 2005; Schröder *et al.* 2006). It has also been shown that silicate stimulates gene expression of sillicatein and collagen (Krasko *et al.* 2000).

1.4.4. Morphogenesis of sterrasters

In euasters, the axial filaments display a radial arrangement, so each ray of the aster contains a branch of axial filament (Simpson *et al.* 1985a). Therefore, sterrasters also have this polyaxonal filament (Rützler & Macintyre 1978; Simpson 1989). While three isoforms of silicatein could be identified in the axial filament of the megascleres of *Geodia cydonium* (silicateins-α, β and γ), only one could be detected in the axial filament of sterrasters: silicatein-α/β (Müller *et al.* 2007). Each aster is produced within a single microsclerocyte in the choanosome (Sollas 1880; Simpson *et al.* 1985a; Simpson 1989) before being transported to the cortex (Dendy 1921; Hoffmann *et al.* 2003). Therefore, we usually find young stages of sterrasters in the choanosome and fully-grown sterrasters in the endocortex. Once the sterrasters are in the endocortex, collagen fibrils fix them (Sollas 1880; Uriz 2006). Further development and maturation of the sterrasters might involve the silicalemma which might expand from the rays in order for the areas between the rays to become filled with silica (Simpson 1989). Seemingly, deposition of the final 5-15 µm layer of silica including the ray tips (rosettes) is a secondary process apparently unrelated to the axial filaments of the rays (Rützler & Macintyre 1978). The depression (hilum) observed in all sterrasters marks the position of the microsclerocyte nucleus (Sollas 1880).

1.5. Function of spicules

To understand the evolution of spicules, one must first question the function of spicules. The first function of spicules in sponges is structural support. Megascleres especially provide a three-dimensional skeleton that gives the sponge body rigidity and its shape. This is important to colonize space and therefore optimize its filter-feeding activity. It is also important in order to withstand hydrodynamic forces (Palumbi 1986). Microscleres might also reinforce and strengthen the tissue (Koehl 1982); this is especially obvious in the thick cortex rich in microscleres found in many Astrophorida (e.g. Geodiidae). Since sponges may have originated in the Early Neoproterozoic, before eumetazoans predators (Martin *et al.* 2000;

Ivantsov 2009), the use of spicules as protection against predators might be an exaptation, a side-effect of structural reinforcement (Sperling *et al.* 2010). Defense against predators come in two ways: i) the spicules may form a natural strong barrier protecting against the teeth of predators (as in the *Geodia* spp.) and/or ii) the spicules make the sponge dangerous to eat, the spicules might enter the gut (Birenheide *et al.* 1993) and/or make the digestion difficult. May it be megascleres or microscleres, they can deter natural predators such as fish (Randall & Hartman 1968; Burns & Ilan 2003), hermit-crabs (Hill *et al.* 2005), or sea urchins (Birenheide *et al.* 1993; Ferguson & Davis 2008). Conversely, other studies show that the skeleton does not always provide protection (Chanas & Pawlik 1996). Some predators do not seem to be bothered: fish sea turtles (Meylan 1988), some hermit-crabs (Waddell & Pawlik 2000a), sea stars (Waddell & Pawlik 2000b), polychaetes (Pawlik 1983) or chitons (Warén & Klitgaard 1991; Todt *et al.* 2009). Either these predators avoid spicules (Pawlik 1983) or ingest everything with no visible effects (Randall & Hartman 1968; Meylan 1988; Birenheide *et al.* 1993). But the outcomes of these feeding experiments are often contradictory: there does not seem to be a general rule. Predator-sponge relations may be strictly species dependant and therefore difficult to compare. Moreover, studies are now showing that when it comes to defense, spicules can act synergistically with other defense mechanims such as secondary metabolites to deter predators or to attract epibionts which themselves deter predators (Hill *et al.* 2005; Jones *et al.* 2005; Ferguson & Davis 2008). For example, although they have a thick solid cortex, Caribbean *Geodia* are eaten by some fish (Dunlap & Pawlik 1996; Hill & Hill 2002). Instead of having a chemical defense (Pawlik *et al.* 1995), they may use secondary metabolites to promote overgrowth of other species better equipped to defend themselves from fish predation (Wilcox *et al.* 2002; Engel & Pawlik 2005).

Other uses of spicules are known. They are not widespread and are probably also exaptations: spicules can be used for buoyancy of gametes (Uriz *et al.* 2003), buoyancy of planktonic larvae (Vacelet 1999), depth regulation in parenchymella larvae (Maldonado *et al.* 1997), protection of gemmules (e.g. amphidiscs) (Hartman 1981), passive capture of prey (Vacelet & Boury-Esnault 1995), or for conducting light to chlorosymbionts (Brümmer *et al.* 2008). Apart from those very specific functions, most of the spicule diversity that taxonomists use to distinguish different species/genera are difficult to relate to specific functions and appear to be non-adaptative traits (Dendy 1921; Hartman 1981).

2. AIMS OF THIS THESIS

At the base of any biological research there has to be solid alpha-taxonomy (the practice and science of classification). This is particularly true for phylogenetics where interpretations of results are fully dependant on proper specimen identifications. My first aim was therefore to properly identify and in some cases describe/revise the taxonomy of the Astrophorida species that were sampled (**Paper I, II & III**). Although I have had specimens from all over the world, I have mainly sampled in the Atlantic Ocean and thus focused my taxonomical research on Atlantic Astrophorida. In this process, I have assessed the importance of DNA data as an auxiliary criterion to help sponge taxonomists in their task (**Paper III, IV & V**). I have also explored ways to store morphological along with genetic data while participating in the making of the sponge DNA barcoding database (**Paper I, II, III & IV**).

To date, molecular phylogenetic studies including Astrophorida species are scarce and offer limited sampling. Previous Demospongiae molecular phylogenetic studies have included only three to six species of Astrophorida (Borchiellini *et al.* 2004b; Nichols 2005; Erpenbeck *et al.* 2007a) while the most complete study, focusing on the Tetractinellida sampled ten, two of which were lithistids (Chombard 1998). Needless to say that phylogenetic relationships within this order are for the most part unknown and hypotheses based on morphology largely untested. My second aim was therefore to reconstruct a resolved and robust phylogeny of the Astrophorida (**Paper IV & V**). Also, the molecular pioneering work of Chombard (1998) on this taxonomical group suggested that some of its families were not monophyletic. I thus knew that in order to properly revise the order I would need to include in my sampling all the Astrophorida families, and as many species as possible (**Paper V**). My phylogenetic null hypotheses were i) the last major revision of the order, taken from the *Systema Porifera* (Hooper & van Soest 2002b) and ii) the molecular phylogeny from Chombard *et al.* (1998). Part of my aim was also to translate our phylogenetic results into a revision of the Astrophorida classification. To succeed in this, I have made attempts to use and compare a phylogenetic classification (following the rules of the *PhyloCode* v.4c, http://www.ohiou.edu/PhyloCode) and a Linnaean classification (following the International Code of Zoological Nomenclature (*ICZN*), http://www.iczn.org/iczn/index.jsp) (**Paper IV & V**).

Looking for a pattern of evolution often goes along with understanding evolutionary processes. The homology/homoplasy of characters in sponges is a longstanding problem of paramount importance (Boury-Esnault 2006). Since most of the Astrophorida taxonomy relies on spicule morphology, our aim was to re-assess i) the homology (**Paper IV & V**) and ii) the homoplasy (**Paper V**) of Astrophorida spicules. As I said before, because of its high spicule diversity, the Astrophorida is a group of choice to investigate and understand spicule evolution.

To summarize, the three aims of this study were:

1. To provide a solid taxonomical basis of Astrophorida species.
2. To reveal phylogenetic relationships within the Astrophorida.
3. To investigate the evolution of Astrophorida spicules.

3. LIST OF PAPERS

Paper I

Cárdenas, P., Xavier, J., Tendal, O.S., Schander, C. & Rapp, H.T. (2007) Redescription and resurrection of *Pachymatisma normani* (Demospongiae, Geodiidae), with remarks on the genus *Pachymatisma*. *Journal of the Marine Biological Association of the United Kingdom*, 87, 1511-1525.

Paper II

Cárdenas, P., Menegola, C., Rapp, H.T. & Díaz, M.C. (2009) Morphological description and DNA barcodes of shallow-water *Tetractinellida* (Porifera: Demospongiae) from Bocas del Toro, Panama, with description of a new species. *Zootaxa*, 2276, 1-39.

Paper III

Cárdenas, P. & Rapp, H.T. A review of Astrophorida with streptasters (Porifera, Demospongiae) from Norway, new records and a new species (manuscript).

Paper IV

Cárdenas, P., Rapp, H.T., Schander, C. & Tendal, O.S. (2010) Molecular taxonomy and phylogeny of the Geodiidae (Porifera, *Demospongiae*, Astrophorida) — combining phylogenetic and Linnaean classification. *Zoologica Scripta*, 39, 89-106.

Paper V

Cárdenas, P., Xavier, J., Reveillaud, J., Schander, C. & Rapp, H.T. Molecular taxonomy and phylogeny of the Astrophorida (Porifera, *Demospongiae*) — an unexpected high level of spicule homoplasy (manuscript).

4. RESULTS AND DISCUSSION

4.1. Taxonomy of the Atlantic Astrophorida species

4.1.1. The Atlantic Astrophorida species

Specimens from the North-East Atlantic (NEA) were collected in Western Norway (Bergen area), Southern Norway (BIOSKAG 2006), Northern Norway (Polarstern ARK-XXII/1a, 2007) and the Greenland Sea (BIODEEP2007, H2DEEP 2008) (Paper I, III, IV & V) using a triangular dredge (Fig. 3A), boxcores, a Van Veen grab, an Agassiz trawl, a Sneli sled (Sneli 1998), remote operated vehicles ('Aglantha' and 'Bathysaurus XL') and the manned-submersible 'Jago'. Specimens from the Western Atlantic were collected by diving and snorkeling in Bocas del Toro, Panama (Paper II). Spirophorida specimens (outgroups for Paper V) were collected at the same time, in the NEA and Panama (Paper II & V). The rest of the specimens used for comparative material or the phylogenetic study came from different collaborators, institutions and campaigns (cf. Acknowledgments in Paper I to V). All in all, I examined more than 600 specimens of Astrophorida.

My taxonomy and nomenclatural studies focused on the Astrophorida from the Caribbean coast of Panama (Paper II) and from the Norwegian coast (Paper I & III). Paper I and III (along with the phylogenetic study of Paper V) resulted in many new records and taxonomical decisions concerning the NEA Astrophorida: two species are recorded for the first time in Norway (*Characella pachastrelloides* (Carter) and *Vulcanella aberrans* (Maldonado & Uriz), Paper III), one species is given a new name (*Geodia hentscheli*, Paper IV), one species is synonymized (*Geodia simplississima* Burton, Paper V), two species are resurrected (*Pachymatisma normani* Sollas, Paper I; *Thenea schmidti* Sollas, Paper III) and one is new for science (*Pachastrella nodulosa*, Paper III). Concerning the Western-Atlantic sponge fauna: four species are recorded for the first time on the Caribbean coast of Panama (*Cinachyrella kuekenthali* (Uliczka), *Ecionemia megastylifera* Wintermann-Kilian & Kilian, *Stelletta fibrosa* (Schmidt), *Stelletta* sp., Paper II), two species are synonymized (*Erylus bahamiensis* Pulitzer-Finali and *Ecionemia dominicana* (Pulitzer-Finali), Paper II), one species is resurrected (*Geodia tumulosa* Bowerbank, Paper II & V) and one is new for science (*Stryphnus raratriaenus*, Paper II).

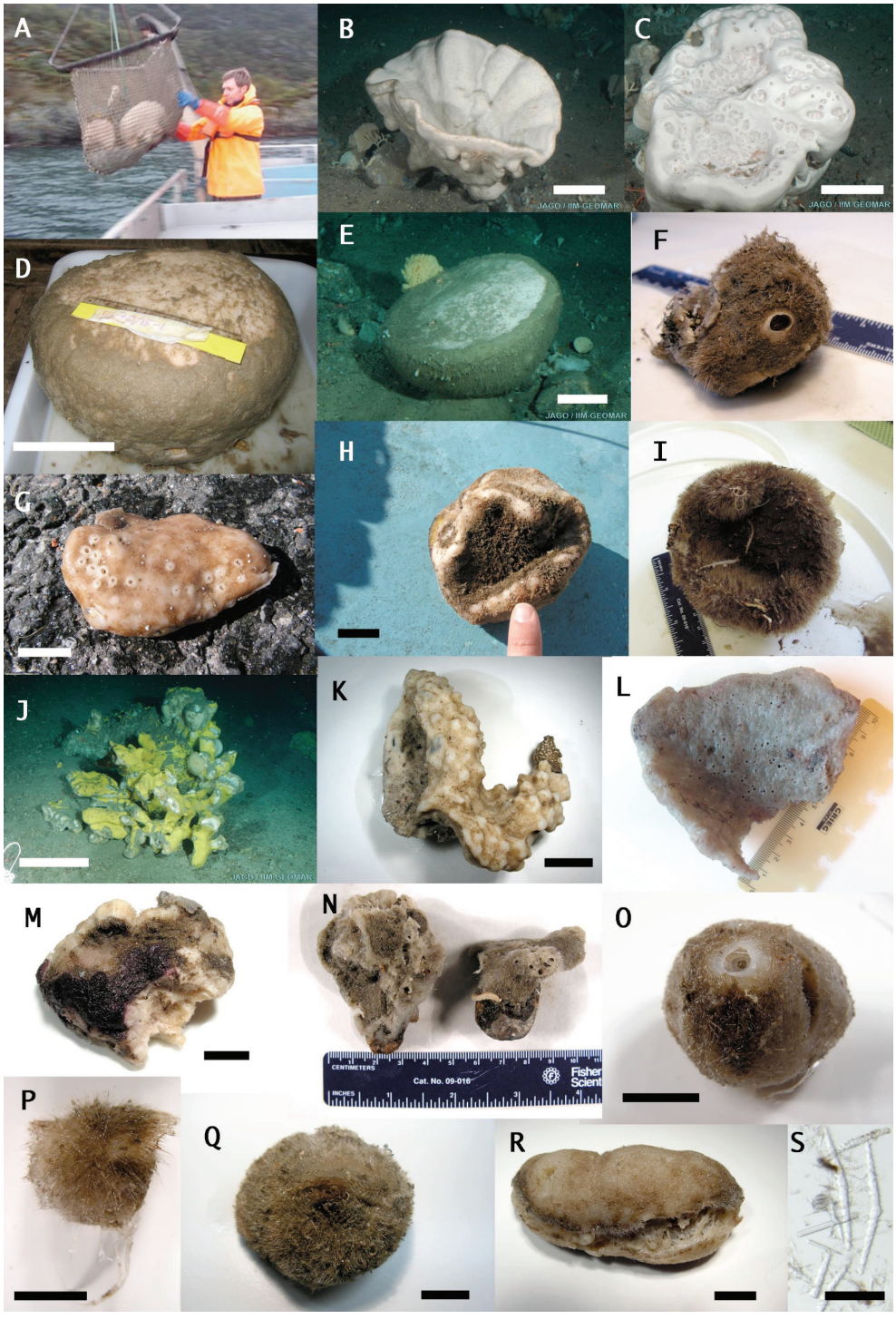
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Figure 3. **A.** H. T. Rapp bringing the triangular dredge with sponge samples on board of the *R/V Brattström* in the Korsfjord, Western Norway. **B.** *Geodia atlantica* (not sampled) in Trænadjupet, Northern Norway (manned-submersible dive). Scale: 10 cm. **C.** *Geodia barretti* (#PS70/27-1(11)) in Trænadjupet, Northern Norway (manned-submersible dive). Scale: 10 cm. **D.** *Geodia macandrewi* (ZMBN 85207) from Northern Norway. Scale: 10 cm. **E.** *Geodia phlegraei* (not sampled) in Trænadjupet, Northern Norway (manned-submersible dive). Scale: 8 cm. **F.** *Geodia hentscheli* (#PC221) from the Schultz Massive seamount, Greenland Sea. **G.** *Pachymatisma normani* (09/05/07) from the Korsfjord, Western Norway. Scale: 1 cm. **H.** *Stelletta normani* (10/03/06) from the Korsfjord, Western Norway. Scale: 2 cm. **I.** *Stelletta raphidiophora* from the Schultz Massive seamount, Greenland Sea. **J.** *Stryphnus fortis* (#PS70/27-1(8)) in Trænadjupet, Northern Norway (manned-submersible dive). It is covered by the encrusting yellow sponge *Hexadella detritifera*. Scale: 40 cm. **K.** *Pachastrella* nov. sp. (ZMBN 85243) from the Korsfjord, Western Norway. Scale: 3 cm. **L.** *Poecillastra compressa* (ZMBN 77932) from Langenuen, Western Norway. **M.** *Characella pachastrelloides* (ZMBN 80248) from the Hjeltefjord, Western Norway. Scale: 1 cm. **N.** *Vulcanella aberrans* (ZMBN 80959) from Trænadjupet, Northern Norway. **O.** *Thenea muricata* (ZMBN 85231) from Marstein, Western Norway. Scale: 1 cm. **P.** *Thenea abyssorum* (ZMBN 85228) from the mid-Atlantic arctic ridge, Greenland Sea. Scale: 1 cm. **Q.** *Thenea valdiviae* (ZMBN 85256) from Freisfjorden, Western Norway. Scale: 1 cm. **R.** *Thenea levis* (ZMBN 85249) from Marstein, Western Norway. Scale: 1 cm. **S.** Spicules of *Alectona millari* (ZMBN 85238) from Sotbakken, Northern Norway. Scale: 500 µm.

Because of a sampling bias towards the NEA/Mediterranean Sea, I focused my attention on reviewing the taxonomy of Astrophorida from that region. All Astrophorida known from the areas we surveyed (Norwegian coast, the Barents Sea and the Greenland Sea) were encountered (Fig. 3), except for *Geodia simplex* Schmidt, 1870, a dubious species. Appendix A reviews the status of all the Astrophorida species from the NEA/Mediterranean Sea according to my examination of specimens (Papers I, III & unpublished results), phylogenetic results (Papers IV & V) and/or the literature. There is a total of 116 NEA/Mediterranean Sea Astrophorida (non-lithistid): 49 Geodiidae (42,2%), 27 Ancorinidae (23,3%), 9 Pachastrellidae (7,7%), 8 Vulcanellidae (6,9%), 8 Theneidae (6,9%), 1 Thrombidae (0,8%), 12 Thoosidae (10,3%) and 2 *incertae sedis* (*Characella*) (1,7%). Astrophorida are certainly more numerous since lithistids were not included in this table. Indeed, they were absent in our arctic-boreal sampling, and not the primary focus of this study. Of these 116 species, 51 (43,9%) are restricted to the NEA while 27 (23,3%) are restricted to the Mediterranean Sea (Appendix A). The remaining 38 species (32,7%) are distributed in both regions. Of the 17 single-area occurrences, 11 species were described from the NEA and 6 species from the Mediterranean. We have encountered and collected 19 (16,4%) species on the Norwegian coast, the Barents Sea and the Greenland Sea (Fig. 3).

4.1.2. Integrative taxonomy

The utility of DNA sequences for taxonomy purposes is well established. DNA brings alternative independent characters in order to reassess the validity of species and their morphological characters. New methods for DNA species delimitation are being developed (e.g. DNA barcoding) while maintaining the importance of morphological or other information (Tautz *et al.* 2003). As a consequence, the 'integrative taxonomy' approach combining all kinds of data (external morphology, spicules, embryology, geography, reproduction, genetic sequences...) is now considered a reliable and efficient way to evaluate the status of a species (Dayrat 2005; DeSalle *et al.* 2005; Padial & De La Riva 2007; Padial *et al.* 2009) while keeping in mind that discordance among lines of evidence does not automatically imply that a species hypothesis is invalid (Padial *et al.* 2009). I have therefore always confronted my molecular results with independent data before taking any taxonomical decision. The Astrophorida molecular phylogenetic analyses have initiated the taxonomical revision of some genera (cf. 4.2.), species or specimens, most of which are discussed in Paper IV and V. The phylogenetic analysis has notably supported the resurrection of *Pachymatisma normani* (Paper I), *Geodia tumulosa* and *Thenea schmidti* (Paper V) and the synonymization of *Geodia simplicissima* (Paper V). It has also cast doubts on the monophyly of *Penares helleri*, *Geodia cydonium* and *Geodia megastrella* Carter (Paper V).

I will here illustrate how DNA and morphological data can complement each other through the example of *Geodia simplicissima,* briefly mentioned in Paper V. It had been originally collected at a fairly shallow depth of 10-75 m, in the Foldenfjord (Northern Norway) (Burton 1931) and, having extensively sampled most of the Norwegian coast, we were surprised never to have found it. Two specimens were finally collected while diving in Trellholmstetta (Western Norway) at a shallow depth of 34 m. Surprisingly, their external morphology (not illustrated by Burton (1931)) and their COI sequences were identical to those of *Geodia barretti*. This was unexpected since COI had clearly discriminated all the other *Geodia* species sampled (Paper V), so we strongly suspected *G. simplicissima* to be a junior synonym of *G. barretti.* To compare *G. barretti* and *G. simplicissima*, thick sections (Fig. 4A-D) and SEM spicule pictures (Fig. 5A-D) were made. Their morphologies were fairly different. The main differences between both species concerned i) the organization of the cortex and ii) the morphology of the sterrasters. The cortex of *G. simplicissima* is more plastic and compressible than in *G. barretti.* One of the main reasons is that sterrasters are

rare, small and underdeveloped. Also, triaenes are smaller, have irregular clads and show additional swellings on the rhabdome. These conspicuous morphological differences raised new doubts about COI being fit to discriminate both species. But the finding of a similar pattern in shallow *Pachymatisma* resurrected our initial hypothesis. Shallow *Pachymatisma* looked very much like deep specimens of *Pachymatisma normani* but they were colored and had a much more flexible cortex. Thick sections (Fig. 4E-H) and SEM pictures (Fig. 5E-H) showed that they also had a thinner cortex with rare and underdeveloped sterrasters, and a thick fibrillar collagen layer under the endocortex. Moreover, their COI was identical to that of *P. normani*. We strongly suspect that environmental parameters are responsible for this major phenotypic modification. Since the influence of silica concentration on spiculogenesis has often been demonstrated (Stone 1970; Elvin 1971; Pé 1973; Yourassowsky & Rasmont 1984; Frøhlich & Barthel 1997; Maldonado *et al.* 1999; Mercurio *et al.* 2000), we think spiculogenesis in *G. barretti* and *P. normani* could have been disrupted due to the lower silica concentrations found at shallower depths.

This example illustrates how DNA taxonomy can represent a powerful complement to traditional morphological taxonomy, especially for the detection of i) morphological polymorphic species (as in the case of *G. simplicissima*) and of ii) morphological cryptic species (Paper I). One of the main advantages of DNA characters of a species being that, in a human time-frame, they are not as much influenced by environmental conditions as sponge phenotypic morphological characters.

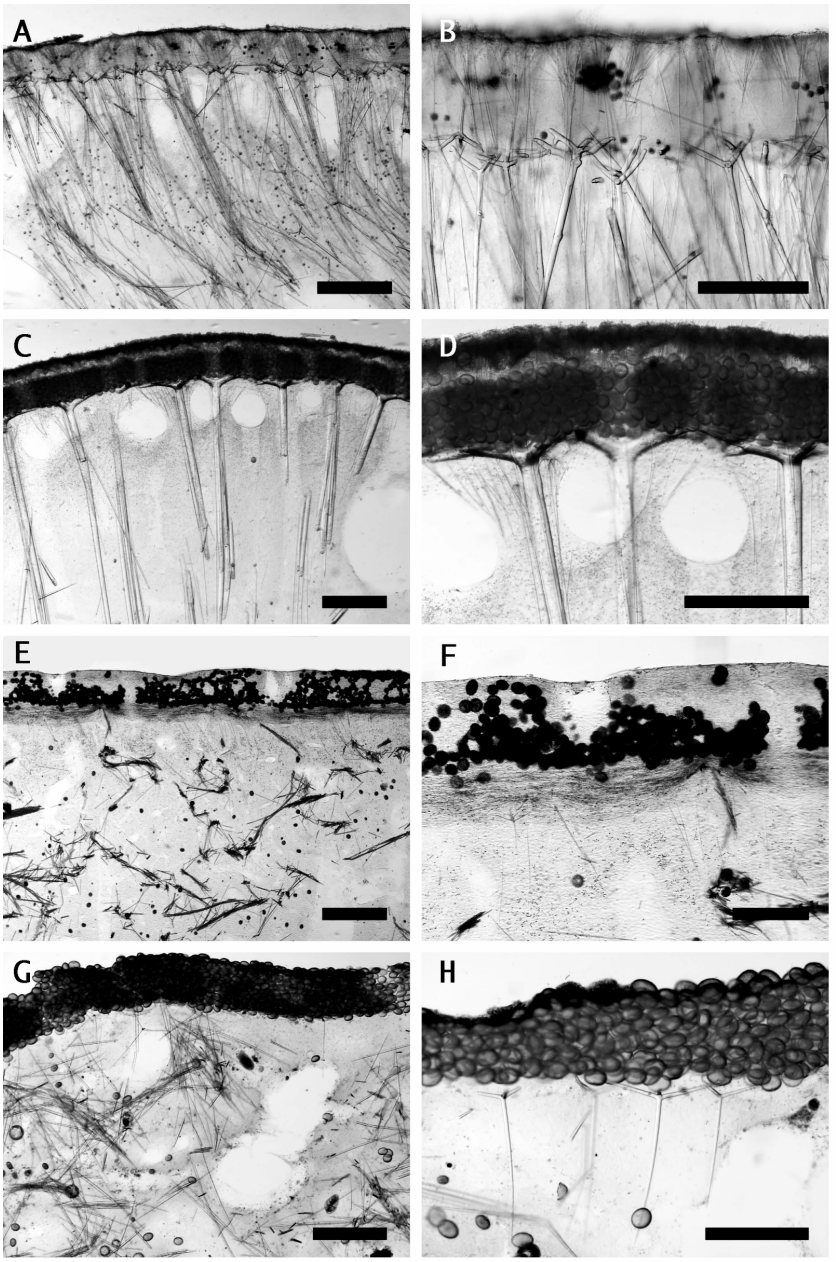


Figure 4. **A.** Thick section of *Geodia simplicissima* (ZMBN 85212) showing cortex and choanosome. Scale: 1 mm. **B.** Close-up on the cortex. Scale: 500 µm. **C.** Thick section of *Geodia barretti* showing cortex and choanosome. Scale: 1 mm. **D.** Close-up on the cortex. Scale: 1 mm**. E.** Thick section of shallow *Pachymatisma normani* (#PC434) showing cortex and choanosome. Scale: 1 mm. **F.** Close-up on the cortex. Scale: 500 µm. **G.** Thick section of deep *P. normani* (ZMBN 77858, neotype) showing cortex and choanosome. Scale: 1 mm. **H.** Close-up on the cortex. Scale: 1 mm.

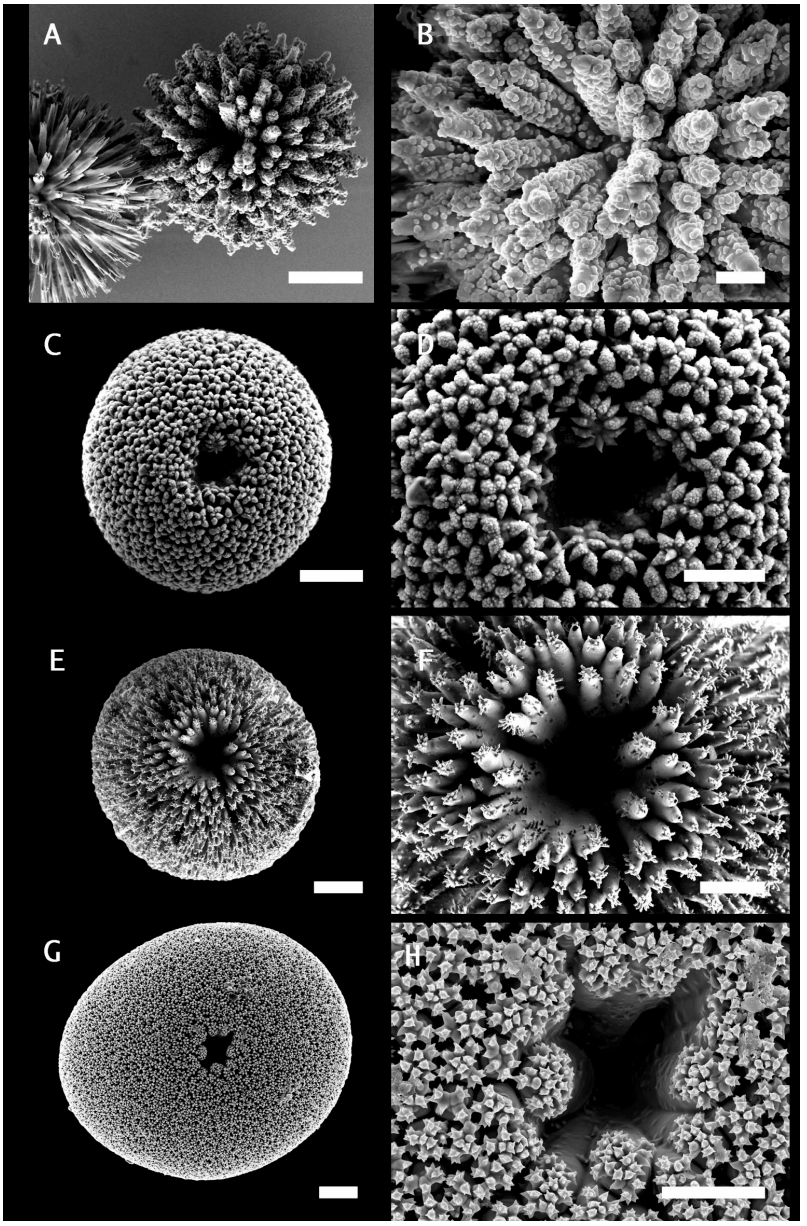


Figure 5. **A.** Sterrasters of *Geodia simplicissima* (ZMBN 85212). Scale: 10 µm. **B.** Close up of a sterraster (ZMBN 85212) showing possible microspheres of silica polymerization. Scale: 2 µm. **C.** Sterrasters of *Geodia barretti* (ZMBN 77922). Scale: 20 µm. **D.** Close up of a sterraster (ZMBN 77922) showing hilum and warty rosettes at the tip of the actines. Scale: 10 µm. **E.** Sterrasters of shallow *Pachymatisma normani* (#PC434). Scale: 20 µm. **F.** Close up of a sterraster (#PC434) showing hilum and spines on the actines. Scale: 10 µm. **G.** Sterrasters of deep *P. normani* (ZMBN 77858, neotype). Scale: 20 µm. **H.** Close up of a sterraster (ZMBN 77858, neotype) showing hilum and rosettes at the tip of the actines. Scale: 10 µm.

4.1.3. Web storage of taxonomical, morphological and genetic data

Following the rise of Genbank, numerous websites enabling to store morphological and taxonomical data have emerged: Zoobank (www.zoobank.org/), MorphDBase (www.morphdbase.de/) or MorphoBank (www.morphobank.org/) (Fig. 6). Thinking morphological data from the sequenced specimens should be stored and accessible to future researchers, I have explored and used two of these web applications: MorphoBank (Paper I) and the Sponge Barcoding Project (SBP) website: www.spongebarcoding.org (Paper II, III & IV) (Fig. 7).

I chose to use MorphoBank because it was specifically designed for morphological phylogenetics and cladistics research. Features I appreciated were that i) every specimen loaded is attached to its collecting information (Fig. 6), ii) the amount of data one can store is unlimited, iii) pictures can be annotated (Fig. 6) and iv) every picture gets a MorphoBank accession number so that it is easily traceable and can be cited in an article (Fig. 6).

Figure 6. Screenshot of an annotated *Pachymatisma normani* picture (M9625) stored in MorphoBank (Cárdenas *et al.* 2007).

Record #173: *Pachymatisma normani*

Taxonomic Information from World Porifera Database

Entrez cross-database search for *Pachymatisma normani*

Specimen Information

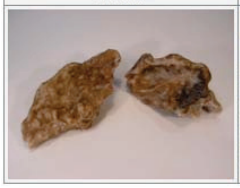
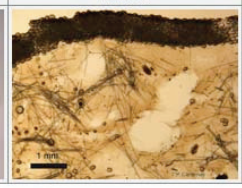
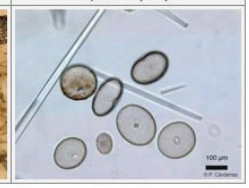
Status		**Submitted**
Submitted as		*Pachymatisma normani*
Collection	**date**	2007-03-21
	location	Korsfjord, Norway (60° 10' 00'' N, 5° 10' 00'' E)
	by	Hans Tore Rapp
Voucher number		ZMBN 77858
Voucher location		Museum of Bergen
Preservation method		Ethanol 96%
Identified by		Paco Cárdenas and Hans Tore Rapp
Morphological description (show/hide)		
Additional information		This is the neotype from the type locality. The holotype from the Norman Collection (Sollas, 1882) is presumably lost.

Reference (show/hide)

Cárdenas, P., Xavier, J., Tendal, O.S., Schander, C. & Rapp, H.T. (2007) Redescription and resurrection of Pachymatisma normani (Demospongiae, Geodiidae), with remarks on the genus Pachymatisma. Journal of the Marine Biological Association of the United Kingdom, 87, 1511-1525.

Cárdenas, P., Rapp, H.T., Schander, C. & Tendal, O.S. (2010) Molecular taxonomy and phylogeny of the Geodiidae (Porifera, Demospongiae, Astrophorida) — combining phylogenetic and Linnaean classification. Zoologica Scripta, 39, 89-106.

Pictures

in situ	section	spicule prep
		

Associated DNA Sequences

Show/Hide	Sequence #174	CO1 Folmer	Genbank EF564322
Download sequence in FASTA format			

Show/Hide	Sequence #178	ITS1 and ITS2	Genbank EF577051
Comment: 5.8S included			
Download sequence in FASTA format			

Show/Hide	Sequence #230	28S, 5' fragment	Genbank EU552087
Comment: D1-C2-D2 domains			
Download sequence in FASTA format			

Search | Specimen List | Login

Figure 7. Screenshot of the submitted record of the neotype of *Pachymatisma normani* in the Sponge Barcoding Project at www.spongebarcoding.org (accessed on 1st March 2010).

Unfortunately, MorphoBank has no link to the genetic data storage websites. In that sense, I found it less practical than the SBP. On the SBP, every voucher has its own webpage (Fig. 7) with all the collecting/identification data, and morphological information. Furthermore, the SBP links the voucher to the GenBank tag sequences (http://www.ncbi.nlm.nih.gov) and to the 'World Porifera Database' (www.marinespecies.org/porifera). On the other hand disadvantages of the SBP are: i) 'sp.' specimens are not accessible to the public and ii) the amount of morphological data stored is limited. One way to make up for these weaknesses is to publish simultaneously the morphological data of the vouchers and their DNA tags (Paper II & III) so that taxonomists can have a full description along with a discussion on the specimen studied.

4.2. Phylogenetic relationships within the Astrophorida

We extracted the DNA from a total of 445 specimens of Astrophorida, 172 of which gave no sequences because i) the specimen had not been properly fixed and the DNA was too degraded (Box 2), ii) the specimen had been stored too long, iii) the specimen was contaminated or iv) co-purified contaminants were blocking the PCR reactions (Paper II). The oldest fixed specimen we managed to get a COI sequence of had been collected in Yucatan (Mexico) on the 19th of October 1985 (24 years before the extraction).

When PCRs did not work although DNA was present, DNA quality was assessed using a Nano-Drop-1000 Spectrophotomoter. This showed us that the Viogene DNA extraction kit was not always very efficient to get rid of co-purified contaminants. Figure 8 shows the example of DNA extracted from *Stryphnus raratriaenus* (a newly described species from Panama: Paper II). We have consistently had PCR problems with species of this genus, possibly because they produce specific secondary metabolites which tend to block the PCR reactions. One can see how the DNA quality increases when we use a standard phenol/chloroform DNA extraction technique or if we add an extra step of DNA cleaning (precipitation, drying, extra washing with 70% ethanol): the 230 nm wavelength decreases, the DNA peak at 260 nm is clearer.

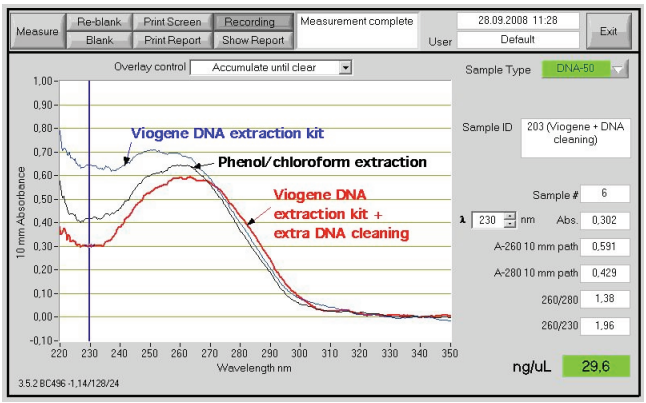
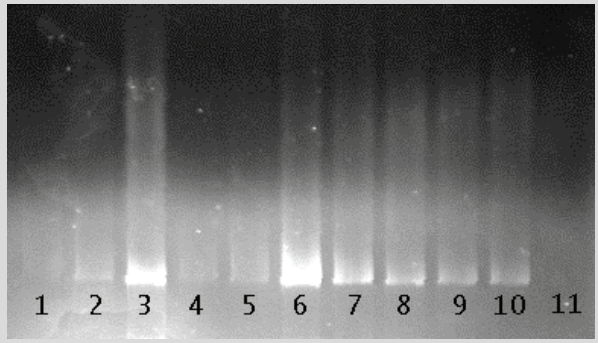


Figure 8. Nano-drop measurement curves of three DNA extractions of *Stryphnus raratriaenus* treated differently.

Box 2: Preservation of sponge material for molecular studies.

A specimen of *Geodia barretti* (#PC250) was collected in the Korsfjord. Small pieces (ca 10 mg) were fixed in various ways (1-11). DNA was extracted one month after, using the Viogene DNA extraction kit. Results can be seen on the DNA agarose gel below.

1. formalin 4% (50 ml)
2. -20°C freezer
3. liquid Nitrogen (-196°C)
4. drying at 60°C
5. ethanol 75% (50 ml)
6. ethanol 96% (50 ml)
7. methanol 75% (50 ml)
8. methanol 96% (50 ml)
9. acetone 75% (50 ml)
10. acetone 96% (50 ml)
11. xylene (50 ml)



The best preservation methods were clearly liquid nitrogen and ethanol 96%. The next best method would be methanol 75%. Similar results were found when we repeated this experiment with *Phakellia ventilabrum* (Axinellidae) and *Sycon ciliatum* (Sycettidae), both from the same area. Ethanol being easier to use in the field, all of our samples were fixed in 96% ethanol.

Astrophorida pairwise percentages of sequence divergence measurements in ITS (Paper I), the COI Folmer fragment and the 28S(C1-D2) partition (Paper IV & V) are summarized in Table 1. The ITS marker has been only studied on two *Pachymatisma* sister-species so the divergence measurements are not comparable with COI and 28S(C1-D2).

Table 1. Pairwise percentages of sequence divergence (from uncorrected 'p' distances) within the Astrophorida in the three molecular markers used in this study.

Molecular marker	**ITS1-5.8S-ITS2**	**COI**	**28S(C1-D2)**
Intra-specific divergence (%)	0-0.47	0-1	0.25-0.97
Inter-specific divergence (%)	0.36	0-18.6	0.12-26.0

All COI and 28S intra-specific distances are nested *within* the range of inter-specific values which makes it difficult to propose a standard sequence threshold to reveal new Astrophorida cryptic species (Hebert *et al.* 2004). However, we should emphasize that all species sequenced in our study are clearly discriminated and identifiable with either COI or 28S(C1-D2), except for some *Thenea* species not discriminated by COI (Paper III & V). 28S(C1-D2) evolves slightly more rapidly than COI (Paper IV) so it seems a better barcoding marker for species identification than COI. In order to confirm this, a larger intra-specific sampling is required. To conclude, using COI in combination with 28S(C1-D2) for the identification of known Astrophorida species is possible but ill suited for a screening procedure in order to detect new Astrophorida species.

The mitochondrial COI and the nuclear 28S(C1-D2) have independent evolutionary histories, but they were nonetheless shown to give congruent phylogenetic relationships among the Geodiidae (Paper IV). For a comprehensive study of the Astrophorida, they were therefore analyzed together in a single matrix containing a total of 152 (potential) Astrophorida specimens (29 genera, 2 sub-genera, 89 species) and 1,527 characters. The resulting maximum-likelihood (ML) tree (Paper V: Fig. 1) is repeated here (Fig. 9) for the readers' convenience. In short, i) the monophyly of the Astrophorida was confirmed (including lithistids, *Alecona* and *Neamphius*), ii) the Euastrophorida and Streptosclerophorida were both found polyphyletic, iii) the Calthropellidae were monophyletic (and found to be a subfamily of the Geodiidae), iv) the Geodiidae, the Ancorinidae and the Pachastrellidae appeared polyphyletic and had to be redefined, v) a new subfamily of the Geodiidae was revealed, the Caminellinae subfam. nov. and finally vi) some genera were found to be polyphyletic (*Ecionemia, Erylus, Poecillastra*, *Penares, Rhabdastrella*, *Stelletta* and *Vulcanella*). Furthermore, amphiasters appeared for the first time to be a synapomorphy for a clade henceforth named *Amphiastrosa* (created under the *PhyloCode*): the clade comprising amphiaster- and euaster-bearing Astrophorida. These results suggested a revised classification of the Astrophorida, presented and discussed in

Papers IV and V. It is briefly summarized below, according to the Linnaean classification, along with changes made to the *Systema Porifera* classification. A morphological key to the Astrophorida families, subfamilies and genera *incertae sedis* is proposed in Box 3.

Revised classification of the Astrophorida (Paper IV & V):

Order Astrophorida Sollas, 1888

Family Thrombidae Sollas, 1888

Thrombus Sollas, 1886

Yucatania Gómez, 2006

Family Thoosidae Rosell and Uriz, 1997 (resurrected and reallocated from the Hadromerida)

Alectona Carter, 1879

Delectona de Laubenfels, 1936

Thoosa Hancock, 1849

Family Theneidae Carter, 1883 (resurrected, new definition)

Annulastrella Maldonado, 2002 (upgraded to the genus level)

Cladothenea Koltun, 1964

Thenea Gray, 1867

Family Vulcanellidae **fam. nov.**

Poecillastra Sollas, 1888 (new definition)

Vulcanella Sollas, 1886 (new definition)

All the taxa below belong (or may belong) to the *Amphiastrosa:*

Family Pachastrellidae Carter, 1875 (new definition)

Brachiaster Wilson, 1925

Pachastrella Schmidt, 1868

Triptolemma de Laubenfels, 1955

Family Geodiidae Gray, 1867 (new definition)

- Subfamily Erylinae Sollas, 1888 (resurrected, new definition)

  Caminus Schmidt, 1862

  ?*Melophlus* Thiele, 1899 (reallocated from the Ancorinidae)

  Erylus Gray, 1867 (new definition)

  Pachymatisma Bowerbank *in* Johnston, 1842

  Penares Gray, 1867 (reallocated from the Ancorinidae, new definition)

- Subfamily Geodinae Sollas, 1888 (resurrected)

  Geodia Lamarck, 1815 (new definition, new synonyms: *Ecionemia* Bowerbank, 1864 (in part); *Isops* Sollas, 1880; *Rhabdastrella* Thiele, 1903; *Sidonops* Sollas, 1889 and *Stelletta* Schmidt, 1862 (in part))

- Subfamily Calthropellinae von Lendenfeld, 1907 (downgraded to sub-family level)
  - *Calthropella* Sollas, 1888
  - *Chelotropella* von Lendenfeld, 1907
  - *Pachastrissa* von Lendenfeld, 1903
  - *Pachataxa* de Laubenfels, 1936
- Subfamily Caminellinae **subfam. nov.**
  - *Caminella* von Lendenfeld, 1894 (resurrected)

Family Ancorinidae Schmidt, 1870

- Subfamily Sanidasterinae Sollas, 1888 (resurrected)
  - *Dercitus* Gray, 1867 (reallocated from the "Pachastrellidae")
  - *Disyringa* Sollas, 1888
  - ?*Ecionemia* Bowerbank, 1864 (in part)
  - ?*Psammastra* Sollas, 1886
  - *Stoeba* Sollas, 1888 (reallocated from the "Pachastrellidae")
  - *Stryphnus* Sollas, 1886 (new synonym: *Asteropus* Sollas, 1888)
  - ?*Tribrachium* Weltner, 1882
- Subfamily Stellettinae Carter, 1875 (resurrected)
  - ?*Ancorina* Schmidt, 1862
  - ?*Cryptosyringa* Vacelet, 1979
  - *Stelletta* Schmidt, 1862
  - *Tethyopsis* Stewart, 1870

Family Corallistidae Sollas, 1888
Family Theonellidae von Lendenfeld, 1903
Family Phymaraphiniidae Schrammen, 1924
} They belong to the *Amphiastrosa*

Family Isoraphiniidae Schrammen, 1924
Family Macandrewiidae Schrammen, 1924
Family Neopeltidae Sollas, 1888
Family Phymatellidae Schrammen, 1910
Family Pleromidae Sollas, 1888
} Probably belong to the *Amphiastrosa*

Characella Sollas, 1886 *incertae sedis* (new definition)
Neamphius de Laubenfels, 1953 *incertae sedis* (reallocated from the Hadromerida)
} Belong to the *Amphiastrosa*

Acanthotriaena Vacelet et al., 1976 *incertae sedis*
Lamellomorpha Bergquist, 1968 *incertae sedis*
Jaspis Gray, 1867 incertae sedis
} Probably belong to the *Amphiastrosa*

Holoxea Topsent, 1892 *incertae sedis* (asters lost)
Ancorella von Lendenfeld, 1907 *incertae sedis* (asters lost)

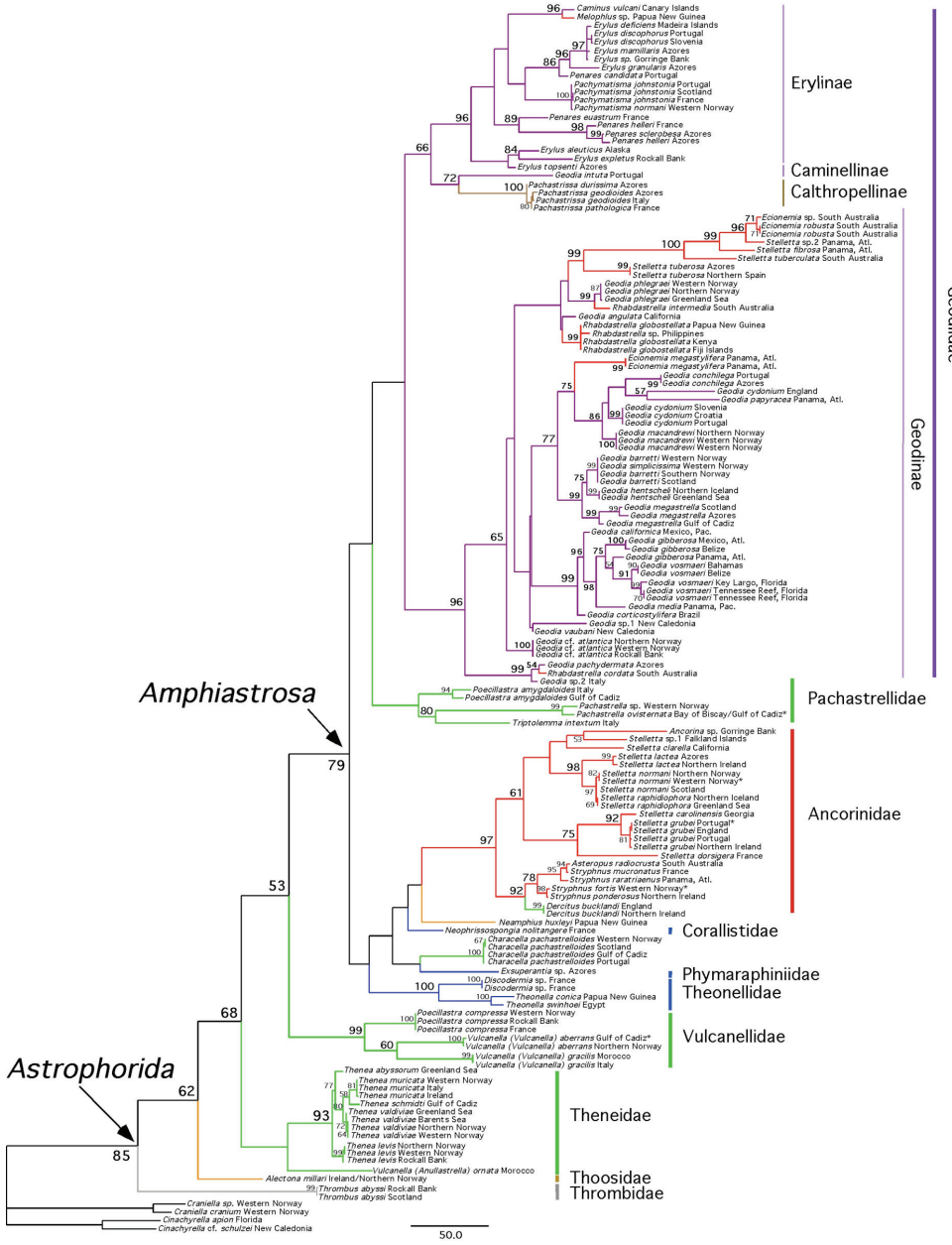


Figure 9. Maximum-likelihood phylogeny of the Astrophorida derived using 28S+COI partial sequences (1,527 pb.) from 152 Astrophorida taxa (89 species) and four Spirophorida outgroups analyzed under the GTR + I + G model. Bootstrap nodal support values > 50% are given at the nodes (2,000 replicates). Sub-family and family names result from the discussion in Paper V.

Box 3: Key to the Astrophorida families, sub-families and genera *incertae sedis* (lithistids not included).

1. Asters absent 2
   Asters present 3

2. Microscleres are spiny microxeas; calthrops with an aborted fourth actine ..... *Ancorella*
   Microrhabds in ectocortex; short-shafted triaenes ..... **Geodiidae** (Erylinae), *Penares* (in part)
   Microscleres trichotriaenes are present ..... **Thrombidae**, *Thrombus* (in part)
   Microscleres include sanidasters; short-shafted dichotriaenes ..... **Ancorinidae** (Sanidasterinae), *Stoeba*
   Microscleres include sanidasters and trichodragmata (sometimes lost); triaenes absent ..... *Holoxea*
   No microscleres; long-shafted plagiotriaene; anatriaenes and oxeas ..... *Stelletta anasteria*

3. Asters are euasters (sometimes modified to toxas) 4
   Asters are streptasters (plesiasters, metasters and/or amphiasters) 5
   Asters are amphiasters and thin-rayed oxyasters (sometimes modified to toxas); excavating sponge ..... **Thoosidae**, *Thoosa*

4. Microscleres include sanidasters; triaenes are long-shafted triaenes (sometimes secondarily lost) or calthrops ..... **Ancorinidae** (Sanidasterinae)[1]
   Microscleres include only euasters (sometimes modified to toxas) 6
   Euasters include sterrasters (sometimes secondarily lost*) in the endocortex and another kind of euasters in the ectocortex; triaenes are long-shafted 7
   Euasters include sterrasters or aspidasters (sometimes secondarily lost*) in the endocortex and spherules or microrhabds in the ectocortex; triaenes are short-shafted; ana/pro/mesotriaenes are absent ..... **Geodiidae** (Erylinae)
   Microscleres do not include sterrasters/aspidasters and sanidasters; triaenes are calthrops (sometimes in combination with long-shafted triaenes) and short-shafted mesotriaenes ..... **Geodiidae** (Calthropellinae)

5. Streptasters are mainly amphiasters 8
   Streptasters are mainly spirasters and plesiasters 9

6. Triaenes present ..... **Ancorinidae** (Stellettinae)
   Triaenes absent ..... *Jaspis*

7. Uniporal oscule leads into a cloaca ..... **Geodiidae** (Caminellinae)
   Uniporal oscule does not lead into a cloaca ..... **Geodiidae** (Geodinae)

8. Megascleres include calthrops (sometimes with an aborted fourth actine) or short-shafted mesotriaenes and/or mesotrider desmas ..... **Pachastrellidae**
   Megascleres are long-shafted triaenes ..... *Characella*
   Triaenes are absent 10

9. Microxeas present; no acanthotriaenes ..... **Vulcanellidae**
   Microxeas present; long-shafted acanthotriaenes present ..... *Acanthotriaena*
   Microxeas absent ..... **Theneidae**

10. Robust diactine or polyactine megascleres; excavating sponge ..... **Thoosidae,** *Alectona*
    Robust diactine/polyactine megascleres absent 11

11. Trichotriaenes present ..... **Thrombidae**
    Trichotriaenes absent 12

12. No megascleres; microscleres include microrhabds ..... **Thoosidae**, *Delectona*
    Megascleres include only oxeas; excavating sponge in its early stage ..... *Neamphius*
    Megascleres include oxeas, strongyles and strongyloxeas; other microscleres are microstrongyles ..... *Lamellomorpha*

* DNA sequencing is necessary to reveal this loss.
[1] The phylogenetic position of *Ancorina* is ambiguous (Paper V). To simplify this key, we considered *Ancorina* to be part of the Sanidasterinae (as suggested by morphological data).

Some clades could not be named under the Linnaean classification because i) they need to be confirmed by independent data or ii) we are missing the type species of the genus or of the family necessary to take a taxonomical decision. Introducing a phylogenetic classification (under the rules of the *PhyloCode* v.4c, January 2010, www.ohio.edu/phylocode) of the Astrophorida (Paper IV & V) enabled us to i) name those clades while waiting for further studies and sampling, ii) communicate and compare our results more efficiently and iii) suggest a new classification based only on clades, which can be tested later with independent data. In a way, the phylogenetic classification established the foundations for future taxonomical revisions and phylogenetic investigations of the Astrophorida.

4.3. The evolution of spicules in Porifera

4.3.1. The Astrophorida, a homoplasy-rich group

Our phylogenetic tree gave us an opportunity to follow the evolution of Astrophorida sponge spicules (cf. discussion in Paper IV & V). Mapping the microscleres and megascleres on the molecular tree was a way to reveal synapomorphies and plesiomorphies for the different taxa and thus investigate the underlying evolutionary processes (Paper IV: Fig. 4 & 6; Paper V: Fig. 2 & S1). Our main result is that independent evolution of the same character state (homoplasy) in spicules is more common than what we expected. The term homoplasy refers to two major processes: convergence and secondary loss (= reversal). In the Astrophorida convergences and secondary losses have happened many times and for all type of spicules, megascleres and microscleres. The main consequence is that few spicule types (and secondary losses) are actually phylogenetically informative, at the order level at least. But before discussing separately each of these processes, I should clarify the term of "secondary loss", which I will be using copiously and which can become ambiguous in some cases.

4.3.1.1. The meaning of secondary loss in phylogenetics and with respect to spicules

Jenner (2002) emphasized that one has to stop considering 'absence' states as empirically empty as opposed to 'presence' states which furnish potential phylogenetic

evidence. By doing so, we prevent these 'absence' states to be optimized as plesiomorphies or apomorphies. An often ignored fundamental fact is that simple can also mean derived. In other words, an 'absence' state is also a 'gain', with the difference that this 'gain' often leaves no trace of its past presence, and is therefore invisible. Identified secondary losses can therefore potentially represent synapomorphies and thus bring new characters with phylogenetic information. But the difficulty of discriminating the different 'absence' states can render morphological studies heavily skewed. Sponge taxonomists have always acknowledged secondary loss of spicules (e.g. Dendy 1921) but they were hardly able to justify it, let alone test it. With the arrival of cladistic theory and a renewal of independent data came the possibility to unveil homoplasy and thereby secondary losses (Jenner 2004; Boury-Esnault 2006). Since these tools are fairly recent, there are fewer records of secondary losses than of convergent evolution.

I consider that there are two main types of spicules losses (Fig. 10): i) a "true" loss when nothing replaces the spicule lost (e.g. loss of sterrasters) or ii) a "semantic" loss by modification of a spicule into another (e.g. microrhabds becoming spherules; sterrasters become aspidasters). A true loss is a reversion so it is first a homoplasy, but if it is identified, it can be thought of as a derived character, an apomorphy. If secondary losses of the same spicule are identified in different clades, the loss becomes a convergent character, so it comes back to being a homoplasic character. On the other hand, a semantic loss obligatorily involves homologous spicules, it first leads to an apomorphy, but if the same transformation takes place in different clades, it is a homoplasy. "True" and "semantic" losses can be "partial" or "total" (see examples below). And, as Maldonado *et al.* (1999) have suggested, these losses can be "permanent" or "temporary" (= reversible). When we will discuss losses, it will always be "true" losses, unless stated otherwise.

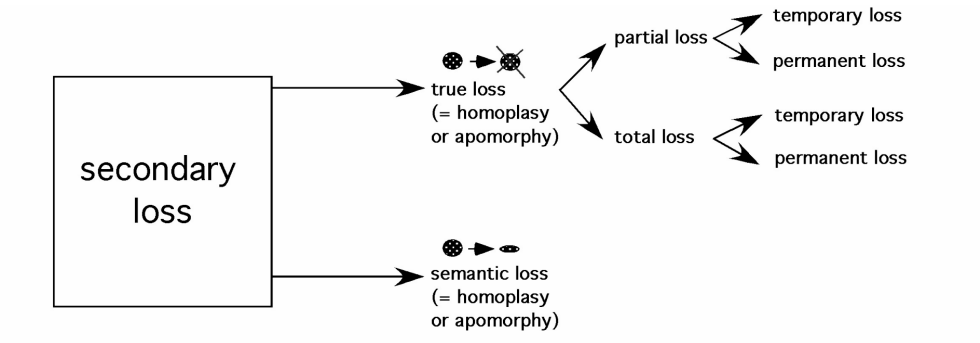


Figure 10. Meanings of "secondary loss".

4.3.1.2. Secondary losses within the Astrophorida

One needs to consider smaller clades to really use spicule categories or secondary losses as synapomorphies. For example, sterrasters as synapomorphy of the *Geodiidae*, trichodragmas as synapomorphy of *Dragmastra*, calthrops as synapomorphy of the *Calthropellinae*, secondary loss of ana/pro/mesotriaenes as a synapomorphy of the Erylinae+Calthropellinae+Caminellinae, secondary loss of sterrasters as a synapomorphy of the *Geostelletta* (group defined under the *PhyloCode*). Or, in order to have unique synapomorphies among the Demospongiae, one can use combinations of spicule characters to support monophyletic clades (e.g. calthrops and euasters (not toxas) for the Calthropellinae).

This high-frequency of homoplasy in the Astrophorida is all the more impressive if we consider that our results are certainly underestimated, especially concerning secondary loss. Many other cases are reported in Astrophorida not sampled in our study. First of all, apart from *Asteropus* (= *Stryphnus*), *Melophlus*, *Neamphius*, *Annulastrella* and *Thrombus* sampled in our study many other Astrophorida have secondarily lost their triaenes: *Holoxea*, *Jaspis*, some *Stelletta* — many of which were originally described as *Jaspis* (e.g. *Stelletta jonesi* (Thomas) — some *Erylus* (*Erylus amissus* Adams and Hooper), *Geodia* (e.g. *G. robusta* (von Lendenfeld), *G. spherastrosa* (Wilson)), *Rhabdastrella* (= *Geodia*) (e.g. *R. distinctus* (Thiele), *R. sterrastraea* (Row)), *Ecionemia* or *Lamellomorpha*. Because of the absence of triaenes, most of these species were originally described in separate genera: *Dorypleres* (= *Stelletta*), *Geodinella* (= *Geodia*), *Asteropus* (= *Stryphnus*) or *Stellettinopsis* (= *Ecionemia*). Partial losses of triaenes are also common: either clads are missing or remain as buds, or triaenes are simply very rare. This is common in *Ecionemia* (e.g. *E. corticata* (Carter)), *Stelletta* (e.g. *S. carolinensis* (Wells *et al.*), *S. stellata* Topsent, *S. tuberculata* (Carter)), *Rhabdastrella* (e.g. *R. intermedia* Wiedenmeyer) and in *Stryphnus raratriaenus* (Paper III). It should be emphasized that total loss of triaenes can lead to ambiguous identifications because Astrophorida are not the only order to produce asters (Fig. 1). Indeed, Hadromerida asters can be challenging to differentiate from Astrophorida asters. This is why we suspect *Jaspis* (Astrophorida) and *Hemiasterella* (Hadromerida) to be polyphyletic, which has been already confirmed by molecular data in the case of the Hemiasterellidae and *Hemiasterella* (Chombard 1998; Nichols 2005). I managed to get a 28S(C1-D2) sequence from a *Jaspis incrustans* (Topsent) collected in the Mediterranean (unpublished data). A blast search (03/03/2010) indicated that it was closer to the Agelasida+Axinellidae (= clade C *sensu* Nichols (2005)) and then to some Hadromerida species. Furthermore, a close observation of sections made in *J. incrustans*

(from the Mediterranean and from the Gulf of Cadiz) and *Hemiasterella* sp. 2 from Nichols (2005) showed that they had very similar skeleton organization. This might suggest that (some) *Jaspis* are closer to some *Hemiasterella* but we definitely need more data to confirm this. Most of these species were originally grouped in the former polyphyletic Coppatiidae Topsent, 1898 (= [Epipolasidae] Sollas, 1888), since taxonomists had difficulty knowing if these species were reduced Hadromerida, Astrophorida or merely primitive types which had never had either triaenes or tylostyles (de Laubenfels 1936; Bergquist 1968).

Secondary losses of asters are easier to reveal when triaenes are left, since Astrophorida and Spirophorida (the only taxa to have triaenes) are fairly easy to tell apart. This is the case in many *Penares* species (e.g. *P. sphaera* (von Lendenfeld), *P. alata* (von Lendenfeld), *P. saccharis* (de Laubenfels)), *Stelletta anasteria* Esteves & Muricy, all *Stoeba* and *Holoxea,* and some *Thrombus*. Here again, losses can be partial, and microscleres in some species (or specimens) can thus be very rare. For example, *Erylus deficiens* Topsent, originally described as a variety of *Erylus discophorus,* has very few aspidasters. Meanwhile, other specimens from the Gorringe Bank, temporarily identified as *Erylus* sp. and phylogenetically close to *E. discophorus* (Paper V), have completely lost their aspidasters (Xavier & van Soest 2007). Losses of either triaenes or asters were more readily identified and accepted because they concerned only one of the two synapomorphic spicules for the Astrophorida group. But some species may have lost triaenes *and* asters, a very likely scenario according to our results, and may have thus been classified in other orders (e.g. Halichondrida). We are therefore convinced that more Astrophorida species remain to be identified and removed from other sponge orders.

4.3.1.3. Convergent evolution within the Astrophorida

The abundant spicule nomenclature, essentially descriptive, tends to blur the primary homology of spicules (Fromont & Bergquist 1990). Therefore, independent characters are often necessary to reveal the true nature of spicules that look alike. An efficient way to reveal convergent evolution is to observe the spicule formation (with SEM or TEM): spiculogenesis or sclerocytes (e.g. Rützler & Macintyre 1978). Another way is to consider the position and orientation of these spicules in the sponge architecture (Paper IV). One can also consider characters not directly related to the spicules studied (other spicule categories present, embryology, biochemistry, histology, molecular phylogenetics…). Finally, one can use

phylogenetic reconstructions methods with morphological and/or molecular data (Paper IV & V).

With respect to convergent evolution, our results are also likely to be underestimated. Within the Astrophorida, similar spicules have been systematically interpreted as inherited by a single common ancestor. Our study (Paper V) nonetheless shows that this it is not always the case. Euasters seem to have appeared twice independently in the Geodiidae and the Ancorinidae. Calthrops have appeared independently at least three times (in the Calthropellinae, *Pachastrella* and *Dercitus*); it has always led to the loss of the typical Astrophorida radial arrangement. Other examples are the sanidasters that may have appeared in *Ancorina* sp. and *Stryphnus* independently; likewise for the discotriaenes in *Alectona* and *Discodermia*, microrhabds in *Ecionemia* and the Erylinae species; toxas in *Erylus*, *Geodia*, *Stelletta*, *Dercitus* and *Thoosa* species; amphiasters in the Thoosidae, Pachastrellidae, lithistids, *Characella*, *Neamphius* and even *Erylus amphiastera* (Wintermann-Kilian & Kilian) etc. I am sure that many more cases of unexpected convergent evolution are to be revealed within the Astrophorida.

The high level of homoplasy found in the Astrophorida may be due to our large sampling. Indeed, homoplasy has been shown to be correlated to the number of terminal taxa in an analysis (Sanderson & Donoghue 1989). In other words, the probability that a character will change somewhere on the tree is related to the total number of internodes and therefore of the number of taxa. So similar phylogenetic studies in other sponge groups should find similar levels of homoplasy. In the following part, we will investigate the Porifera literature in search of these homoplasies.

4.3.2. The Porifera, a homoplasy-rich phylum

4.3.2.1. Convergent evolution in Porifera

Numerous examples of spicule convergent evolution have been revealed by comparative morphology, and more recently by molecular phylogenetic data. For example: calthrops in the Homosclerophorida and the Astrophorida; trichodragmas in some *Stelletta* (Ancorinidae), Tetillidae (Spirophorida), *Spinularia* (Polymastiidae), Desmacellidae (Poecilosclerida), some *Haliclona* (*Gellius*) (Haplosclerida), *Dragmacidon, Dragmaxia* and some *Axinella* (“Axinellidae”) (Donadey *et al.* 1990); toxas in different Astrophorida, some

Haliclona (*Gellius*) (Haplosclerida), and some Poecilosclerida; euasters within the Astrophorida, the Hadromerida (see above) and the Chondrosida (*Chondrilla*) (Fig. 1); sterrasters (Geodiidae) and selenasters (Placospongiidae) (Rützler & Macintyre 1978); sanidasters in the Ancorinidae and in *Negombo* (Halichondrida), and spinorhabds (Podospongiidae, Poeciloasclerida); didiscorhabds (*Didiscus*, Halichondrida) and discorhabds (*Latrunculia*, Poecilosclerida) (Hiemstra & van Soest 1991); pseudo-rotules of the Spongillina ("Haplosclerida") and the "amphiasters" found in the Thrombidae (Astrophorida); amphidiscs of the Euplectellidae (Hexactinellida) and birotula in the Iotrochotidae (Poecilosclerida). Examples of convergent evolution are of course also found in the Calcarea: e.g. pseudosagittal triactines in Heteropiidae and some *Sycon* (Sycettidae) (Dohrmann *et al.* 2006). A few studies are now showing that convergent evolution is also present within sponge species: reduction in spines on the verticillate acanthostyles is assumed to have developed twice independently in widely separated populations of the *Astrosclera willeyana* Lister complex (Wörheide *et al.* 2002). Even siliceous spicules may have evolved independently twice: in the Silicea and in the Homosclerophorida (Maldonado & Riesgo 2007; Philippe *et al.* 2009). We should however note that convergent evolution appears surprisingly less common within the Hexactinellida (Dohrmann *et al.* 2008) but maybe because large molecular phylogenies have not been done yet on this group.

Other sponge characters involving the skeleton have been shown to arise through convergent evolution. The polyphyly of "lithistids" inevitably demonstrates that desmas have been acquired many times in Demospongiae evolution (Paper V). Likewise for the polyphyly of "sclerosponges" and their coralline skeletons which appeared in the Hadromerida and in the *Axinellida+Agelas* clade (Chombard *et al.* 1997). Spongin skeletons have evolved at least twice in the Demospongiae: in the Myxospongia/G2 and in the Keratosa/G1 (Maldonado 2009). Other skeleton frameworks have been acquired independently: axially compressed and extra-axially plumo-reticulate skeletons are present in Axinellidae, Raspailiidae and some Hadromerida (e.g. *Trachycladus*) (Erpenbeck *et al.* 2007c), the polyphyletic 'Jenkinidae' (Calcaronea) all share a thin-wall and an inarticulate choanoskeleton (Dohrmann *et al.* 2006); dictyonal framework may have appeared at least twice in the Hexactinellida (Dohrmann *et al.* 2008).

Sponge evolution shows that even more complex characters can be acquired independently. Oviparity for instance, has been acquired twice in different groups: Myxospongia/G2 and within the Democlavia/G4 (Borchiellini *et al.* 2004b), and we know that viviparity has been re-acquired independently in some Spirophorida (e.g. *Craniella*), all

Poecilosclerida (except for the Raspaillidae) and some Halichondrida (Sollas 1882a; Borchiellini *et al.* 2004b).

4.3.2.2. Secondary loss in the Porifera

Loss of spicules in general is less documented and scattered than spicule convergence because there are no comprehensive molecular phylogeny for orders rich in spicule diversity like the Hadromerida and the Poecilosclerida. Loss of megascleres is even less documented since their morphological diversity outside the Astrophorida is lower than for microscleres, but they are occasionally suspected: e.g. the loss of tuberose tyles in *Crambe* (Maldonado & Uriz 1996). Loss of microscleres, on the other hand, is well documented in other Porifera groups: many Tetillidae (Spirophorida) have secondarily lost their sigmaspires (e.g. some *Craniella*); independent losses of chelae is also hypothesized in the Poecilosclerida (e.g. Tedaniidae, Latrunculiidae and Desmacellidae) (Hajdu *et al.* 1994; Erpenbeck *et al.* 2007a); independant loss of gemmules in the Spongillidae may well have happened several times (Meixner *et al.* 2007). Total loss of spicules may have happened at least twice in the Demospongiae (Maldonado 2009), and more within the Homosclerophorida (e.g. some *Corticium, Oscarella*) (Solé Cava *et al.* 1992). All in all, it would seem that secondary loss appears more common for microscleres than for megascleres in the Demospongiae.

Secondary losses in the Porifera do not only concern spicules. In the Calcarea, molecular phylogenetic analysis indicated independent secondary loss of important and complex characters such as the cortex or even symmetry (Manuel *et al.* 2003). Spongin may have been lost at least twice: in the Tetractinellida and in *Suberites* (Borchiellini *et al.* 2004b). Even one of the possible synapomorphies of the Porifera, choanocyte chambers, have been secondarily lost in some Cladorhizidae (Poecilosclerida) (Vacelet & Boury-Esnault 1995).

4.3.2.3. Spicule function, adaptivity and homoplasy

Spicules have functions (cf. 1.3.5.) so the evolution of (some) spicules is obviously under selective pressures from the environment. Persistence across geological time of the same spicule morphologies is here to further support their purpose (Uriz 2006). And the remarkable fact that octocorals, plathelminthes, mollusks, echinoderms and ascidians have come up with similar spicules (Kingsley 1984), which may have similar functions of support

and/or protection (Koehl 1982; West 1998; López-Legentil *et al.* 2006; Clavico *et al.* 2007) should convince us, if needed, that (some) sponge spicules are truly adaptative.

One last phenomenon that also supports the usefulness of spicules is the compensation process, which I have observed many times, and that is hardly presented in the literature. Many sponges might compensate a secondary loss of a spicule, whether it be temporary or permanent, partial or total. 1) A semantic loss may be compensated by modifying the skeleton organization. Astrophorida which have lost long-shafted triaenes but gained calthrops may have lost a way to organize the choanosome and support the cortex. Topsent (1902) rightly noticed that the appearance of calthrops (in Calthropellinae, Pachastrellidae and *Dercitus*) was always correlated with a multiplication of their number in the choanosome (Fig. 11A-B); maybe because the loss of the triaene rhabdome induced a loss of skeletal support. 2) Sponges can compensate a true loss by producing more spicules of another category: in *Erylus deficiens*, the disappearance of aspidasters seems to be balanced by an abundance of microrhabds in the cortex in order to supposedly strengthen it (Fig. 11E-F). 3) Sponges can compensate by incorporating material from the environment: in *Rhabdastrella* (= *Geodia*) *aurora* (Hentschel), sand grains are extremely abundant in the cortex and functionally replace the triaenes, few in number and irregular in shape (Bergquist 1968). We have also observed this in *Ecionemia* (= *Geodia*) sp. (Fig. 11C) and *Stelletta* (= *Geodia*) *tuberculata*, both from South Australia, and wonder if these sand grains have also replaced the lost sterrasters. 4) Sponges can also compensate by producing more of another tissue: shallow-water *P. normani* which have very few sterrasters produce a thick fibrillar collagen layer under the cortex (Fig. 4F). This is also maybe how spongin appeared, because sponges needed to compensate the loss of their spicules with a new supporting skeleton (Maldonado 2009). 5) Finally, other sponges without spicules can use other living organisms as a scaffold to gain support: *Hexadella detritifera* Topsent can use the large megascleres on the hispid surfaces of large Astrophorida (Figs. 3J, 11D).

So spicules are adaptative, and a primary cause of convergence may be functional adaptation to similar environments (Patterson 1988). Because of their obvious larger role in the skeleton support, megascleres may be more adaptative than microscleres, and therefore under more selective pressures from the environment. Most microscleres are so small and often randomly distributed, that they seem to play a minor role in the skeleton framework (Dendy 1921). For example, it is hard to imagine which selective pressures act on the asters of Astrophorida species: how is an oxyaster better/worse than a strongylaster for the sponge's survival or fitness? Therefore, if we admit that less selective pressure acts on them, these

characters would be free to evolve in any direction. Darwin (1859) considered such "free" characters as more fitted for classification and phylogeny because logically their evolution will not be the result of environmental selection, which favors homoplasy. But our results suggest on the contrary that microscleres are very homoplasic characters. Furthermore, functional adaptation to similar environments does not explain the facility of secondary loss (in megascleres and microscleres). So an alternative cause of homoplasy is required. It has been shown that homoplasy is positively correlated to the limitations on the number of characters states (Donoghue & Ree 2000). Given a particulate rate of evolution (= character change), the fewer the evolvable states the more homoplasy is expected, and vice versa (Donoghue & Ree 2000). As shown in other organisms (Wake 1991; Donoghue & Ree 2000), we therefore propose that spicule formation constraints are a primary cause of homoplasy in spicules. These design constraints limit the number of different spicules one can make. For example, there may only be so many evolving combinations you can have from a long-shafted triaene, hence the multiple independent appearances of calthrops. Spicule design constraints can be genetic, cellular or biochemical. And spicule formation constraints may be the primary cause of secondary loss.

To conclude, spicule homoplasy may be due to 1) functional adaptation to similar environments and 2) spicule formation constraints, which limit what a spicule can look like. Both options can cause spicule convergence, but the second one may be the main cause of secondary loss, and may explain why this process is so banal in microscleres. We should note that a spicule can be lost even if it is adaptative: it is the case of triaenes and sterrasters implying that spicule formation constraints may have more influence on the evolution of spicules than the environment. What kind of constraint could be favoring such losses?

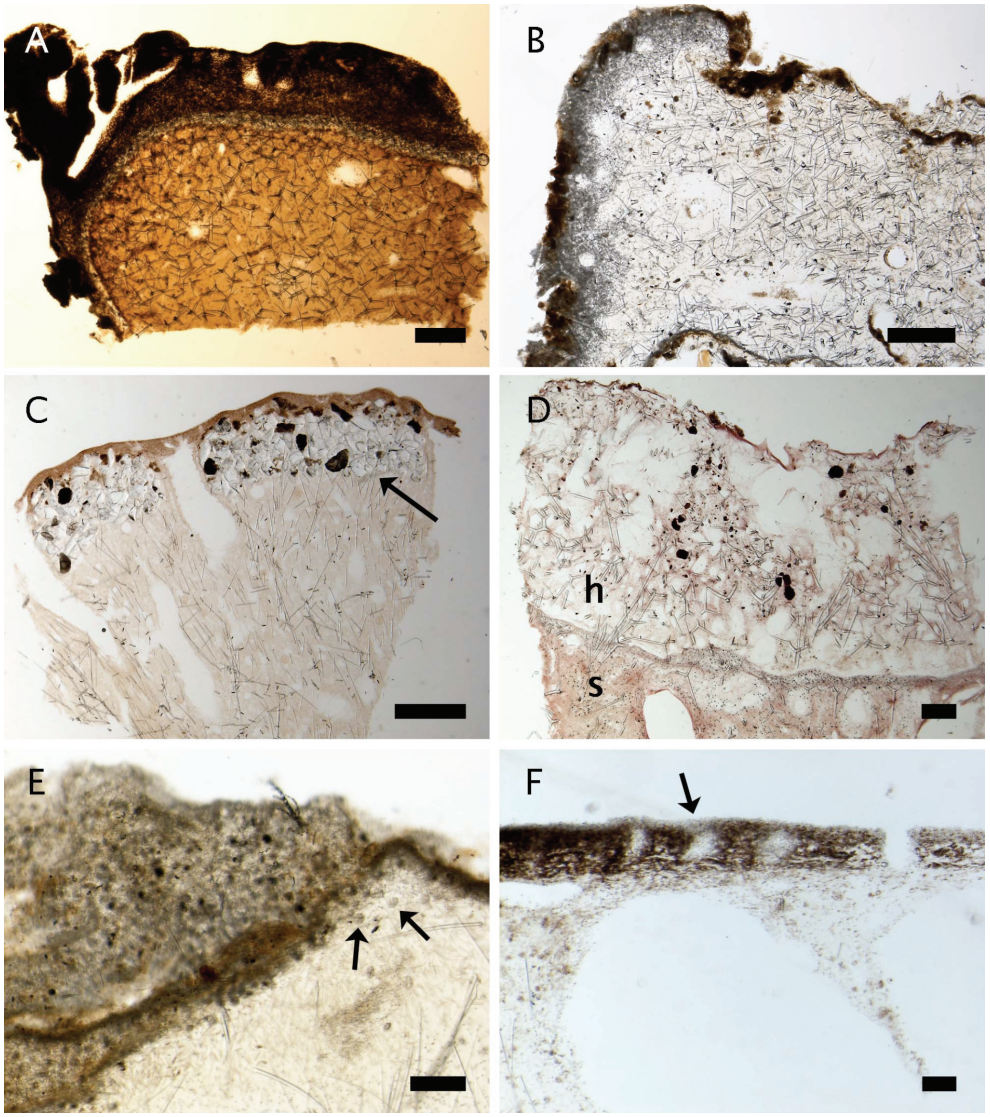


Figure 11. **A.** Thick section of *Dercitus bucklandi* Mc 2649). Scale: 1 mm. **B.** Thick section of *Pachastrissa pathologica* (MNHN DT754, type). Scale: 1 mm. **C.** Thick section of *Ecionemia* sp. (S1020). Arrow points at sand grains in cortex. Scale: 1 mm. **D.** Thick section showing *Hexadella detritifera* (h) growing on *Stryphnus fortis* (s) (ZMBN 82977). Scale: 1 mm. **E.** Thick section of *Erylus discophorus* (#PC82). Arrows point at aspidasters. Scale: 100 µm. **F.** Thick section of *Erylus deficiens* (ZMAPOR 20419). Arrow points at thick layer of microrhabds. Scale: 100 µm.

4.3.3. Secondary loss of sterrasters

We have mentioned possible cause(s) of the multiple secondary losses of sterrasters in the Geodiidae (cf. discussion in Paper V) and I will take the opportunity of this discussion to speculate further on how these losses might have been favored. A rough mapping of shallow/deep-water species on the Astrophorida phylogenetic tree (Paper V: Fig. 2) led us to suggest that Geodiidae ancestors were probably deep-water species, which implies that sterrasters appeared in a deep-water environment. The rigid cortex formed by sterrasters obviously confer a role of protection against predators (Hill & Hill 2002). One personal observation concerning a common two-sponge symbiosis in the Florida Keys further suggests this (Wilcox *et al.* 2002). *Geodia vosmaeri* (Sollas) has been shown to lack secondary metabolites to defend itself against predators but it might promote growth of other sponges, such as *Amphimedon erina* (de Laubenfels) that produce such chemicals (Pawlik *et al.* 1995). We noticed that sterrasters tended to disappear where *A. erina* was present, as if *G. vosmaeri* could afford a thinner cortex when overgrown by its protecting symbiont (Fig. 12).

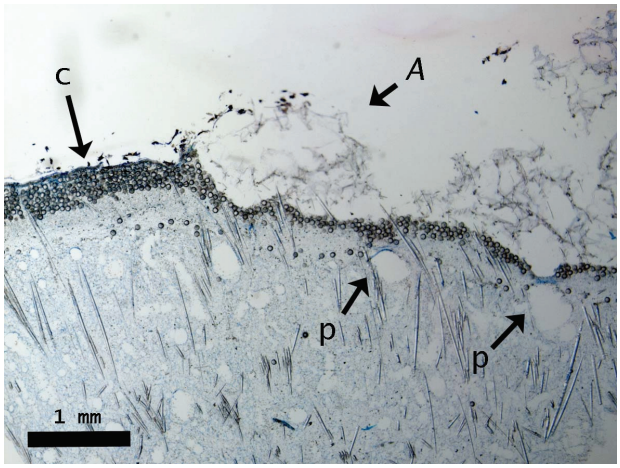


Figure 12: Stained thick section of the cortex of *Geodia vosmaeri* (ZMBN 85213) overgrown by *Amphimedon erina* (A) in the Florida Keys. p: uniporal pores of *G. vosmaeri*; c: cortex with sterrasters.

So sterrasters may have originally been selected for because of the survival advantage they brought to the ancestor of the Geodiidae. If this is true, then sterrasters could have appeared when or after the appearance of predators. This does not contradict their first occurrence in the fossil record of the Lower Cambrian (Reitner & Mehl 1995). On the other hand, we notice that most of the secondary losses of sterrasters have occurred in ancestors of

shallow-water species from tropical or temperate waters (never boreal or arctic). It is therefore tempting to propose that secondary loss of sterrasters has been favored in tropical to temperate shallow-waters. This would imply that environmental parameters such as lower pressure, higher water temperature and/or lower silica concentration could be responsible for the loss of these sterrasters. Silica concentration is particularly well known for its effect on spicule morphology (cf. 1.3.3.). Silica concentration may trigger or not spicule production in the different sclerocytes and thereby modify the set of spicules produced (Jørgensen 1944; Maldonado *et al.* 1999). Vacelet (1988) notes that all sponges that lost their mineral skeleton are actually sponges from the littoral zone, maybe because of the low silica concentrations due to the expansion of diatoms in the photic zone (Maldonado 2009). Seemingly, lithistids in shallow water tend to loose their desmas (Vacelet 1988; Maldonado *et al.* 1999; pers. obs.) and shallow deep-water Geodiidae species have a disturbed spiculogenesis (cf. 4.1.2.).

Following this, we can propose that shallow-waters Geodiidae may have stopped producing sterrasters because of a lack of silica. The silica concentration may not have been high enough to stimulate the transcription and/or translation of silicatein-α/β, the specific silicatein producing sterrasters (Krasko *et al.* 2000; Müller *et al.* 2007). Or, some Geodiidae may have still been able to produce a kind of sterraster, but not fully formed, as the ones observed today in our shallow *G. barretti* and *P. normani* (Fig. 5A, 5E), in the (shallow) *Erylus mamillaris*/*discophorus* complex (Fig. 13) or in some shallow *Rhabdastrella* species from tropical waters (e.g. *R. aurora*, *R. cordata*). In those cases, silicatein-α/β may still be produced but it is the final maturation and fusion of the ray tips that is disturbed, which suggests the role of yet another mechanism. Some species may have tried to compensate the loss of sterrasters with foreign material or other spicules (Fig. 11C, 11F). Permanent loss of sterrasters may happen if some gene involved in the production of sterrasters (e.g. silicatein-α/β gene or a gene controlling it) mutated. This would not matter for Geodiidae that already survived the phenotypical loss of their sterrasters. Once this mutation would spread in the population, we would have a permanent secondary loss of sterraster and possibly new species. The fact that the cortex with few and underdeveloped sterrasters of the shallow *P. normani* and *G. barretti* (Fig. 4A-B, E-F) look surprisingly like the cortex (Fig. 11E) and aspidasters of *E. discophorus* (Fig. 13) — a more southern shallow species, placed in the sister-group of *Pachymatisma* (Paper V) — suggests that the *E. mamillaris/discophorus* complex may have originated, like in our hypothetical scenario, from a deep-water ancestor that moved to shallower waters.

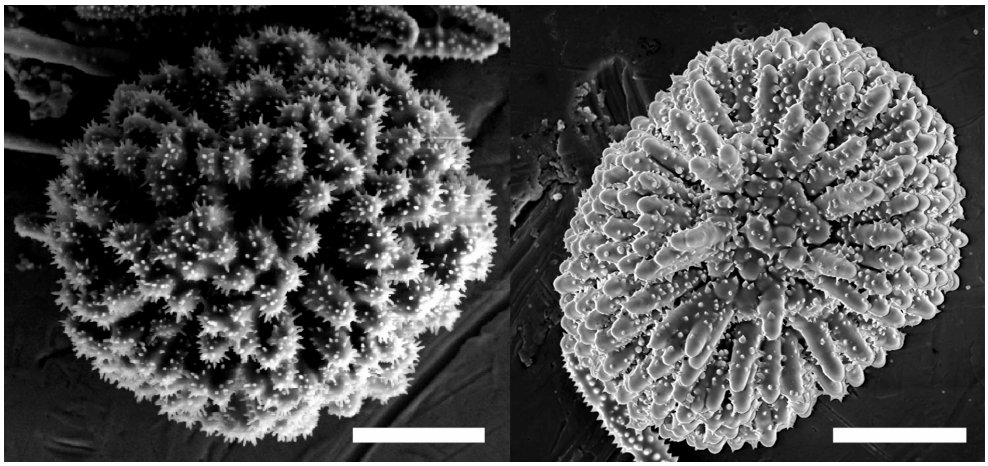


Figure 13. SEM observation of aspidasters of *Erylus discophorus* from Portugal (#PC81). Scale: 10 µm. Compare with underdeveloped sterrasters of shallow *Geodia barretti* (Fig. 7A) and *Pachymatisma normani* (Fig. 7E).

Theoretically some species may have managed to come back to deep-water and gain their sterrasters back (= reversible evolution). However, this last scenario is never met in our phylogenetic tree (Paper V: Fig. 2). The few deep-water species from our study to have lost their sterrasters (*Stelletta* (=*Geodia*) *tuberosa* (Topsent), *Penares sclerobesa* Topsent, *Pachastrissa* spp.) could represent species with shallow-water ancestors, which then came back to deep-water environments but had lost the possibility to produce sterrasters *de novo*. Or they could represent species that lost their predators or found other ways to deter them (e.g. secondary metabolites), so that the sterrasters were not so useful anymore.

To conclude, silica concentration may represent one of these spicule formation constraints causing secondary loss. It does not act on the function of a spicule but only on its formation: it represents a limiting factor conditioning the presence or absence of a spicule.

5. CONCLUSION

- My first aim was to provide a solid taxonomical basis of Astrophorida species. A full revision was obviously beyond the scope of this study but I have however during this Ph.D. managed to study specimens from all the Astrophorida families, from tropical, temperature and boreo-arctic regions. My taxonomy papers focused on Astrophorida from the Caribbean coast of Panama and the Norwegian coast. These studies resulted in many new records, three species were synonymized, two species were resurrected and two were new to science (*Stryphnus raratriaenus* and *Pachastrella nodulosa*). The study of so-called 'cosmopolitan species' (e.g. *Poecillastra compressa, Pachastrella monilifera, Thenea muricata*) or 'common' species (e.g. *Geodia gibberosa*, *Geodia cydonium*) shows that more work is required, as well as additional genetic data to fully revise these species. Appendix A of this thesis represents a solid basis to pursue this endeavor in the NEA/Mediterranean region. Integrative taxonomy has proven to be a powerful method to detect putative cryptic species or synonyms.

- Before this study, the evolutionary relationships within the Astrophorida order were for the most part unknown. This is the first comprehensive molecular phylogeny of the Astrophorida. We obtained a well resolved tree that suggested phylogenetic relationships between 89 species of Astrophorida from nine families of sponges. The taxonomic translation of this tree was a complete revision of the Astrophorida for which we proposed a new classification. With the adding of the eight families of lithistids, the Thoosidae and *Neamphius huxleyi*, the Astrophorida became a larger order than previously considered, comprising ca 820 species. 28S(C1-D2) and COI have been efficient markers in revealing deep and shallow nodes but some questions remain regarding poorly supported clades, *incertae sedis* taxa (e.g. *Characella*, *Neamphius*) and missing taxa in our study (e.g. *Holoxea, Jaspis, Tethyopsis, Psammastra, Tribachium, Chelotropella, Pachastrissa, Thoosa*, many lithistid families…). Furthermore, we should not forget that our phylogenetic reconstruction resulted in a hypothetical tree, parts of which are congruent with morphological data, but which needs to be tested with independent molecular data.

- Our study is far from being the first study to show the misleading nature of spicules and to question their utility in sponge taxonomy (Solé Cava *et al.* 1992; Klautau *et al.* 1994;

Schönberg & Barthel 1998; Erpenbeck *et al.* 2006a), especially with the numerous studies on the phenotypical plasiticity of spicules and the recent outburst of cryptic species identification. But this is maybe the first study where homoplasic spicules (megascleres as well as microscleres) are shown to be so widespread and common. We were able to reveal this homoplasy because of the spicule diversity of the Astrophorida and because of our large sampling. Our results show for the first time the banality of spicule secondary loss (especially for microscleres) and its potential as a synapomorphy (e.g. in the Erylinae+Calthropellinae+Caminellinae, in *Geostelletta*). We further discussed the cause of this high homoplasy levels and concluded that it may be due to 1) functional adaptation (especially concerning megascleres) and 2) spicule formation constraints. These design limitations (such as silica concentration) may be a major cause of secondary loss in spicules.

As the eminent French sponge taxonomist Emile Topsent (1925) once put it: " La détermination des *Geodia* est actuellement très difficile." (= The identification of the *Geodia* is today very difficult). Well, we might say it is even harder now with all these absent lost characters to take into account. But at least we can be satisfied that we have learned a great deal more about their evolution.

6. FUTURE PERSPECTIVES

- A revision of the boreo-arctic *Geodia* (Tendal, Klitgaard, Cárdenas & Rapp, in prep.) and the NEA *Erylus mamillaris*/*discophorus* complex (Xavier & Cárdenas, in prep) are currently pursued. Doing so, DNA tagging of revised species will be continued in order to give non-specialists (e.g. ecologists, environmentalists, biochemists) a reliable barcoding identification tool.

- Future molecular phylogenetics on the Astrophorida should focus on i) sampling and sequencing the missing taxa with the same markers and ii) start to sequence additional markers to confirm some important nodes, relationships and clades. It will also be interesting to see how well our phylogenetic classification stands when the sampling and/or the molecular data increases. Sponge-associated microorganisms are probably as old as the sponges themselves and maintained through vertical transmission (Taylor *et al.* 2007). There is today a growing interest to understand the relationships between sponge bacterial communities and their hosts (Taylor *et al.* 2007). The phylogenetic tree we obtained could therefore also be used for coevolution studies between Astrophorida and their symbionts. Co-phylogeny work has been pioneered by Erpenbeck et al. (2002) in the Halichondrida so co-phylogenies between specific Astrophorida bacterial groups and their hosts could be envisaged. Furthermore, a Demospongiae phylogenetic tree — including our phylogenetic results on the Astrophorida — is being put to use in an environmental microbial study which aims at understanding the relationships between the microbial community patterns of 13 sponge species (of which six are Astrophorida) from the cold-water coral reefs of Norway and their hosts (Hoffmann, Cárdenas, Rapp, Boetius & Ramette, in prep.). Another way to further benefit from our Astrophorida phylogeny would be to calibrate some of the nodes (take advantage of the rich lithistids fossil record, and the early Cambrian Tetractinellida fossils) and use a relaxed clock model in order to have divergence time estimates. We would then have a better idea when spicules were secondary lost and if we can correlate these losses to geological/geochemical events.

- A major research theme follows my study on secondary loss in sponges. It is the effect of silica concentrations with respect to secondary loss and its potential role in sponge speciation. Indeed, we already suspect silica to be a key element in important sponge evolutionary events (Maldonado *et al.* 1999; Maldonado 2009). Seemingly, by directly

influencing the phenotype of sponges, silica concentrations might have initiated many of those gains/modification/secondary losses of spicules, which could have been fixed in separate populations thereby producing new species.

7. REFERENCES

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Appendix A. North-East Atlantic (A) and Mediterranean (M) species of Astrophorida (Astrophorida lithistids not included), along with their taxonomic status. We follow here the Astrophorida family classification resulting from Paper V. In bold, species examined in this study.

Family	Species	Main synonyms	Taxonomic status	Distribution	Basis of status and synonymy
Geodiidae	***Caminella intuta*** (Topsent, 1892)	*Isops maculosus* Vosmaer, 1894 *Caminella loricata* von Lendenfeld, 1894	valid	A, M	Vosmaer, 1933; Paper V
	Caminus vulcani Schmidt, 1862		valid	A, M	Cárdenas et al., 2010
	Erylus candidata (Schmidt, 1868)		valid [1]	A, M	Paper V
	Erylus aspidodiscus Topsent, 1928		valid	M	Topsent, 1928 [2]
	Erylus corsica Pulitzer-Finali, 1983		valid? [3]	M	Pulitzer-Finali, 1983 [2]
	Erylus deficiens (Topsent, 1927)		valid? [4]	A, M	Cárdenas et al., 2010; Paper V
	Erylus discophorus (Schmidt, 1862)	*Erylus cantabricus* (Ferrer-Hernández, 1912)	valid	A, M	Cárdenas et al., 2010; Paper V
	Erylus expletus Topsent, 1927		valid	A	Cárdenas et al., 2010; Paper V
	Erylus granularis Topsent, 1904		valid [1]	A	Paper V
	Erylus mamillaris (Schmidt, 1862)		valid	A, M	Cárdenas et al., 2010; Paper V
	Erylus nummulifer Topsent, 1890		valid [1]	A	
	Erylus oblongus Topsent, 1828		valid? [5]	A	
	Erylus papillatus Topsent, 1927		valid	A	Topsent, 1927 [2]
	Erylus papulifer Pulitzer-Finali, 1983		valid? [3]	M	Maldonado, 1992
	Erylus topsenti von Lendenfeld, 1903	*Erylus chavesi* Topsent, 1904	valid [1]	A	Paper V
	***Erylus* sp.** (Gorringe Bank)		valid? [4]	A	Xavier et al., 2007; Paper V
	Geodia anceps (Vosmaer, 1894)		valid	M	Maldonado, 1992
	Geodia atlantica Stephens, 1915	?*Isops pallida* Vosmaer, 1882 ?*Geodia* cf. *atlantica* Stephens, 1915	valid [1]	A	Paper V
	Geodia barretti Bowerbank, 1858	*Geodia simplicissima* Burton, 1931	valid	A, M?	Paper V
	Geodia canaliculata (Schmidt, 1868)		valid [1]	M	Topsent, 1938 [2]
	Geodia conchilega Schmidt, 1862	*Geodia pergamentacea* Schmidt, 1870 *Pachymatisma intermedia* (Schmidt, 1868) [1]	valid	A, M	Paper V
	Geodia cydonium (Jameson, 1811) (British Isles)	*Cydonium mülleri* Fleming, 1828 *Geodia zetlandica* Johnston, 1842	valid	A	Sollas, 1880; Paper V
	Geodia* aff. *cydonium (Jameson, 1811) (Mediterranea Sea, Portugal)	*Geodia placenta* Schmidt, 1862 *Geodia gigas* Schmidt, 1862	valid	A, M	Topsent, 1894; Topsent, 1934; Paper V
	Geodia divaricans Topsent, 1928		valid	A	Topsent, 1928 [2]
	***Geodia echinastrella* Topsent, 1904**		valid? [1]	A	Topsent, 1904 [2]
	Geodia geodina (Schmidt, 1868)		valid [1]	A, M	Topsent, 1938
	Geodia macandrewi Bowerbank, 1858	*Geodia normani* (Sollas, 1888)	valid	A	Paper V; O. Tendal for synonymy (pers. com).

	Geodia megastrella Carter, 1876		species complex?	A	Paper V
	Geodia nodastrella Carter, 1876		valid	A, M	
	Geodia globus Schmidt, 1870		valid?	A	Burton, 1946 [2]
	Geodia hentscheli Cárdenas et al., 2010	*Geodia mesotriaena* (Hentschel, 1929)	valid [1]	A	Cárdenas et al., 2010; Paper V
	Geodia pachydermata (Sollas, 1886)		valid? [6]	A, M	Vacelet, 1996
	Geodia phlegraei Sollas, 1880	*Geodia parva* Hansen, 1885 *Isops sphaeroides* Vosmaer, 1882 [1] *Isops pyriformis* (Vosmaer, 1882) [1]	valid	A	Cárdenas et al., 2010; Paper V
	Geodia ramosa (Topsent, 1928)		valid	A	Burton, 1956
	Geodia senegalensis Topsent, 1891		valid [1]	A	
	Geodia simplex Schmidt, 1870		dubious	A	O. Tendal (pers. com.)
	***Geodia spherastrella* Topsent, 1904**		valid [1]	A	Topsent, 1904 [2]
	Geodia tuberosa Schmidt, 1862		valid?	M	von Lendenfeld, 1894
	Geodia tuberosa (Topsent, 1892)	*Stelletta tuberosa* Topsent, 1892	valid	A	Paper V
	Pachastrissa durissima **(**Topsent, 1892)		valid	A	Paper V
	Pachastrissa geodioides (Carter, 1876)	*Calthropella simplex* Sollas, 1888	valid	A, M	Paper V
	Pachastrissa inopinata (Pulitzer-Finali, 1983)		valid?	M	Pulitzer-Finali, 1983 [2]
	Pachastrissa pathologica (Schmidt, 1868)		valid [1]	M	Paper V
	Pachastrissa stelligera (Schmidt, 1868) [7]	*Calthropella recondita* Pulitzer-Finali, 1972	valid	A, M	
	Pachymatisma normani Sollas, 1888*		valid	A	Cárdenas et al., 2007
	Pachymatisma johnstonia Bowerbank *in* Johnston, 1842	*Caminus osculus* Grübe, 1872	valid	A, M?	Cárdenas et al., 2007
	Penares euastrum (Schmidt, 1868)	*Erylus stellifer* Topsent, 1892	valid [1]	A, M	
	Penares helleri (Schmidt, 1864)		species complex?	A, M	Paper V
	Penares sclerobesa Topsent, 1904		valid	A	Paper V
Ancorinidae	***Ancorina cerebrum*** Schmidt, 1862	*Ancorina immunda* (Schmidt, 1862) *Ancorina verruca* Schmidt, 1862 *Ancorina wageneri* (Schmidt, 1862)	valid [1]	M	
	Ancorina radix Marenzeller, 1889	*Ancorina coronata* (Topsent, 1892)	valid	A, M	
	Ancorina multistella (von Lendenfeld, 1907)		valid	A	
	***Ancorina* sp.** (Gorringe Bank)		valid	A	Xavier et al., 2007; Paper V
	Dercitus bucklandi (Bowerbank, 1858)	*Dercitus niger* Carter, 1871	valid	A	Paper V
	Dercitus lesinensis (von Lendenfeld, 1894)		valid		Maldonado, 2002
	Dercitus plicatus (Schmidt, 1868)		valid	A, M	Maldonado, 2002
	Dercitus dissimilis (Sarà, 1959)		valid	A	Maldonado, 2002
	Jaspis incrustans (Topsent, 1890)		valid? [8]	A, M	Topsent, 1928

	Jaspis johnstoni (Schmidt, 1862)	*Astropeplus pulcher* Sollas, 1888 *Coppatias inconditus* Topsent, 1892	valid	A, M	
	Holoxea furtiva Topsent, 1892		valid	A, M	
	Stelletta dorsigera Schmidt, 1862		valid	A, M	Paper V
	Stelleta grubei (Schmidt, 1862)		valid	A, M	Paper V
	Stelletta hispida Buccich, 1886		valid	A, M	Uriz, 1981
	Stelletta inermis (Topsent, 1904)		valid	A	Topsent, 1904 [2]
	Stelleta lactea Carter, 1871		valid	A, M	Paper V
	Stelletta latiancora Topsent, 1928		valid? [9]	A	Topsent, 1928 [2]
	Stelletta normani Sollas, 1880		valid	A	Paper V
	Stelletta mediterranea (Topsent, 1893)		valid? [10]	M	Topsent, 1893, 1894
	Stelletta pumex (Nardo, 1847)		valid?	M, A?	
	Stelletta raphidiophora Hentschel, 1929		valid	A	Paper V
	Stelletta simplicissima (Schmidt, 1868)		valid [1]	M	
	Stelletta stellata Topsent, 1893		valid	M	
	Stelletta ventricosa Topsent, 1904		valid	A	Topsent, 1904 [2]
	Stryphnus fortis (Vosmaer, 1885)	*Stryphnus rudis* Sollas, 1888	valid [1]	A	Paper V
	Stryphnus ponderosus (Bowerbank, 1866)	*Stelletta aspera*, Carter 1871 *Seiriola compacta* Hanitsch, 1889 (in part)	valid	A, M	Paper V
	Stryphnus mucronatus (Schmidt, 1868)	*Stelletta carbonaria* Schmidt, 1880	valid	A, M	Paper V
incertae sedis	***Characella pachastrelloides*** (Carter, 1876)*	*Characella sollasi* Topsent, 1890	valid	A	Paper IV, Paper V
	Characella tripodaria (Schmidt, 1868)	*Poecillastra armata* Hanitsch, 1895 *Sphinctrella linaresi* Ferrer-Hernández, 1914	valid? [1]	M	Maldonado, 1996; Paper IV
Pachastrellidae	***Pachastrella amygdaloides*** Carter, 1876		valid	A, M	Paper V
	Pachastrella* aff. *amygdaloides Carter, 1876		valid [11]	M	
	Pachastrella chuni von Lendenfeld, 1907		valid	A	Paper IV
	Pachastrella echinorhabda Pulitzer-Finali, 1972		valid	M	Pulitzer-Finali, 1972 [2]
	Pachastrella monilifera Schmidt, 1868		valid [1]	A, M	Paper IV
	Pachastrella nodulosa sp. nov.*		valid [1]	A	Paper IV
	Pachastrella ovisternata von Lendenfeld, 1907		valid	A, M	Maldonado, 1993, Paper IV
	Triptolemma intextum (Carter, 1876)	?*Triptolemma parasiticum* (Carter, 1876)	valid	A, M	Paper V
	Triptolemma simplex (Sarà, 1959)		valid	M	Sarà, 1959
Vulcanellidae	***Poecillastra compressa*** (Bowerbank, 1866)	*Poecillastra stylifera* (von Lendenfeld, 1907) *Poecillastra scabra* (Schmidt, 1868) ?*Poecillastra crassiuscula* (Sollas, 1886)	valid	A, M	Paper IV; Paper V
	Poecillastra rudiastra Pulitzer-Finali, 1983		valid	M	Maldonado, 1996 [2]
	Poecillastra saxicola (Topsent, 1892)		valid	M	
	Poecillastra symbiotica Topsent, 1902		valid	A	Topsent, 1904 [2]

	Vulcanella aberrans (Maldonado & Uriz, 1996)*		valid	A, M	Paper IV; Paper V
	Vulcanella cribrifera (Sollas, 1886)		valid	A	Sollas, 1886 [2]
	Vulcanella gracilis (Sollas, 1888)		valid	A, M	Paper V
	Vulcanella horrida (Schmidt, 1870) *sensu* Topsent, 1892		valid [12]	A	Topsent, 1904; Maldonado, 1996
Theneidae	***Annulastrella ornata*** (Sollas, 1888)		valid	A	Paper V
	Annulastrella verrucolosa (Pulitzer-Finali, 1983)		valid	M	Maldonado, 2002 [2]
	Thenea abyssorum Koltun, 1964		valid	A	Steenstrup & Tendal., 1982; Paper IV; Paper V
	Thenea bojeadori von Lendenfeld, 1907	*Thenea microclada* von Lendenfeld, 1907 *Thenea megastrella* von Lendenfeld, 1907	valid	A	Lévi, 1959; Cruz, 2002
	Thenea levis Ledenfeld, 1907		valid	A	Steenstrup & Tendal., 1982; Paper IV; Paper V
	Thenea muricata (Bowerbank, 1858)	*Thenea wallichi* (Wright, 1870) *Thenea intermedia* Sollas, 1888	valid	A, M	Steenstrup & Tendal., 1982; Paper IV; Paper V
	Thenea schmidtii Sollas, 1886		valid	A, M?	Paper V
	Thenea valdiviae Lendenfeld, 1907		valid	A	Steenstrup & Tendal., 1982; Paper IV; Paper V
Thoosidae	***Alectona millari*** Carter, 1879		valid	A, M	Paper V
	Alectona verticillata (Johnson, 1899)		valid	A	Johnson, 1899 [2]
	Alectona sp. 1 (Azores)		valid	A	J. Xavier (pers. com.)
	Alectona sp. 2 (British Isles, Norway)		valid	A	J. Xavier (pers. com.)
	Delectona alboransis Rosell, 1996		valid	M	
	Delectona ciconiae Bavestrello et al., 1996		valid	M	
	Delectona madreporica Bavestrello et al., 1997		valid	M	
	Thoosa circumflexa Topsent, 1891		valid	A	
	Thoosa istriaca Müller, 1979		valid? [13]	M	
	Thoosa letellieri Topsent, 1891		valid	A	
	Thoosa mollis Volz, 1939		valid	M	
	Thoosa tortonesei Sarà, 1958		valid	M	
Thrombidae	***Thrombus abyssi*** (Carter, 1873)		valid [1]	A, M	Paper V

* New or resurrected species for Norway after this study.
[1] Type material examined.
[2] Species only known from the type specimen(s).
[3] *E. expletus*, *E. corsica* and *E. papulifer* all look similar with respect to spicules and external morphology. They all share similar toxas and we doubt they are different species. A comprehensive revision of these three species is necessary (Maldonado, 1992).
[4] It could be part of a same polymorphic species: *Erylus discophorus* (Paper V).
[5] This species is identical to *E. nummulifer* except for the oblong shape of its aspidasters and rare oxyasters I.

[6] There has been some debate whether the NEA species was really conspecific with the holotype collected in Florida (Vacelet, 1996).
[7] It has been moved from *Calthropella* to *Pachastrissa* because oxeas have been found in some specimens (Topsent, 1895; Voultsiadou and Vafidis, 2004). Oxeas are absent in the specimen from Marseille that we examined.
[8] Topsent (1928) doubted of his own species since all the *Jaspis* he could find had spiny oxyasters. Molecular data is needed to revise both species of *Jaspis*.
[9] The description given by Topsent (1928) makes it possible that this species is conspecific with *Geodia tuberosa* (Topsent, 1892) (formerly *Stelletta tuberosa*) with anatriaenes. A re-examination of the type is necessary.
[10] The only difference between *S. mediterranea* and *S. lactea* seems to be the presence of anatriaenes in the former. In our opinion, this is a weak diagnostic character since the presence of anatriaenes can be very much depend on the environment and/or be overlooked quite easily. *S. lactea* has been furthermore identified many times in the Mediterranean Sea, including in Banyuls, the type locality of *S. mediterranea* (Boury-Esnault, 1971; Pouliquen, 1972).
[11] Several specimens of an unidentified *Pachastrella* were found in deep-sea material from the MEDECO 2007 collection (unpublished). Compared with *P. amygdaloides*, they have i) a different color (green in ethanol), ii) triactinal calthrops with characteristic bow-shaped actines and iii) very rare streptasters.
[12] It is a valid species (Maldonado, 2002) but we question the conspecificity of specimens from the NE Atlantic with the holotype from Florida. For example, the holotype has calthrops whereas specimens from the NE Atlantic have short-shafted ortho/dichotriaenes.
[13] This needs comparison with *Thoosa mollis* Volz, 1939 (van Soest et al., 2010).