Sponge Ecology in the Molecular Era

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Abstract

Knowledge of the functioning, health state, and capacity for recovery of marine benthic organisms and assemblages has become essential to adequately manage and preserve marine biodiversity. Molecular tools have allowed an entirely new way to tackle old and new questions in conservation biology and ecology, and sponge science is following this lead. In this review, we discuss the biological and ecological studies of sponges that have used molecular markers during the past twenty years and present an outlook for expected trends in the molecular ecology of sponges in the near future. We go from (1) the interface between inter- and intraspecies studies, to (2) phylogeography and population level analyses, (3) intra-population features such as clonality and chimerism, and (4) environmentally modulated gene expression. A range of molecular markers has been assayed with contrasting success to reveal cryptic species and to assess the genetic diversity and connectivity of sponge populations, as well as their capacity to respond to environmental

changes. We discuss the pros and cons of the molecular gene partitions used to date and the prospects of a plentiful supply of new markers for sponge ecological studies in the near future, in light of recently available molecular technologies. We predict that molecular ecology studies of sponges will move from genetics (the use of one or some genes) to genomics (extensive genome or transcriptome sequencing) in the forthcoming years and that sponge ecologists will take advantage of this research trend to answer ecological and biological questions that would have been impossible to address a few years ago.

1. Introduction

Coastal benthic ecosystems are in danger worldwide as a result of human activities. Consequently, assessment of benthic biodiversity and population vulnerability is a crucial ecological concern for marine biologists. Studies of marine biodiversity and vulnerability benefited massively from the incorporation of molecular tools and, as a result, knowledge of the functioning, health state, and capacity for recovery of marine benthic organisms and assemblages has improved vastly during the last decades (Bickford *et al.*, 2006; DeSalle and Amato, 2004; Haig, 1998; Sweijid *et al.*, 2000).

Molecular sponge ecology is a relatively new discipline, which has resulted from applying molecular tools to both traditional and new ecological issues. These tools allowed an entirely new way to interrogate organisms and to tackle old and new questions in sponge ecology. The health state and capacity for adaptation to environmental changes of sponge populations has been estimated by analyzing several genetic descriptors such as gene diversity, gene flow, departures from Hardy-Weinberg equilibrium, inbreeding, and changes in effective population size, among others (Charlesworth, 2009; Grosberg and Cunningham, 2001; Hellberg *et al.*, 2002; Pearse and Crandall, 2004). These descriptors are calculated from data on allele frequencies or sequence differences, and from the partitioning of their variation within and among populations.

For the accurate assessment of population descriptors, neutral molecular markers with an adequate degree of polymorphism are necessary. At the beginning of the molecular ecology era, the markers commonly used for ecological issues were polymorphic enzymatic proteins (allozymes), which proved suitable for assessing population differentiation and adaptation to particular environmental conditions. While allozymes have been very useful in many sponge studies (reviewed in Borchellini *et al.*, 2000; Solé-Cava and Boury-Esnault, 1999; Van Oppen *et al.*, 2002), they present important practical problems such as the requirement for fresh tissue, troubles with the interpretation of the electrophoresis gels, and the difficulty to compare across studies. These drawbacks, together with new technological developments such as polymerase chain reaction (PCR), led researchers in the field of molecular ecology to

move from analyses of proteins to genes, and sponge molecular ecology followed this trend.

Sequence data from several gene partitions of mitochondrial and nuclear DNA have been used for ecological issues involving marine benthic invertebrates. In addition to mere allele frequency data, sequences have the advantage of containing useful phylogenetic information. Mitochondrial DNA in particular has been and still is of prime importance in phylogeography and population genetics (Avise, 2000, 2009). However, sponges feature a low level of intra-species variability in mtDNA that has hindered the application of this marker (Duran *et al.*, 2004a, Wörheide *et al.*, 2005). It remains unclear why poriferan mtDNA displays low rates of evolution (Lavrov *et al.*, 2005), but this fact restricts the applicability of some of the most popular markers for studies of sponge population genetics. Nevertheless, only a restricted subset of mitochondrial genes (COI in particular) has been assayed so far and more research on other genes is necessary.

Finding a nuclear substitute for mtDNA is problematic because of technical hitches such as allele resolution, the prevalence of paralogy, recombination, and longer coalescent times compared to mitochondrial genes (Palumbi *et al.*, 2001; Zhang and Hewitt, 2003). Internal Transcribed Spacers (ITS) separating conserved regions of the rDNA genes have been used for studies at phylogenetic and population levels (e.g., Duran *et al.*, 2004b, Wörheide *et al.*, 2002a, 2002b). However, it has been recognized that intra-genomic polymorphisms (IGP), due to a lack of homogenization of the multiple copies of the rDNA clusters, can greatly limit the application of this marker to population genetics of sponges, and that levels of IGP should be determined and taken into account in any study (Duran *et al.*, 2004b; Wörheide *et al.*, 2004). Clearly, in sponges, development of new nuclear markers, preferably single copy genes with high variability (introns), is necessary for advancement in the fields of population genetics and demography (Wörheide *et al.*, 2005). New technologies will surely increase our ability to develop large numbers of markers in nonmodel organisms such as sponges (Thompson *et al.*, 2010).

Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs), are among the most variable and ubiquitous types of DNA sequence in the genome (Li *et al.*, 2002). Given their high mutation rate, they make possible fine-scale analysis of the genetic relationships among populations (Bowcock *et al.*,

1994) and, coupled with new analytical tools (e.g., Csilléry *et al.*, 2010), they appear to be the best choice for studies on population differentiation, gene flow, and clonality in sponges. They can provide useful demographic parameters to answer ecological questions and can help to make risk assessment and predictions on the fate of sponge populations submitted to exploitation or to harmful conditions (e.g., Dailianis *et al.*, 2011). However, the species-specific nature of microsatellites makes it necessary to develop them *de novo* for each new target species, in a time-consuming procedure involving the preparation and screening of genomic libraries. This drawback is likely responsible for the scarce number of sponge species (8) for which microsatellite markers have been developed so far: *Crambe crambe* (Schmidt) (Duran *et al.*, 2002), *Halichondria panicea* (Pallas) (Knowlton *et al.*, 2003), *Scopalina lophyropoda* Schmidt (Blanquer *et al.*, 2005), *Hymeniacidon sinapium* de Laubenfels (Hoshino and Fujita, 2006), *Spongia lamella* (= *S. agaricina*) (Schulze) (Noyer *et al.*, 2009), *S. officinalis* Linnaeus (Dailianis and Tsigenopoulos, 2010), *Ephydatia fluviatilis* Topsent (Cigliarelli *et al.*, 2008), and *Paraleucilla magna* Klautau, Monteiro and Borojevic (Guardiola *et al.*, 2011).

The microsatellite markers developed for sponges have allowed a number of recent studies at the intra- and interpopulation level, uncovering patterns of genetic structure at several scales, and allowing the study of clonality and chimerism (Blanquer *et al.*, 2009; Blanquer and Uriz, 2010, 2011; Calderón *et al.*, 2007; Dailianis *et al.*, 2011; Duran *et al.*, 2004c, Guardiola *et al.*, 2011; Hoshino *et al.*, 2008; Noyer, 2010). Doubtlessly, in recent years the application of microsatellites has revitalized the field of sponge molecular ecology. With the new technologies of massive sequencing, loci containing tandem repeats (microsatellites) can be easily obtained and optimized by sequencing a small part of the genome (Agell and Uriz, 2010; Jennings *et al.*, 2011). These new technologies will surely fuel the development of microsatellite markers in the forthcoming years, so that we expect an explosive increase of research on molecular ecology and biodiversity of non-model organisms such as sponges.

Undoubtedly, many sponge ecological studies can benefit from using molecular approaches to strengthen their conclusions. In particular, one of the key issues in the field of the sponge molecular ecology is the assessment of how sponges respond to environmental changes, whether weak or strong, cyclic or stochastic. The responses

of individuals and populations to a changing environment and the causes underlying adaptation are major topics in the field of molecular ecology (Carroll *et al.*, 2007). Mobile organisms can respond to suboptimal environmental conditions by migrating to a more favorable area. However, sponges live fixed to the sea bottom as adults and cannot migrate; sponges must respond to new conditions only physiologically, by acclimating their metabolism for survival. The ability of sponges to persist in a given area is determined by their genetic constitution. Under adverse conditions, selective mortality may occur in sponge populations resulting in local genetic adaptation, or even populations may become locally extinct. In the two cases, genetic markers can assist in evaluating the impact of the environmental changes on sponge assemblages (Hutchings *et al.*, 2007).

Although there is little doubt about the benefits of applying molecular techniques to improve and expand the field of sponge ecology, classical ecological approaches have been decisive for the formulation of appealing questions that can be approached by using molecular tools. Ecological and biological issues related to reproduction (e. g. McKinnon *et al.*, 2004), growth, resistance/vulnerability to man-induced perturbations, responses to natural changes, chemically or physically mediated interactions, competition, facilitation, commensalism, trophic and larval ecology (Palumbi *et al.*, 2008), are still poorly known in the Porifera. Thus, classical physiological and ecological approaches continue to be necessary in sponge studies as a source of new questions and hypothesis that can nowadays be addressed with an array of molecular tools.

In this review, we will consider the main aspects where molecular tools have contributed to the advancement of our knowledge on sponge ecology. We will go from (1) the interface between inter- and intraspecies studies, to (2) phylogeography and population level analyses, (3) intra-population features such as clonality and chimerism, and (4) environmentally modulated gene expression. We leave out of this review, on purpose, the extensive recent literature on molecular markers applied to studies of sponge symbiont assemblages, which will be dealt with elsewhere (Thacker and Freeman, this volume). We will end this retrospective with a prospect of future directions and developments that we foresee that the field will witness in the forthcoming years.

2. Where molecular markers alert us about hidden sponge diversity: cryptic speciation and its ecological repercussions

2.1. Role of molecular markers in the discovery of cryptic sponge species

We are in a time of accelerated biodiversity loss, with many species disappearing even before they are identified (Williams and Hilbert, 2006) while, at the same time, other species are moved from their native areas to new ranges where they can threaten local biota (Kaiser and Gallagher, 1997). Assessment of local and introduced biodiversity is urgent and mandatory before issues of conservation and protection can be addressed. Yet this is a difficult task, in particular in groups that lack sufficient morphological characters and whose taxonomy is difficult, such as sponges. This is the first aspect in which genetic tools have come to the rescue in recent years.

Molecular tools are especially powerful in disclosing previously undetected taxonomic diversity, such as that represented by the so-called cryptic species, which cannot usually be resolved efficiently with only morphological characters. The message stemming from the application of molecular tools is that diversity has been grossly underestimated in benthic invertebrates in general (Knowlton, 2000) and in sponges in particular (Wörheide *et al.*, 2005). The finding of cryptic species is a common outcome whenever sponges have been investigated using genetic markers. Often, studies that focused on population genetics of a single species have revealed a previously misperceived species complex whose status needed clarification as a first step (e.g., Blanquer and Uriz, 2007; Miller *et al.*, 2001; Zilberberg *et al.*, 2006a; Xavier *et al.* 2010a).

The use of molecular markers has proved that populations of marine sessile invertebrates in general (e.g., Bierne *et al.*, 2003; Palumbi *et al.*, 1997) and sponges in particular (e.g., Blanquer *et al.*, 2009; Blanquer and Uriz, 2010; Duran *et al.*, 2004b; Nichols and Barnes, 2005), have strong spatial structure and restricted gene flow (see subchapter 3), which are favorable conditions for reproductive isolation (Knowlton, 1993) and, thus, speciation. Cryptic species, besides hindering biodiversity assessment, create problems for non-taxonomic research. Even if they are very close morphologically, cryptic species often show contrasting physiological, reproductive and/or other biological traits (Blanquer and Uriz, 2008b), so that their

misidentification may cause serious inconsistencies in biological and ecological studies.

Widespread geographic distributions have been often reported in the old literature for many sponge species because of the lack of clear morphological differences (e.g., in spicule shape and size) among individuals inhabiting distant areas. The idea of “cosmopolitan” sponge species, however, is at odds with what we know about the limited dispersal capacity of most sponge larvae (e.g., Boury-Esnault *et al.*, 1993; Mariani *et al.*, 2005; Uriz *et al.*, 1998; Uriz *et al.*, 2008). Molecular analyses soon made claims of cosmopolitanism fall into disrepute as examples of over-conservative systematics (Boury-Esnault and Solé-Cava, 2004; Klautau *et al.*, 1999; Lazoski *et al.*, 2001; Miller *et al.*, 2001; Wörheide *et al.*, 2002b), and the existence of several cryptic sibling species was demonstrated in most cases. Thanks to the extensive use of molecular approaches to improve species identification, the number of cryptic sponge species is steadily increasing (e.g., Blanquer and Uriz, 2007; Klautau *et al.*, 1999; Pérez *et al.*, 2011; Solé-Cava *et al.*, 1991a, 1991b; Solé-Cava and Boury-Esnault, 1999).

Several markers have proved useful for assessing the taxonomic status of sponge morphotypes or species complexes (See Cárdenas *et al.*, this volume) and thus to detect cryptic species. Allozymes, besides being suitable markers for studies of population genetics in sponges, also resulted useful for establishing species boundaries (e.g., Barbieri *et al.*, 1995; Bavestrello *et al.*, 1992; Boury-Esnault *et al.*, 1992; Klautau *et al.*, 1994; Muricy *et al.*, 1996a, 1996b; Sarà *et al.*, 1993; Solé-Cava and Thorpe, 1986, 1994; Solé-Cava *et al.*, 1991a, 1991b, 1992; reviewed in Solé-Cava and Boury-Esnault, 1999). But both mitochondrial and nuclear sequences have replaced allozymes for species delimitation in the last years. Internal transcribed spacer sequences were used to study taxa purportedly to be widely distributed, uncovering the existence of several species (e.g., Wörheide *et al.*, 2002b). The 5'-end or Folmer partition (Folmer *et al.*, 1994) of the mitochondrial gene cytochrome oxidase subunit 1 (COI), which has been proposed as the standard marker for DNA barcoding (e.g., Hebert *et al.*, 2003), has proved to be suitable to discriminate species in many cases (e.g., Blanquer and Uriz, 2007) because of its extraordinarily low intraspecies variability in sponges as compared to other groups (e.g., Duran *et al.*, 2004a; López-Legentil and Pawlik, 2009). A slightly longer fragment of the same

gene, including the I3-M11 partition, was claimed to improve resolution and phylogenetic signal compared to the Folmer partition (Erpenbeck *et al.*, 2006a; López-Legentil and Pawlik, 2009). Of course, more solid results are obtained when several genes are used and congruent patterns appear. Thus, multiple nuclear and mitochondrial markers have been used in some studies to detect or confirm species differentiation (Blanquer and Uriz, 2007; Xavier *et al.*, 2010a; Zilberberg *et al.*, 2006a; Reveillaud *et al.*, 2011a, 2011b). Occasionally, cryptic species were first detected during studies of population genetics, using allozymes or microsatellites, and then confirmed by mitochondrial and nuclear sequences (e.g., Blanquer and Uriz, 2007). In retrospect, morphological characters matching the new species boundaries could be found in some cases (Blanquer and Uriz, 2008a; Muricy *et al.*, 1996a).

2.2 Representative case examples

Cryptic speciation in sponges has been uncovered using molecular markers in at least 23 species-complexes resulting in ca. 50 cryptic species (Table 1). Solé-Cava and coworkers first applied allozymes to establish the species boundaries of *Calcarea* and *Demospongiae* across geographical clines. Solé-Cava and Thorpe (1986) described two new species within the *Suberites ficus* (Johnston) complex while Solé-Cava *et al.* (1991a) studied two geographically distant populations of the allegedly cosmopolitan species, *Clathrina clathrus* (Schmidt) and *C. cerebrum* (Haeckel). In both cases, populations of the two species from the South West Atlantic (Brazil) and the Mediterranean Sea showed high levels of genetic divergence, which allowed the authors to consider them different species with a disjointed geographical distribution (Table 1). Allozymes proved also useful to discover a new cryptic species phenotypically intermediate between the well-known *Axinella verrucosa* Brøndsted and *A. damicornis* (Esper) (Solé-Cava *et al.*, 1991b).

Genetic (allozyme electrophoresis) and cytological studies of the aspiculate homoscleromorph *Oscarella lobularis* (Schmidt) from the Mediterranean revealed the existence of several species, including two cryptic polymorphic species: *O. lobularis* and *O. tuberculata* (Schmidt) (Boury-Esnault *et al.*, 1992). The phenotypic differences between these two species are hard to find but an accurate histological

study (Muricy *et al.*, 1996a) found several cytological diagnostic characters such as amount of collagen, types of vacuolar cells and types of symbiotic bacteria.

Muricy *et al.* (1996b), by using eleven allozyme loci, distinguished four species within the “*Plakina trilopha*” complex, which were difficult to identify on the basis of their spicule characteristics. Indeed, the values of the “*I*” index of genetic identity (Nei, 1978) and the presence of diagnostic alleles for each of the several morphotypes provided evidence of the presence of four species (Muricy *et al.*, 1996b). The “true” *Plakina trilopha* Schulze was widely distributed in the Mediterranean but the other three species in the complex were found only in certain caves and vertical walls at single sites (each species) around Marseilles (France). These caves represent exceptional habitats (Vacelet *et al.* 1994) that may have kept sponge populations isolated. The authors hypothesized that these *Plakina* species likely evolved by independent colonization events in the different caves of the region, followed by reproductive isolation of the subpopulations due to restricted water circulation in the caves and low dispersal capabilities of their larvae (Muricy *et al.*, 1996b).

Perhaps the most paradigmatic instance of cryptic speciation in sponges has been the “*Chondrilla nucula*” complex. The type material is from the Adriatic and it was considered cosmopolitan until it was used precisely to illustrate the problems with overconservative systematics. Klautau *et al.* (1999) using 10 allozyme loci showed the existence of five genetic clades in samples from the Atlantic and the Mediterranean. The authors tentatively retained the original name for the Mediterranean clade and assigned letters to the other 4 groups. Morphological characters (spicule sizes) did not correlate with species boundaries defined genetically. Zilberberg and coworkers (2006a) further analyzed several forms belonging to what was formally *Chondrilla “nucula”* Schmidt in the Caribbean and Brazilian coasts using allozymes. Although focused on the assessment of asexual reproduction, this study found two of the species defined by Klautau *et al.* (1999) and two more species belonging to this complex, one in the Caribbean and one in Brazil. Duran and Rützler (2006) analyzed the 5'-end partition of COI in individuals of what they called *Chondrilla cf. nucula* inhabiting two contrasting ecosystems in the Caribbean: mangal swamps and coral reefs. Each habitat was occupied by a distinct morphotype, which differed among them in color and general shape: the lighter

colored, thinner morphotype from the coral reefs and the darker and thicker morphotype from the mangal swamps. Five out of 12 haplotypes found were specific of mangrove habitats while another five were exclusive of coral reefs (Duran and Rützler, 2006). An AMOVA based on haplotype frequencies statistically supported high genetic isolation between the two habitats present in the same localities. Moreover, populations from the same habitat (either mangrove or reef) separated by more than 1000 km had a similar haplotype composition. This system represented the first instance of ecological speciation in sponges. The 5'-end partition of COI also allowed differentiating between the Alaskan populations of *Halichondria panicea* and those from the NE Atlantic. The resulting phylogenetic tree clearly showed two clades, which represented two separate species (Erpenbeck et al., 2004).

Complete mitochondrial genomes have been recently used to identify cryptic species of *Halisarca* Johnston (Ereskovsky et al., 2011). *H. dujardini* Johnston, and *H. harmelini* Ereskovsky, Lavrov, Boury-Esnault, and Vacelet, showed identical mitochondrial genomes as for their gene content and gene arrangement but differed in size by ~1,300 bp (6.8%). The overall genetic distance between coding sequences of the two species was much greater than previously reported for species of non-bilaterian animals (Ereskovsky et al., 2011). This genetic difference calls for caution about claims of highly conserved mitochondrial genomes in sponges, based only on the cytochrome oxidase gene.

Other gene fragments have also been assayed with different success. Wörheide et al. (2002a) used the ITS1-5.8S-ITS2 region to analyze several populations of the purportedly circum-Pacific coralline demosponge *Astrosclera willeyana* Lister. Despite the small number of differences between sequences, these authors concluded that, under a strict version of the phylogenetic species concept, the populations from the Red Sea, the GBR, and Fiji/Vanuatu represented distinct species, of which the Fiji/Vanuatu species would correspond to *A. willeyana* s.s. (but see Wörheide, 2006, for a lack of differentiation among two of the species using another marker). The species status for these three populations according to the molecular markers used corroborated the three previously detected groups based on morphological characters (Wörheide, 1998). However, Nichols and Barnes (2005) using ITS markers failed to resolve the phylogenetic relations among representatives of the genus *Placospongia* Gray from West Pacific, Caribbean and Indo-Pacific populations. Although discrete lineages were found in the several geographical

regions, cryptic species could not be established. These authors stated that, because of the intra-genomic variation of ITS, the phylogenetic structure in their dataset reflected duplication events rather than relationships among individuals. Using a partial sequence of the COI and a ITS1-5.8S-ITS2 nuclear fragment, Cárdenas *et al.* (2007) showed that *Pachymatisma johnstonia* (Bowerbank) and *P. normani* Sollas, two astrophorid species that had been considered synonym were indeed good species, undistinguishable on the basis of spicule shapes and sizes.

Molecular markers can confirm species boundaries but sometimes reveal lack of speciation. Lazoski *et al.* (2001) using thirteen allozymes discovered that the Atlantic populations of *Chondrosia reniformis* Nardo from Bermuda and Brazil were indeed a separate species from the Mediterranean *C. reniformis*. However, populations of *C. reniformis* from the Atlantic, separated by up to 8.600 km of distance, showed remarkable genetic similarity, albeit with significant population structure (see subchapter 3).

Cryptic speciation was unexpected for the only known Mediterranean *Scopalina* (*S. lophyropoda*) despite the few diagnostic characters available for the species (Blanquer and Uriz, 2008a). However, microsatellites developed for *S. lophyropoda* failed to amplify some populations along the distribution area reported for the species (from the Adriatic to the Canary Islands), pointing to the possible presence of a species complex (Blanquer and Uriz, 2007). Mitochondrial COI (5'-end, Folmer partition) and 16S rDNA, together with sequences of the nuclear 28S rDNA, confirmed that the populations that did not amplify were indeed three new species (Blanquer and Uriz, 2007, 2008a). One of them (*S. blanensis* Blanquer and Uriz) shared habitat and could even be found in contact with the true *S. lophyropoda* (Fig. 1). The other two (*S. ceutensis* Blanquer and Uriz and *S. canariensis* Blanquer and Uriz), however, seemed to be restricted to geographically distant areas: North Africa (Mediterranean) and Canary Islands (North Atlantic), respectively.

Sometimes the cryptic species resulting from a species complex show a disjointed distribution. Several Atlanto-Mediterranean populations of the sponge *Cliona celata* Grant, until then considered a cosmopolitan species, were analyzed using mitochondrial COI and Atp8 synthetase, as well as the nuclear 28S rDNA gene (Xavier *et al.*, 2010a). The phylogenetic reconstructions indicated the existence of four well-supported clades with a clear gap between intra and inter-clade

divergences. Consequently, *C. celata* resulted to be a complex of four cryptic species, with contrasting distributions: two species occurring along the Atlantic European coasts and the other two in the Mediterranean and Macaronesian islands. These results confirmed previous findings obtained with allozyme markers, which led to the suggestion of splitting the Mediterranean “*C. celata*” into two species (Barbieri *et al.*, 1995). These results also showed that the boring and massive growth forms of this excavating sponge are truly different growth stages or ecological phenotypes of the same species.

Reveillaud *et al.* (2011a) used the Folmer partition of the mitochondrial COI gene, the D3-D5 region of the nuclear large ribosomal subunit (28S rDNA) and the second intron of the nuclear ATP synthetase, beta subunit gene (ATPS) to establish the boundaries of the three Atlanto-Mediterranean species of *Hexadella* Topsent and to investigate the presence of cryptic species within this genus. Phylogenetic analyses revealed several divergent clades for the deep-sea sponges, congruent across the mitochondrial and nuclear markers. One clade contained specimens from the Irish, the Scottish, and Norwegian margins and from the Greenland Sea (*Hexadella dedritifera* Topsent), another clade contained specimens from the Ionian Sea, the Bay of Biscay and the Irish margin (*Hexadella cf. dedritifera*), and a third clade corresponded to a new Mediterranean deep-sea species (*Hexadella* sp.). Furthermore, another cryptic shallow-water species (*Hexadella cf. pruvoti* Topsent) was also revealed in the Mediterranean Sea and in the Gorrige Bank (North Atlantic). The ATPS marker, first applied to sponges by Bentlage and Wörheide (2007), proved its applicability for species delimitation in this group in the representatives of the genus *Hexadella*. Reveillaud *et al.* (2011b) using the M1M6 and I3M11 partitions of the COI, and 28S rDNA sequences, in combination with sponge morphology, detected an under-estimated biodiversity of the genus *Plocamionida* Topsent along 3,000 km of European margins, with three additional valid species besides *Plocamionida ambigua* (Bowerbank).

Although most molecular studies have detected genetic variation in sponges associated with even subtle morphological differences that had been considered without diagnostic value in traditional sponge taxonomy (e.g., color: Blanquer *et al.*, 2008b; Boury-Esnault *et al.*, 1992; Klautau *et al.*, 1999; Knowlton, 2000; Miller *et al.*, 2001), a few studies have reported the lack of genetic variability in sympatric

morphotypes that differed in color and shape (e.g., Boury-Esnault *et al.*, 1992; López-Legentil and Pawlik, 2009; Solé-Cava and Thorpe, 1986). For instance, the Caribbean sponge, *Xestospongia muta* (Schmidt) has three main morphotypes, which are characterized by their digitate, rough, or smooth external surface, respectively. The haplotype network of their populations based on the I3-M11 partition of COI indicated that the high degree of morphological differentiation did not reflect genetic boundaries in *X. muta*, and that gene flow occurs between these morphotypes (López-Legentil and Pawlik, 2009). The genus *Xestospongia* De Laubenfels seems to harbor species with wide intraspecies phenotypic variation. No correlation between external morphology and sterol chemotypes (Kerr and Kelly-Borges, 1994), which had been considered to have chemotaxonomic value (Bergquist *et al.*, 1990), has been reported for the Indo-Pacific species of *Xestospongia*. This seems to represent a particular case where environmental conditions (mainly currents) may determine the shape of the outer surface of the individuals while populations living in contrasting habitats are genetically connected maybe because of a relatively wide dispersion of gametes in these oviparous sponges.

Another example of high intraspecies phenotypic plasticity unrelated with genetic differentiation is *Callyspongia vaginalis* (Lamarck), which shows three morphotypes differing in color and external surface in the Caribbean. The genetic distances among these three morphotypes and the close species *C. fallax* Duchassaing and Michelotti (all of them with oxeas as the only spicule type) were assessed by partitions of two mitochondrial (COI and 16S) and two nuclear (18S and 28S rDNA) genes (López-Legentil *et al.*, 2010). None of these genetic markers provided evidence for differentiation among the morphotypes of *C. vaginalis* or between these and the congeneric *C. fallax*. Morphological characters (spicule sizes and spongin fiber characteristics) showed differences not linked to genetic patterns. As in *Xestospongia muta*, *C. vaginalis* seems to maintain a high degree of phenotypic plasticity, and their morphological characteristics did not indicate reproductive boundaries.

2.3. Conclusions

Molecular markers can reveal hidden sponge biodiversity and, thus, improve the consistency of ecological studies. They also assist taxonomists in assessing the taxonomic value of phenotypic characters, distinguishing those that are evolutionarily fixed from those that result from environmental plasticity. Morphological characters that have been traditionally disregarded for species identification can turn out to be adequate diagnostic characters for discriminating some species complexes, and the reverse is also true. In some cases (e.g., *Chondrilla* Schmidt, *Plakina* Schulze, and *Scopalina* Schmidt), an “a posteriori” scrutiny of the phenotypic characters of the cryptic species revealed differences in color, skeletal arrangement, amount of spongin surrounding the skeletal tracks, or sponge surface features. At the same time, although the main tale is one of hidden diversity, suspected cryptic species may result in mere morphotypes, as revealed by genetic divergence below the “between-species” differentiation threshold.

It should also be noted that the lack of genetic differences derived from a single marker must be interpreted with care, since the same gene may show contrasting mutation rates in different lineages of the Porifera. Hence, the choice of a suitable marker strongly depends on the evolutionary context of each single taxon (Heim *et al.*, 2007) and, as a consequence, several markers should be assayed before a decision on the taxonomic status of a given species is taken. A foreseeable trend in the near future is the incorporation of more markers and the use of a combined, multilocus approach to assess sponge biodiversity.

According to the many examples of cryptic sponge species with disjointed distributions reported in the literature, sponge speciation seems to have occurred mainly in allopatry even at small geographical scales (hundreds of km). Allopatric speciation has been proposed for the two species of the *Clathrina clathrus* complex (Solé-Cava and Boury-Esnault, 1999), as well as for *Scopalina lophyropoda*, *S. ceutensis*, and *S. canariensis* (Blanquer and Uriz, 2008b). In these cases, the most plausible scenario for their speciation is that derived from isolation by distance, as a result of a strong larval philopatry, which is a shared trait of most sponge species (e.g., Uriz *et al.*, 1998; Uriz *et al.*, 2008). Speciation of *S. lophyropoda* and *S. blanensis*, despite their currently overlapping habitat, might have also originated in allopatry, with *S. blanensis* diverging at the Central Mediterranean and then recolonizing the western Mediterranean coasts (Blanquer and Uriz, 2008b).

Interrupted gene flow among populations due to physical barriers produces genetic divergence and the consequent speciation. However, ecological reproductive barriers may also bring about speciation. Understanding the role of ecological factors in speciation will require an integrated knowledge on ecological, evolutionary, and behavioral aspects, as well as on the selective pressures operating in natural populations. Until now, there has been little evidence of limited interbreeding within sponge populations related to ecological niche differentiation. The cryptic speciation within the *Chondrilla* cf. *nucula* complex in two disjointed habitats, mangal swamps and coral reefs, illustrates the only example so far of ecologically driven speciation in sponges (Duran and Rützler, 2006).

A combination of physical and ecological barriers may be underlying the intense species radiation of the Homoscleromorpha *Plakina* in caves of a small geographical area of the western Mediterranean (Muricy *et al.*, 1996b). The physical isolation of caves, which in some respects can be considered as islands (e.g., Vacelet *et al.*, 1994) and the particular ecological conditions of these biotopes such as trophic depletion, reduced water movement and light (e.g., Martí *et al.*, 2004) adds to the poor larval dispersal of sponges in general (e.g., Mariani *et al.*, 2005) and can boost sponge speciation.

The new clades that are arising using genetic techniques represent a substantial increase in the number of sponge species currently known. The detection of cryptic species by molecular methods will continue to improve our knowledge of the true diversity in the world oceans in the forthcoming years. There is an active dispute about the relative merits of molecular-based and traditional descriptions of species (e.g., Bucklin *et al.*, 2011; Cook *et al.*, 2010; Packer *et al.*, 2009; Schlick-Steiner *et al.*, 2007). In our view, phenotypic descriptions should complement molecular-based species descriptions to make them practically available in ecological studies. Moreover, studies on the biological, biochemical, and ecological aspects of cryptic species are also recommended to approach a multidisciplinary “species concept” (Blanquer *et al.*, 2009; Cárdenas *et al.*, this issue; Erpenbeck *et al.*, 2004, 2006b; Loukaci *et al.*, 2004; Manuel *et al.*, 2003; Pérez *et al.*, 2011). Several biological aspects such as the extent and timing of the reproduction period, investment in reproduction, recruitment success, growth, and competitive abilities, can be different among cryptic species (e.g., Blanquer *et al.*, 2008b). Disregarding true sponge

biodiversity by ignoring the presence of cryptic species in an ecosystem will unavoidably lead to incorrect conclusions in ecological studies.

3. Population genetics and phylogeography

Population genetics focuses on the genetic characteristics that shape populations and influence their success or failure at ecological and historical time scales. The health state of populations and their capacity to adapt to environmental changes can be estimated by several genetic descriptors such as gene diversity, effective population size, gene flow, kinship, inbreeding, and the extent of the asexual reproduction, among others. Predictions obtained from theory are compared with empirical results obtained from actual populations to make inferences about patterns, processes, and cause-and-effect relationships in the biological world (Hamilton, 2009). On the other hand, understanding the historical events that have contributed to the current geographical distributions of populations is the main goal of phylogeography (Avice, 2000). The topics of population genetics merge with those of phylogeography and intraspecific phylogeny (Avice, 2004) as both address genetic differentiation among populations, but they focus on processes that occur at contrasting time scales (i.e., present-day recurrent processes, historical events, and evolutionary time scales) and, consequently, the molecular markers that are suitable to provide the necessary information for each issue may not be the same. Studies of population genetics of sponges lag behind other marine taxa, especially regarding the development and application of updated molecular markers. It has been suggested that sponge populations may not reach equilibrium rapidly and, consequently, highly variable molecular markers are required, together with particular evolutionary models (Wörheide *et al.*, 2004).

3.1. Choice of variable molecular markers at the intraspecies level

A range of molecular markers has been assayed with contrasting success in genetic studies of sponge populations. The divergent results of the several studies draw attention on the contrasting behavior that a gene may show in different species

and made it clear that new markers are still needed for answering the diverse ecological questions.

- Allozymes

Allozymes proved to be markers with high intraspecies variability in sponges, and were thus the molecular marker of choice for studying population structure and differentiation in sponges until the end of the past century, (e.g., Lazoski *et al.*, 2001; Miller *et al.*, 2001; Thorpe and Solé-Cava, 1994) and continue to be used today (Whalan *et al.*, 2005, 2008). Allozyme electrophoresis has also proven very useful to detect cryptic species and false cosmopolitanism (reviewed in Borchellini *et al.*, 2000; Solé-Cava and Boury-Esnault, 1999, see subchapter 2).

- Gene sequences

Nuclear gene sequences

The internal transcribed spacer regions (ITS1 and ITS2) have been widely used for phylogeographic studies of sponges (Duran *et al.*, 2004b, Lopez *et al.*, 2002; Nichols and Barnes, 2005; Wörheide *et al.*, 2002a, 2002b, 2004) likely because of the availability of rDNA PCR primers, which can be used across a range of taxa. Conserved and variable regions alternate in these genes, which facilitates the design of PCR primers in conserved regions, flanking more variable sequences (Nichols and Barnes, 2005). However, these genes were not sufficiently variable in all the targeted species. For instance, individuals of *Placospongia* from both sides of the Isthmus of Panama show little divergence in the targeted ITS region (Nichols and Barnes, 2005) as did individuals of the coralline sponge *Astrosclera willeyana* from the Red Sea, the Great Barrier reef and, and Fiji/Vanuatu (Wörheide *et al.*, 2002a). Moreover, ITS's may show intragenomic polymorphism (IGP) in sponges (Duran *et al.*, 2004b, Hoshino *et al.*, 2008; Van Oppen *et al.*, 2002; Wörheide *et al.*, 2004), which complicates the interpretation of phylogeographic results using these markers. Single-strand conformation polymorphism (SSCP) methods (e.g., Lobo-Hadju *et al.*, 2004) or cloning must be used to screen IGP in sponges before using ITS as markers for population genetics, because moderate ITS paralogy can be tolerable for phylogenetic studies, but less so for population level studies (Wörheide *et al.*, 2004).

Other gene partitions have been also assayed. An intron of the ATP beta

synthetase gene was used (in combination with ITS) for a phylogeographic and population genetic study of two calcareous sponges in the Pacific and Indo-Pacific regions (Bentlage and Wörheide, 2007; Wörheide *et al.*, 2008), and seems to be a promising tool.

Mitochondrial gene sequences

Mitochondrial genes are widely used in population genetics and phylogeographic studies of marine organisms because they are maternally inherited without recombination, have shorter coalescence times, and are expected to undergo lineage sorting three times faster than nuclear markers (Avice *et al.*, 1987; Palumbi *et al.*, 2001). However, the mtDNA gene partitions that have been used with success for other invertebrates (e.g. COI), are extremely conserved in sponges (e.g., Knowlton, 2000) and they have been rather proposed for use as markers for medium and low-level phylogenies in lineages that diverged up to 200 MYA ago (e.g., Erpenbeck *et al.*, 2002).

The several studies that have used mitochondrial markers, in particular the 5' region (Folmer *et al.*, 1994) of the cytochrome C oxidase subunit I (COI), to determine the genetic structure of sponge populations, revealed very low levels of variability of this gene even over broad geographic scales (tens of thousands of Km, Wörheide, 2006), although population structure could be demonstrated in general (DeBiasse *et al.*, 2010; Duran *et al.*, 2004a; Duran and Rützler, 2006). The I3-M11 partition of the same gene seems to show a slightly higher intraspecies variability in sponges than the Folmer partition (Erpenbeck *et al.*, 2006a; López-legentil and Pawlik, 2009), and thus it seems more suitable for sponge population level studies. In some cases, where COI sequences indicated significant differences among targeted “populations”, they were in fact revealing cryptic species (e.g., Xavier *et al.*, 2010a).

Another mitochondrial gene, the NADH dehydrogenase subunit 5 (nad5), has recently been assayed for intraspecific genetic diversity in two sponge species (Hoshino *et al.*, 2008). As in the case of COI, the nad5 gene showed very low genetic diversity that hinders its applicability in this group.

Amplified fragment length polymorphisms (AFLPs)

AFLPs are reliable and relatively easy to obtain markers used for studies of population genetics in several invertebrate groups (Mueller and Wolfenbarger, 1999). In sponges, however, AFLPs have only been used to differentiate *Ephydatia fluviatilis* and *E. mülleri* when gemmules, which harbor the diagnostic spicules, are not present. AFLPs have been proposed to use in combination with other markers in population level studies in those areas where the two species are in sympatry, to avoid overestimation of genetic differentiation among populations of one species due to erroneous species attribution (Gigliarelli *et al.*, 2008).

Microsatellites.

Because of the intra-genomic variability of ITS, the low resolution of sponge-mtDNA sequences even over long geographical distances, and the methodological constraints of allozymes, microsatellites appear to be the best choice among the currently available markers for sponge studies of population differentiation, genetic diversity, gene flow, clonality and other population genetic descriptors. Microsatellites or SSRs (simple sequence repeats) are small DNA stretches consisting of a repeated core sequence of a few base pairs. Because they are highly polymorphic in the number of repeats (and thus in length), and co-dominant, they have been extensively used since the 1990s in population assignments, paternity analyses and fine-scale dispersal analyses of terrestrial organisms (Webster and Reichart, 2005). The major drawback of this technique has been the cumbersome effort needed to generate a statistically relevant number of such polymorphic loci in non-model organisms (Zane *et al.*, 2002). Consequently, they have been only occasionally used up to now in sponge studies. There are at present 8 sponge species for which microsatellites have been developed (listed in subchapter 1).

3.2. Genetic differentiation at large and regional geographical scales

Genetic differentiation among sponge populations seems to be the rule in studies at broad scales. Benzie and coworkers (1994) studied allozyme variation at six polymorphic loci in four dictyoceratid species (*Phyllospongia lamellosa* (Esper), *P. alcornis* (Esper), *Carteriospongia flabellifera* (Bowerbank), and *Collospongia auris* (Bergquist, Cambie & Kernan) in the western Coral Sea (Pacific Ocean). The allele

frequencies showed that the populations of these species were in Hardy-Weinberg equilibrium, presumably as a result of random mating in local populations. Genetic differentiation was found for all the populations of all studied species, two of which followed the isolation by distance model (Table 2). The study allowed the authors to detect a barrier to gene flow between some populations caused by the South Equatorial Current since the genetic divergence found among populations North and South of this current was higher than expected from the geographic distances between them.

As said above (see subchapter 2), Klautau *et al.* (1999) demonstrated with the use of 10 allozyme loci the false cosmopolitanism and sibling speciation in the *Chondrilla nucula* complex. In the same work, they analyzed the differentiation of 7 populations of one of the genetic forms (*Chondrilla* sp. B) along 2700 Km of Brazilian coastline and found that they were highly structured, indicating low gene flow along the coast studied.

Lazosky *et al.* (2001) analyzed both interspecies and intraspecies variation of 13 allozyme polymorphic loci in Mediterranean and Atlantic (Bermuda and Brazil) populations of the purportedly cosmopolitan species *Chondrosia reniformis*. The low genetic identities of Atlantic and Mediterranean sponges were compatible with the presence of two cryptic species. However, the West Atlantic populations of *C. reniformis* were genetically similar over a distance of >8000 Km, and a high gene flow scenario was suggested in what was a completely atypical result for sponges. The interpretation of this result, however, should be taken with caution. Genetic homogeneity was calculated based on Nei's *I* index (Nei, 1978) and it was high for both Mediterranean (>0.96) and West Atlantic populations (>0.88). These values, however, are well within the range of those found in intraspecies comparisons in sponges (Solé-Cava and Boury-Esnault, 1999). On the other hand, analyses of genetic differentiation (*Fst*) showed that the populations have a significant genetic structure in both areas ($p < 0.0001$) in spite of high genetic similarity.

In a study of a species of *Haliclona* Grant with allozymes, Whalan *et al.* (2005) investigated the genetic structure of populations at several spatial scales. Although only two loci were included in the study, the results were consistent with panmictic populations within reefs, but significant differentiation was found at intermediate

(between reefs separated by hundreds of m) and large (between areas 400 Km apart) scales.

Nuclear ribosomal internal transcribed spacers (ITS) have often been used for sponge phylogeographic studies. Duran *et al.* (2004b) analyzed sequence variation in the nuclear ribosomal internal transcribed spacers (ITS-1 and ITS-2) in eleven populations of the sponge *Crambe crambe* across the species distribution range in the western Mediterranean and Atlantic Ocean. They reported the first confirmed instance of intragenomic variation of ITSs in sponges. Phylogeographic, nested clade and population genetic analyses revealed highly structured populations affected by restricted gene flow and isolation-by-distance. The authors speculated about a recent expansion of the species distribution range to the Macaronesian region from the Mediterranean, and stated that the pattern observed was not likely to be the result of a natural biogeographic relationship between these zones but of a man-mediated introduction.

ITS sequences were much more variable in populations of two species of *Hymeniacidon* Bowerbank (*H. flavia* Sim and Lee, and *H. sinapium* de Laubenfels) from Japan than NADH dehydrogenase subunit 5 (*nad5*) mitochondrial DNA sequences (Hoshino *et al.*, 2008), which only showed two haplotypes per species along their respective distribution ranges. Several significant genetic structures were detected in the nested clade analysis (NCA) for *H. flavia*, indicating restricted gene flow with isolation by distance, while *H. sinapium* showed very little genetic variation. The authors speculated about a recent introduction via natural or man-mediated processes to the Western Pacific of *H. sinapium*, which may have experienced a bottleneck as a result of founder effects during introduction, or a severe population decline followed by rapid range expansion. The geographic genetic structure of *H. flavia* suggests low dispersal ability of its larvae, whereas higher larval dispersal was suggested for *H. sinapium*.

Bentlage and Wörheide (2007) developed a new nuclear marker for sponges (the second intron of the nuclear ATP synthetase beta subunit gene, ATPS β -III), and analyzed it together with internal transcribed spacer (ITS) sequences to uncover phylogeographic patterns of the coral reef sponge *Pericharax heteroraphis* in the southwest Pacific. Variation among ITS sequences was low in contrast to ATPS β -III, indicating a better performance of the newly developed marker for population studies.

A statistical parsimony network suggested a past population subdivision with subsequent range expansion for GBR alleles. The authors expressed concern about the small sizes of most sampled populations but, based on the pairwise F_{st} values among pooled regional populations, they reported a high degree of differentiation between Indonesia and the GBR, Queensland Plateau and Vanuatu. Moreover, Vanuatu was strongly differentiated from the Queensland Plateau, central and southern GBR, whereas the differentiation between Vanuatu and the northern GBR was considerably smaller.

Wörheide *et al.* (2008) studied the genetic divergence among Indo-Pacific populations of the calcareous sponge *Leucetta chagosensis* Dendy by using two nuclear markers (ITS 1 and 2) and the same intron (ATPS β -III), used in the previous study (Bentlage and Wörheide, 2007). A deep phylogeographic structure was found, congruent across the ITS and ATPS β -III markers. One phylogeographic clade contained specimens from the Indian Ocean and Red Sea, another clade was composed of individuals from the Philippines, and two other clades consisted of sponges from NW Pacific and SW Pacific with an area of overlap in the Great Barrier Reef/Coral Sea. Gene flow was low among most regional populations, which showed isolation by distance along the Equatorial Current in the South-western Pacific. Overall, the results pointed towards stepping-stone dispersal with some putative long-distance exchange, consistent with expectations from low dispersal capabilities. Both founder and vicariance events during the late Pliocene and Pleistocene were speculated to be partially responsible for generating the deep phylogeographic structure found.

Duran *et al.* (2004a) performed the first study of population structure in sponges using COI sequence data (5'-end or Folmer partition). 8 populations of the poecilosclerid *Crambe crambe*, separated by distances from 20 to 3000 Km, were analyzed. As mentioned, low variability of this gene was found (only two haplotypes). Nevertheless, the different frequencies of these haplotypes revealed genetic structure and low gene flow between populations separated by tens of Km.

The phylogeographic study of the purportedly circum-Pacific species *Astrosclera willeyana* across the Indo-Pacific using the Folmer partition of COI (Wörheide, 2006) is a paradigmatic example of the low variability of the sponge mtDNA. Only three COI haplotypes with a maximum p-distance of 0.42% were identified across the Indo-

Pacific populations spanning more than 20,000 Km. The haplotype distribution, however, was uneven, as all Pacific individuals had one of the haplotypes with the exception of a single population featuring a second haplotype. The Red Sea population consisted of individuals with the third haplotype found.

Whalan *et al.* (2008) used the Folmer partition of COI for analyzing the population structure of the species *Rhopaloeides odorabile* Thopsom, Murphy, Bergquist, and Evans in the central GBR. Sampling distances ranged between 100 m and 140 Km. Moreover, they analyzed the same samples with three polymorphic allozyme loci and compared the results with both markers. Populations did not show structure for any of the two markers and no evidence for genetic differentiation between inner- and mid-reef sites was revealed. Nuclear and mtDNA markers indicate large-scale genetic admixture in this species, although there was some evidence for small, localized, genetic differences between some populations, which the authors attributed to reef-specific hydrodynamics.

López-Legentil and Pawlik (2009) compared the two above-mentioned partitions of COI in seven populations of *Xestospongia muta* from Florida, the Bahamas and Belize and found higher nucleotide diversity in the I3-M11 partition than in the 5'-end partition. Pairwise tests of genetic differentiation among geographic locations based on *Fst* values showed significant genetic differentiation between most populations, but this genetic differentiation did not follow the isolation by distance model. These authors explained the differentiation found by the patterns of ocean currents, although they did not discard that the limited dispersal of larvae contributed to the differentiation found. The authors advised to consider local hydrological features in future plans for management and conservation of sponges in coral reefs.

The genetic population structure of the common branching sponge, *Callyspongia vaginalis*, along more than 450 Km of the Florida reef system, from Palm Beach to the Dry Tortugas, was assessed by using sequences of the Folmer partition of the COI gene (DeBiasse *et al.*, 2010). No clear pattern of genetic differentiation was revealed. The strong structure of populations from most sampling locations was attributed to larval philopatry as in other sponge species. However, in a few cases, non-significant pairwise *Fst* values were found between relatively distant sampling sites. The genetic connectivity between populations far away from each other led the authors to suggest that some long distance larval dispersal may occur via ocean

currents or larval transport within sponge fragments as reported for the Mediterranean *Scopalina lophypoda* (Maldonado and Uriz, 1999).

Sequence variation in the I3-M11 partition of the mtDNA COI gene was analyzed in ten populations of the Atlanto-Mediterranean demosponge *Phorbas fictitius* (Bowerbank) (Porifera: Poecilosclerida) at a regional scale comparing mainland (Iberian) and insular (Macaronesian) populations, and at a local scale focusing on different island of the Azores archipelago (Xavier *et al.*, 2010b). Genetic differentiation based of F_{st} estimates was found among most populations at both scales revealing highly structured populations. This confirms the presumably low dispersal potential of this species and the geographical isolation of the studied populations. However, the authors found evidence of long distance dispersal events between some populations. Only two haplotypes were shared by mainland and insular localities. Phylogenetic and network analyses indicate a separation of insular (Macaronesian) and mainland (Iberian) populations. The phylogenetic analysis pointed to the Macaronesian Islands as the species origin area with posterior expansion to mainland locations via current-mediated dispersal of larvae or sponge fragments. This study adds to the growing evidence of structured populations in the marine realm and highlights the importance of the Macaronesian islands on the evolutionary history of the Northeast Atlantic marine biota (Xavier *et al.*, 2010b).

The levels of genetic divergence among populations of *P. fictitius* using the I3-M11 partition (Xavier *et al.*, 2010b) were of the same order of magnitude than those of *Xestospongia muta* populations using the same COI partition (López-Legentil and Pawlik, 2009), and much higher than the values found in Folmer's COI partition in several species at similar and even larger spatial scales (Duran *et al.*, 2004a; Wörheide, 2006). These studies therefore support that this alternative partition of the COI gene is more suitable than the 5'-end partition to infer intraspecific patterns in sponges, as already shown for interspecies relationships by Erpenbeck *et al.* (2006). However, the finding of suitable polymorphic gene partitions for sponge population studies is not resolved and new genes need to be explored. Recently, partial sequences of the ATP synthase 6 (ATP6) and the cytochrome oxidase 2 (CO2) genes and two spacers: one located between ATP6 and CO2 and the other between the NADH dehydrogenase subunit 5 (ND5) and the small subunit ribosomal RNA genes have been assayed simultaneously in taxonomical distant sponges (Rua *et al.*

2011) with contrasting success for alpha-level systematics, phylogeography and population genetics.

The use of microsatellites allowed detection of marked population structure of the species *Crambe crambe* at local and regional scales (Duran *et al.*, 2004c) with more accuracy than sequence data (Duran *et al.*, 2004a, 2004b). Eleven populations were analyzed at six loci in locations placed along the Atlanto-Mediterranean distribution range of the species. High levels of between-population structure were found and a significant isolation-by-distance pattern was observed. A strong genetic structure was also found within sampled sites. Patterns of allelic distribution between populations suggest the possibility of a recent colonization of the Atlantic range from the Mediterranean Sea as already proposed by Duran *et al.* (2004b) using sequence data.

The genetic structure of the Mediterranean sponge *Scopalina lophyropoda* (Schmidt) was analyzed at several spatial scales (from tens of meters to thousands of Kms) by using seven specific microsatellite loci (Blanquer and Uriz, 2010). The genetic diversity of *S. lophyropoda* was structured at the three spatial scales studied: within populations, between populations of a geographic region, and between isolated geographic regions, although some stochastic gene flow might occur among populations within a region. The genetic structure followed an isolation-by-distance pattern according to the Mantel test. However, several of the genetic descriptors gave unexpected results. Despite philopatric larval dispersal (Uriz *et al.*, 1998) and fission events in the species (Blanquer and Uriz, 2010), heterozygote excess was found in many populations, and the contribution of clonality to the population genetic makeup was minor. The heterozygote excess and the lack of inbreeding were envisaged to be the result of either sperm dispersal, a strong selection against mating between relatives to avoid inbreeding depression or a high longevity of genets combined with recruitment events by allopatric larvae.

The population genetics of two emblematic Mediterranean bath sponges has been studied recently by microsatellites. Seven populations along the western Mediterranean and the Portugal coasts of *Spongia lamella* were analyzed by using seven microsatellite loci (Noyer, 2010). Inbreeding was the main characteristic for all loci and populations, which was attributed to mating among relatives or to the existence of breeding subunits within populations. Although the results should be

taken with care because of the high rate of null and unsized alleles and the low number of individuals in some populations, partitioning of the molecular variance (AMOVA) showed that genetic data were spatially structured with significant differences within populations and among populations of each region. Genetic structure was found in all the populations examined, which followed an isolation-by-distance model.

In contrast, a genetic study on the Mediterranean bath sponge *Spongia officinalis* (Dailianis *et al.*, 2011) reports a high genetic diversity in most populations despite the species' harvesting and the recurrent massive mortality episodes (Pérez *et al.*, 2000) that decimate its populations. Population genetic analysis along the species distribution range (from eastern Mediterranean to the Strait of Gibraltar) using eight microsatellite loci showed low levels of genetic structure, not correlated to geographic distance, inside geographic sectors (western and eastern Mediterranean). Anthropogenic and natural mechanisms were speculated to be involved in enhancing larval dispersal, resulting in an unusual connectivity among sponge populations at a regional scale. Specimens were also analyzed using the 5'-end partition of COI to verify whether the several morphotypes of the species described (Vacelet, 1959) were indeed cryptic species. COI sequences indicated that only one species is presented throughout the Mediterranean, except in the Gibraltar zone where another cryptic *Spongia* sp. could be present (Dailianis *et al.*, 2011).

3.3. Small-scale genetic structure

The few studies that assessed the genetic structure of sponges at small spatial scales (i.e., among populations separated by tens of m or within populations at the scale of a few meters) have been performed using microsatellite markers. A small-scale study of the population structure of the sponge *Crambe crambe* from a single rocky wall (inter-individual distances from 0 to 7 m) was done using six microsatellite markers and autocorrelation analysis on mapped individuals (Calderón *et al.*, 2007). The results showed a strong genetic similarity of sponges separated by less than 100 cm. Even when the effect of clonality was removed from the analysis, the trend of genetic relatedness was significant within the first distance classes (30–40 cm). On the contrary, genetic similarities in sponges 2–7 m apart were within the same range

as sponges from other walls of the same locality, or from other Mediterranean localities. Estimated mean dispersal distances per generation were ca. 35 cm, and neighborhood sizes were estimated at ca. 33 sponges. This indicated that, although some or many of the larvae could disperse away from the population of origin, enough propagules settled in the close vicinity of their mother sponges so as to build a marked genetic structure at very small spatial scales. Interestingly, the results strongly pointed to the existence of some degree of self-fertilization in this population

The sponge larval philopatry reported from behavioral studies (e.g., Uriz *et al.*, 1998) seems to be reflected in the inability of larvae to overcome subtle barriers such as unidirectional currents or small submarine walls (e.g., Blanquer *et al.*, 2008b; Guardiola *et al.*, 2011). The Mediterranean sponge *Scopalina lophyropoda* is a clear instance of strongly restricted gene flow even among populations separated by tens of meters. Blanquer *et al.* (2009) mapped and characterized genetically all the individuals of three populations placed on three vertical walls separated ca. 100 m each and analyzed the contribution of sexual and asexual reproduction, and the breeding and mating system, to the spatial genetic structure (SGS) using seven microsatellites. SGS was analyzed at increasing distances by autocorrelation analysis. Significant autocorrelation and thus SGS was found at the smaller distances analyzed (from one to 10 m), which underpins the larval philopatry reported in behavioral studies (Uriz *et al.*, 1998). All these patterns, however, contrasted with the lack of inbreeding detected in the populations, which is in agreement with data on other marine modular invertebrates (Ayre and Miller, 2006; McFadden, 1997) and confirms that strong SGS does not necessarily imply inbreeding.

Microsatellites resulted informative markers to establish the genetic structure of very close subpopulations of the allochthonous calcareous sponge *Paraleucilla magna* at the Blanes littoral (NE of the Iberian Peninsula). This is the only genetic study so far of an introduced sponge species, which has colonized the Mediterranean from the Atlantic in recent years. Low but statistically significant genetic differentiation was found among the three subpopulations of *P. magna* established the first study year despite they were placed less than 100m apart, and the short time after their establishment (Guardiola *et al.*, 2011). Several estimators of genetic differentiation allowed to confirm the differentiation among populations. F_{st} values were similar to those reported for *S. lophyropoda* in the same localities. Populations showed a

heterozygote deficit, attributable to inbreeding, which is in agreement with the species' patchy distribution and an extreme philopatry of their larvae (Frotscher and Uriz, 2008; Lanna *et al.*, 2007). The authors speculated about a founder effect as the cause of this genetic pattern since these small populations were recently established in the study area (ca. 10 years ago).

3.4. Temporal genetic structure

The only study of temporal genetic differentiation across three consecutive years has been performed on the allochthonous calcareous sponge *Paraleucilla magna* (Guardiola *et al.*, 2011). The species is annual (Frotscher and Uriz, 2008) so that the building of the yearly populations relied exclusively on recruits resulting from those larvae released from the previous cohort. The population genetic features allowed the authors to predict either high genetic variation in populations across time, if allopatric recruitment occurs, or low genetic differentiation across years if the yearly cohorts result from philopatric larvae. Low but statistically significant differentiation of the three populations occurred across years. These results also showed heterozygote deficit and allele instability in the populations over the three years, which are consistent with a recent establishment of these populations in the study area. However, one population disappeared in the second study year, likely as a result of recruitment failure, and the other two remained slightly differentiated, while the three populations were in place again in the third study year, and showed significant *F_{st}* values. Overall, the population descriptors pointed to this species as a good opportunistic colonizer but highly sensitive to stochastic events affecting recruitment and thus with low-medium predicted impact on native communities.

3.5. Conclusions

The studies considered in this review highlighted some shortcomings of gene sequence data used, thus far, for the assessment of sponge population genetics. Mitochondrial genes (in particular the Folmer partition of the COI gene) are too

conserved for reliably detecting intraspecies genetic patterns, and even the I3-M11 partition of this same gene is not variable enough for detecting microevolutionary processes of sponge populations. On the other hand, ITS sequences have moderate intraspecies variability, but can show intragenomic variation that must be investigated before these markers can be used. New sequence markers for sponges, such as the ATPS β -III intron, proved useful for revealing differentiation at large geographical distances and deserve further attention. Microsatellites have now replaced the use of allozymes, but there are only a few studies using microsatellites in sponges as yet. Nevertheless, these studies proved that microsatellite markers were highly polymorphic in sponges, and are a good choice to analyze geographic and temporal patterns at a wide range of scales, including the intrapopulation level. However, the high variability associated with microsatellite loci means that sample sizes must be large (Ruzzante, 1998). Microsatellites in sponges also tend to consist of imperfect repeats and to show amplification failures or null alleles that complicate the interpretation of results. More and better microsatellite markers need to be developed, although the initial phase of screening and optimizing markers for each study species represents an important cost in terms of time and money.

Overall, our screening of works that formally analyzed population differentiation at different scales and with different markers (Table 2) shows an overwhelming majority of positive results. In general, as Boury-Esnault and Solé-Cava (2004) stated, we can conclude that sponge populations are genetically structured, which is in accordance with the short-lived type of larva they feature. Isolation by distance has not been substantiated in all studies that have analyzed it, though (Table 2). This points to the importance of sporadic phenomena such as episodes of long-range dispersal or to the relevance of hydrological features in shaping the distribution of sponge populations. It is also noticeable that, with intriguing exceptions (e.g., *Scopalina lophyropoda*), the use of microsatellite markers has always shown a deficit of heterozygotes in the populations, while allozymes tended to indicate that populations are in Hardy Weinberg equilibrium (but see Whalan *et al.*, 2005). More research is necessary on more species to ascertain whether inbreeding is indeed a common feature of sponge populations or whether the results obtained are an artifact of the marker used (e.g., null alleles in microsatellites).

Despite the drawbacks associated with the selected marker or sampling

procedures, the information gathered from studies of population genetics of key species is widely applicable in conservation issues. The genetic fitness of populations and their connectivity should become pivotal aspects in management and design of marine protected areas and should also be considered in regulation policies for exploited natural resources (Bell, 2008; Cognetti and Maltagliati, 2004; DeSalle and Amato, 2004; Palumbi, 2003). Selective protection or removal of target key species (often emblematic animals or plants) often produces ecosystem disequilibria (VanBlaricom and Estes, 1988). Thus, protection of whole habitats or, at least, of as many species as possible, is desirable. Consequently, studies of population genetics of “not-so-emblematic” species such as sponges and other invertebrates are increasingly taken into consideration in conservation policies.

4. Contribution of asexual phenomena to population structure: clonality and chimerism in sponges.

Sponges, as other benthic invertebrates, can combine sexual and asexual reproduction. The former implies the production of small dispersive propagules (larvae) that potentially allow colonization of new habitats. In contrast, asexual reproduction often results in the production of larger propagules with lower dispersal abilities. However, long dispersing propagules, such as floating buds, drifting fragments, and other external gemmules, can also be produced asexually. The ecological and evolutionary significance of these diverse reproductive mechanisms is far reaching. Asexual reproduction allows rapid colonization of an area or rapid recovery after disturbance, at the cost of genetic diversity. Sexual reproduction is often restricted temporally (e.g., seasonally) and is a slower process, but it allows new adaptive combinations of the genetic pool; long-distance dispersal of larvae may allow colonization of new areas.

In other groups of invertebrates such as cnidarians, asexual reproduction generally dominates population structure locally, while sexual propagules ensure gene flow between populations (McFadden, 1997). However, this pattern cannot be extrapolated to sponges and it remains unclear under which circumstances one or another strategy should be favored. Likely, the pattern may change with the particular

biological characteristics of the species and their habitats.

General ecological theory predicts that asexual reproduction may be adaptive in extreme and disturbed habitats, allowing the population to grow quickly while environmental conditions are favorable and to repopulate quickly after disturbance, while sex will be favored in biotically complex and undisturbed environments (Bell, 1982; Trivers, 1985). However, asexual propagation can also be a mechanism for maintaining or increasing the abundance of well-adapted genotypes in a relatively unchanging habitat (Carvalho, 1994; Maynard-Smith, 1978). This seems to be the case of Antarctic sponges, which exhibit a high production of asexual buds independently of their taxonomy (Sarà *et al.*, 2002; Teixidó *et al.*, 2006). On the other hand, sexual reproduction with its associated genetic recombination is advantageous in moderately changing environments where a substrate of genetic variability, upon which selection could act, is necessary (Bengtsson and Ceplitis, 2000, Bürger, 1999; Charlesworth, 1993; Sebens and Thorne, 1985; Williams, 1975). In benthic cnidarians, asexual reproduction has been related to stable habitats and to little predation pressure (Ayre, 1984; Karlson, 1991). However, Coffroth and Lasker (1998) found in a gorgonian the highest amount of reproduction by fragmentation at intermediate levels of disturbance. In fact, sexual and asexual reproductive strategies can successfully coexist in time and space in the same sponge individuals (Bautista-Guerrero *et al.*, 2010; Johnson, 1978; Leong and Pawlik, 2010; Sarà and Vacelet, 1973), or occur in different seasons (Ereskovsky and Tokina, 2007), thus reflecting that these two strategies are evolutionarily stable in a range of environmental situations.

4.1 Sponge asexual propagules.

Asexual propagules have been reported in representatives of all classes of Porifera (Ereskovsky and Tokina, 2007). They are diverse in shape, location within the sponge, and function (Fig. 2) and in most cases contribute to increase the clonality of sponge populations. Ecologically, they can be divided into dispersive (those that can travel to some distance) and non-dispersive propagules (basically recruiting in the same population that originates them). Among the former, external

buds, commonly produced as projections at the sponge surface, are the most widespread (Merejkowsky, 1879). The calcareous *Sycon* spp (Connes, 1964) and *Clathrina blanca* (Miklucho-Maclay) (Johnson, 1978), the hadromerids *Tethya* spp (Connes, 1967; Gaino *et al.*, 2006) and *Polymastia penicillus* (Montagu) (Plotkin and Ereskovsky, 1997), the axinellid *Axinella damicornis* (Esper) (Boury-Esnault, 1970), the poecilosclerid *Mycale contarenii* (Martens) (Corriero *et al.*, 1998; De Vos, 1965), the astrophorid *Thenea muricata* (Bowerbank) (Uriz, 1983), and the hexactinellid *Lophocalyx* spp. (Schulze, 1887), all are examples that often show external small, roundish buds at the end of spicule bundles. In other cases, (e.g *Aplysina* Nardo *Anoxycalyx* Kirparick, and *Rosella* spp.) the propagules consist of irregular outgrowths of large size (from mm to cm) whose stalks progressively constrict off, the propagule finally breaking free, moving to some distance, and attaching to the substrate as new individuals (Vacelet, 1959, Sánchez, 1984, Teixidó *et al.*, 2006). In stable environments, these abundant propagula can result in sponge stands dominated by the asexually reproduced species (Fig. 3 b).

A particular process is found in sponges without rigid skeletons but with high amounts of collagen such as some chondrosids (e.g., *Chondrosia reniformis*, Bonasoro *et al.*, 2001; *Chondrilla "nucula"* complex, Zilberberg *et al.*, 2006a) and homosclerophorids (e.g., *Oscarella lobularis*, Sarà and Vacelet, 1973), which, when placed in overhangs, produce outgrowths that elongate towards the floor, resulting in teardrop-shaped fragments tethered by thin strands of tissue that finally break off, the fragment attaching to the underlying substrate.

External bubble-like buds have been recently reported for the homosclerophorids *Oscarella lobularis* and *O. tuberculata* Schmidt (Ereskovsky and Tokina, 2007). These buds float in the water column and can develop very fast into functional sponges, so they could disperse farther than denser external buds that will tend to sink faster. Bubble-like buds are also seasonally found in *Prosuberites epiphytum* Lamarck from the Mediterranean Sea (authors' pers. obs.) and might have gone unnoticed in other sponge species.

Internal buds (gemmules) were first described in freshwater sponges (Brien, 1967) but have been also reported for marine demosponges. They have been studied in detail in the hadromerid *Stylocordyla borealis* (Loven) (= *S. chupachups* Uriz, Gili, Orejas and Pérez-Porro) (Sarà *et al.*, 2002), but they are particularly

characteristic of the spirophorid genera *Craniella* Schmidt, *Cinachyra* Sollas, *Cinachyrella* Wilson, and *Tetilla* Schmidt (e.g., Chen *et al.*, 1997). These gemmules (Fig. 3 c) are small and appear to be released through the sponge canals (e.g., Sarà *et al.*, 2002). Their dispersal capabilities are expected to be in general lower than those of sexually produced larvae. Moreover, sponge buds have been rarely found in the water column, so that their contribution to the species dispersal can only be assumed except where genetic studies of the species populations are performed.

Among asexual processes that *a priori* do not result in dispersal, individual fissions are frequent in encrusting species (Fig. 1). In contrast, casting off fragments in massive and branching sponges, in particular under rough sea conditions, (Battershill and Bergquist, 1990; Tsurumi and Reiswig, 1997; Wulff, 1985, 1986, 1991, 1995) may contribute to a certain extent to the species dispersal. These easy to fragment species can exploit this condition, e.g., to survive after predator attacks or physical disturbances (Leong and Pawlik, 2010). Spontaneous fission is common in thinly encrusting sponges (Ayling, 1983) and has been analyzed in several species, such as *Crambe crambe* (Garrabou and Zabala, 2001; Teixidó *et al.*, 2009; Turon *et al.*, 1998), *Hemimycale columella* (Bowerbank) (Garrabou and Zabala, 2001), *Halichondria okadai* (Kadota) (Tanaka, 2002), *Scopalina lophyropoda* (Blanquer *et al.*, 2008), *Hymedesmia* spp. and *Microciona* spp (authors' unpubl. res.).

The internal gemmules attached to the substrate of some hadromerids such as *Suberites domuncula* and *Suberites ficus* (Johnston) (Connes, 1977) do not contribute either to the species dispersal. These gemmules are covered by spongin and only develop in direct contact with the water once the adult sponge dies. The internal gemmules of the excavating clonaid genera *Aka* de Laubenfels, *Pione* Gray (Rosell and Uriz, 2002; Schönberg, 2002), *Cliona* Grant (Rosell and Uriz, 2002; Rützler, 1974) and *Thoosa* Hancock (Bautista-Guerrero *et al.*, 2010; Volz, 1939) are reinforced by collagen and spicules and seem to play a primary role similar to that of *Suberites* gemmules, i.e., to ensure the genotype persistence. These gemmules are comparable to the statoblasts of the freshwater Spongillids, which only develop once the produced sponge disappears (Brien, 1967; Saller, 1990).

4. 2. Clonality in sponge populations

Field monitoring studies have assessed the importance of asexual phenomena (fission, fragmentation, budding) in the population dynamics and life history of sponges (e.g., Ayling, 1983; Blanquer *et al.*, 2008b; Leong and Pawlik, 2010; Pansini and Pronzato, 1990; Teixidó *et al.*, 2006; Turon *et al.*, 1998; Wulff, 1985, 1986, 1991). However, they usually cover a restricted time frame and may lead to wrong interpretations of the relative importance of sexual and asexual reproduction on the long term (see below). Determining the extent of clonality is necessary, not only because of its biological implications, but also because clonality causes biases in population genetic parameters (genetic diversity, linkage disequilibrium, inbreeding coefficients, effective population sizes). While some works used histocompatibility techniques to define clones in sponges (Neigel and Avise, 1983; Neigel and Schmahl, 1984), recent studies have tackled the issue using genetic tools. In some cases, data on clonality are obtained in population genetic studies (e.g., Davis *et al.*, 1996; Duran *et al.*, 2004c; Miller *et al.*, 2001; Whalan *et al.*, 2005), but a few works addressed specifically the analysis of clonality at low spatial scales (Blanquer *et al.*, 2009; Calderón *et al.*, 2007; Zilberberg *et al.*, 2006a, 2006b).

For the genotyping of clones, hypervariable markers are needed. In this sense, allozymes and microsatellites have been the markers of choice in sponges. When identical multilocus genotypes (MLG) are found, the probability that they derive from sexual reproduction can be calculated (e.g., Stenberg *et al.*, 2003) and, if negligibly small, the groups of shared MLG are considered the result of asexual processes. Care should be taken, however, in the presence of inbreeding, as the probability of finding identical MLG by chance as a result of sexual reproduction increases significantly with respect to a randomly mating population with the same allele frequencies (Duran *et al.*, 2004b). The contribution of asexual reproduction (C_{asex}) can be measured as the complement (in percentage) of the ratio of different genotypes in a population, $(1-(N_c/N))*100$, where N_c is the number of different MLGs, and N is the total number of sponges analysed (adapted from Ellstrand and Roose, 1987). C_{asex} represents the maximal possible contribution of asexuality, as differences at loci not genotyped and repeated generation of the same MLG through sexual reproduction (even if unlikely) would render the true clonality smaller than the one estimated by C_{asex} .

Davis *et al.* (1996) analysed the genetic composition of sponges (*Halisarca laxus* (Lendenfeld)) epibiont on clump-forming solitary ascidians (*Pyura spinifera* (Quoy and Gaimard)) using six allozymes. They found that, in most cases, a single sponge clone is found on each ascidian clump, implicating an asexual colonization mechanism. Only 63 different genotypes were found among 172 sponge samples ($C_{\text{asex}} = 63\%$).

The extent of clonality in sponge populations can vary widely among closely related species. Miller *et al.* (2001), in an allozyme study of several morphs belonging to the genus *Latrunculia* du Bocage in New Zealand, found extensive cryptic speciation (with up to 8 different species) and evidence for asexual reproduction in each of these 8 species, although the contribution of clonality to the population structure was variable among species (C_{asex} indices from 18 to 63%). Identical MLGs (9 allozymes) were found only within sites, mostly comprising groups of 2 to 4 individuals, but up to 20 sponges shared a single MLG in Wellington.

Duran *et al.* (2004c) studied 11 populations of the poecilosclerid *Crambe crambe* in its Atlanto-Mediterranean range using 6 microsatellite loci. The work was primarily addressed at studying interpopulation structure but, in spite of a sampling planned to avoid clonemates (individuals collected at least 5 m apart), genotypically identical individuals for the 6 loci were found in three of the populations. A total of 13 groups of MLG, each comprising 2 or 3 sponges, were found. In most cases the pattern found was highly unlikely to be the result of sexual reproduction even taking into account the heterozygote deficit in the calculation of clonal probabilities. The authors suggested that fragmentation with subsequent dispersal of fragments, rather than simple fission, could explain the finding of clones more than 5 m apart.

The extent of asexual reproduction was highly variable in four species of the genus *Chondrilla* Schmidt, between species and among populations of the same species, in a study along the Caribbean and Brazilian shores using allozyme markers (Zilberberg *et al.*, 2006a). The C_{asex} index ranged from 27 to 52% in three of the species, while in the fourth it was 7% in one population and 39% in another, which was interpreted as reflecting differences in stability of the respective habitats. Most clonemates were found at short distances, indicating fission as the likely mechanism of clone production, and that asexuality has a role in filling in the available habitat rather than in dispersal. In addition, some individuals in overhangs shared MLG with

those just below, indicating a role of the production of teardrop fragments in the clonal structure of the species. In another study with allozyme markers, Zilberberg *et al.* (2006b) found that 52 coral-excavating *Cliona delitrix* Pang sponges from 13 coral heads separated from a few to several hundred meters had all unique genotypes ($C_{\text{asex}} = 0$). The starting hypothesis was that coral breakage and associated sponge fragmentation would be a mechanism for the spread of this bioeroding species. However, the results indicated that population maintenance is due to sexual reproduction alone in the area surveyed.

Calderón *et al.* (2007) genotyped six microsatellite loci in 177 specimens of the sponge *Crambe crambe* located in a single wall (all interindividual distances < 7m) to ascertain small scale genetic structure and clonality in this species. Given previous knowledge on the discrete fission rates (Turon *et al.*, 1998) and the larval behavior of this species (Mariani *et al.*, 2006; Uriz *et al.*, 1998), it was expected that clonality would have a minor role in the establishment of this population and that little genetic structure would be found at the scale of a few meters. The results were not coherent with expectations, 76 of the individuals had non-unique MLG, forming 24 clones of size 2 to 8 sponges. The contribution of asexual reproduction (C_{asex}) was, therefore, ca. 30%, and this pattern could not be explained by sexual reproduction. The inter-clone distances were less than one meter, with a mean of ca. 20 cm. In addition, strong genetic similarity of sponges located less than 50 cm apart was found even after the effect of clonality was removed, indicating that both sexual and asexual processes acted at small scales and contributed to the establishment of the species.

In another microsatellite-based study, Blanquer *et al.* (2009) analyzed small-scale genetic structure in *Scopalina lophyropoda* in the NW Mediterranean. In this case, field studies have revealed a dynamic system of fissions and fusions (Blanquer *et al.*, 2008b) and, consequently, an important contribution of clonality to the make-up of the populations was expected. However, the analysis of 7 microsatellites in 3 populations revealed that the extent of clonality was minor ($C_{\text{asex}} = 11\%$), and only two clones of 4 and 3 individuals, respectively, were proven to be the result of asexual phenomena. Nevertheless, strong genetic structure, attributable to larval philopatry, was found at small scales (less than 5 meters). The lack of clonality detected can be in part explained by the finding (Blanquer and Uriz, 2011) that this sponge had a prevalence of chimeric individuals (see below). Thus, if there are

different MLGs in a single individual, the assessment of fission events using genetic markers becomes unreliable.

No clones were reported to influence the genetic structure of the populations of several species belonging to the taxonomically distant taxa Calcaronea (within the class Calcarea) and Haplosclerida and Dictyoceratida (within Demospongiae). In a study of *Haliclona* sp. at spatial scales ranging from meters to hundreds of Km, Whalan *et al.* (2005) found no evidence that asexual reproduction is important for the maintenance of populations. Although limited to only two polymorphic allozyme markers, the levels of genetic diversity were high, and the number of identical MLGs found was coherent with chance expectation in sexually reproducing populations given the allele frequencies found. In the Mediterranean populations of the introduced calcareous sponge *Paraleucilla magna*, clonality was found to be almost inexistent ($C_{\text{asex}} = 1.5\%$, Guardiola *et al.*, 2011). No clonality was reported in the genetic studies of the Mediterranean bath sponges *S. officinalis* (Dailianis *et al.*, 2011) and *S. lamella* (= *S. agaricina*) (Noyer, 2010), which is accordance with the lack of asexual buds and the unbreakable (elastic) consistence of the two species, which makes fragmentation unlikely.

In conclusion, from the scarce number of works that applied genetic tools to the characterization of clonality in sponges, a wide variability in the contribution of asexual reproduction to the structure of populations can be concluded. A second lesson that can be gleaned from these studies is that, in many cases, predictions from field studies and conventional population dynamics were not supported by genetic data (Blanquer *et al.*, 2009; Calderón *et al.*, 2007; Zilberberg *et al.*, 2006b). Field studies are in general restricted temporally and miss the historical perspective, which is particularly important in long-lived, slow-growing species whose population structure is built up over the years. In this sense, molecular techniques allow an analysis of clonality that keeps the history of past events, providing an assessment of the long term contribution of sexual and asexual processes to the demography of the populations under study, rather than a temporally restricted snapshot (Calderón *et al.*, 2007). More studies on species with different morphologies and under different environmental conditions are called for to reveal the full extent of the importance of clonality in sponges.

4. 3. Sponge chimerism

The existence of fusion processes between sponges (e.g., Blanquer *et al.*, 2008; Turon *et al.*, 1998) raises the interesting possibility of chimera formation. Histocompatibility assays and natural observations (Neigel and Avise, 1983; Neigel and Schmahl, 1984) indicated that contact between genetically distinct (allogeneic) sponges results in a rejection reaction, while contact between clonally-derived fragments (isogeneic) often leads to fusion. However, fusion between sibling larvae has been reported (e.g., Maldonado, 1998; Van de Vyver, 1970), but this capability to fuse between allogenes is reported to be lost during ontogeny (Gauthier and Degnan, 2008; Ilan and Loya, 1990; Maldonado, 1998). The genetic mechanism responsible for allorecognition is not known at present in sponges and is an important field for future research.

The biological and ecological implications of chimerism have been widely discussed (e.g., Grosberg, 1988a; Pineda-Krch and Lehtila, 2004; Rinkevich, 2005). Among the selective advantages, escape in size from predation and exploitation of joint genomic fitness have been proposed. On the other hand, somatic and germ cell parasitism can occur, sometimes leading to resorption of one of the partners. While these phenomena have been intensely studied in other invertebrate groups such as ascidians (reviewed in Rinkevich, 2005), much less is known in sponges, although takeover of the germ cell population of chimeric sponges by one of the fusion partners has been proposed (Gauthier and Degnan, 2008).

Only two studies have addressed the study of chimerism using genetic tools. In a grafting experiment with *Niphates erecta* Duchanssaing and Michelotti, Neigel and Avise (1985) compared the responses (either fusion or non-fusion) with genotypic composition at three allozyme loci. Although compatibility and genetic results were largely concordant, in the sense that most acceptances occurred in individuals with identical genotypes (and the converse was true for non-fusions), a few grafts between sponges with different allozyme genotypes resulted in allogeneic fusion. In the only genetic study of chimerism in a natural population of a sponge, Blanquer and Uriz (2011) using seven microsatellite loci found a high incidence of multichimerism in *Scopalina lophyropoda*, with a total of 36 multilocus genotypes (MLG) in 13 sponges sampled at four points of their bodies. In some cases, each intra-individual sample had a different MLG. Interestingly, larvae produced by the colonies had

genotypes compatible with the transmission of different MLG, so no germ line dominance seemed to occur.

4.4. Conclusions

The few studies on sponge clonality using molecular tools indicated that the contribution of asexual reproduction to the genetic structure of the sponge populations is lower than previously assumed given the many instances of asexual propagules reported for these animals. In particular, no true clones were found in genetic studies of massive sponges such as *Spongia officinalis*, *S. lamella*, and *Paraleucilla magna* or in the excavating *Cliona delitrix*. Populations of encrusting species would seem more prone to harbor clones resulting from fission, because the released fragments remain attached to the substrate and thus they do not leave the population of origin (e.g., *Crambe crambe*). However, this cannot be generalized to all the encrusting species, since an example exists of a thinly- encrusting, highly fissionable species with minimal numbers of clonemates in their populations (i.e., *Scopalina lophyropoda*). On the other hand, sponge clonality is expected to be higher in relative stable habitats such as deep bottoms or the Antarctic shelf, where sponge distribution use to be very patchy (Rice *et al.*, 1990) and some producers of asexual propagules dominate (Teixidó *et al.*, 2006).

All the instances so far studied address the role of clonality at small scales (within populations), and no evidence has been found for the potential role of widely dispersing asexual propagules (e.g., floating buds). These studies are of course more challenging methodologically, as the probability of encountering asexual propagules far from the source is very small. However, a convenient starting point could be the analysis with genetic markers of population structure of actively fragmenting sponges with potential for relatively long levels of dispersion, such as some branching forms (Wulff, 1985, 1991).

Natural occurrence of sponge chimerism has been but recently proved unambiguously in a sponge species (Blanquer and Uriz, 2011). Whether this characteristic is exclusive of *Scopalina lophyropoda* or it exists in other species remains to be explored. Sponge chimerism could help to understand the long-term

success of relatively small (as for the number individuals) sponge populations, which are highly structured and patchily distributed, by minimizing genetic drift. The study of chimerism in sponges is in its infancy, and no doubt more work using molecular markers will cast light on the prevalence and ecological relevance of this mechanism in natural populations.

5. Differential expression of functional genes.

One of the main topics in marine ecology is the study of the responses of individuals, populations and assemblages to environmental changes. These changes exert selective pressures at ecological time scales. The outcome will depend on the species ability to adjust its physiology and biology to new scenarios.

The identification of cellular changes associated with the acclimatization of species to particular habitats and environmental changes can be assessed by analyzing differential expression of selected genes, and can help in predicting the organisms' ability to survive perturbations. Differential gene expression in individuals under contrasting environmental conditions or experimental treatments has been analyzed through protein quantification, northern blot, real time qPCR, microarrays or, recently, massive sequencing tools. In sponges, nevertheless, only the first three approaches have been used so far. As in other organisms, gene expression studies allow detecting potential adaptive traits in sponge individuals living in contrasting habitats or under adverse environmental conditions. Moreover, these studies have also contributed to the identification of particular genes involved in basic biological functions such as biomineralization, mating or development, which have decisive implications in the ecology of the species.

Studies of gene and protein expression in sponges have proliferated since the nineties (Fig. 4). The identification of functional genes from clone libraries was the first approach to discover new genes in sponges (e. g. Schröder *et al.*, 2000; Ugarkovic *et al.*, 1991). Subsequently, the expression of the identified genes was analyzed under experimental conditions, mainly in laboratory settings (e.g., Krasko *et al.*, 1999; 2000; Schröder *et al.*, 2000), and only rarely in the field. The biological function of the expressed genes was speculated based on sequence similarity with genes of known function in model organisms.

5.1. Genetic and environmental regulators of sponge biomineralization

Biomineralization refers to the biologically controlled process of precipitation of mineral salts. The skeletal elements of animals are the immediate result of biomineralization. The process involves expression of genes that code for protein/enzymes facilitating mineral precipitation and skeleton shaping, and greatly depends of environmental factors such as silicon or calcium concentration, temperature, and water alkalinity. Sponge skeletons can be siliceous (in the form of hydrated silica) and calcareous (crystallized as calcite or aragonite). However, one of the most pursued aim in the studies of sponge gene expression has been to find out the mechanisms involved in the formation of sponge siliceous skeletons: the proteins/genes responsible, the associated genes, the environmental conditions inducing gene expression, and the localization of the process, whether intra-, extra-cellular, or both (e.g., Müller *et al.*, 2008a, 2008b; Schröder *et al.*, 2005b; Uriz *et al.*, 2000). Sponge mineral skeletons play important biological and ecological roles in both siliceous and calcareous sponges (Uriz, 2006; Uriz *et al.*, 2003). They allow sponges to organize their aquiferous system in a three dimensional plan, thus enhancing sponge accessibility to the resources of the water column and diminishing the negative potential effects of sedimentation. It has been speculated that the calcareous skeletons of sponges and other organisms might have arisen to detoxify cells from an excess of calcium ions incompatible with life (Lowenstam and Margulis, 1980). Thus, the current sponge skeletons, which play decisive structural and defensive roles in present day sponges, may be an example of an exaptation mechanism (i.e., fixation of a character that had one function in an ancestral form and a new function in a descendant form) (Uriz, 2006). The genetic basis and the environmental control of the sponge biomineralization have received a great deal of attention in the last 20 years, particularly in siliceous sponges.

Shimizu *et al.* (1998) first elucidated the structure of proteins involved in silification of the sponge *Tethya aurantium* (Pallas) (silicateins). The cDNA sequence of the most abundant silicatein (alpha) revealed that these proteins were highly similar to members of the cathepsin L and papain family of proteases. Later, Cha *et al.* (1999) experimentally demonstrated the enzymatic activity of sponge silicateins

and their involvement in directing silica polymerization *in vitro*. Subsequent research, however, demonstrated that the process is far from simple and that external factors influenced considerably the process.

Schröder *et al.*, (2005b) studied spicule formation in cell aggregates (primmorphs) of *Suberites domuncula*. These authors showed that spicule formation initiates intracellularly independently of the Si concentration in the water, but silicatein expression strongly increased in sponge primmorphs after cultivation at high concentrations of silicate (60 $\mu\text{mol/L}$). This agreed with the higher spicule thickness and strength of sponges living in silicate-rich environments such as bathyal depths, upwelling areas, Antarctic waters, and particular silicon-rich habitats from the Pacific and North Atlantic (e.g. Maldonado *et al.*, 1999). They also reported that a cluster of three genes, which contained the silicatein- α gene, underwent coordinated expression after treatment with silicic acid. Since the expression of these genes occurred synchronously, it was suggested that the three were involved in the formation of spicules (Schröder *et al.*, 2005b). Although the first studies on sponge silification focused on representatives of the Class Demospongiae, a silicatein-related protein has also been found in the spicules of the hexactinellids *Monorhaphis chuni* Schulze (Müller *et al.*, 2008a) and *Crateromorpha meyeri* Gray (Müller *et al.*, 2008b). Thus, in both Demospongiae and Hexactinellida, cathepsin-related silicatein enzymes appear to form part of the siliceous matrix of spicules.

Mn-sulfate in the water induced formation of weak spicules with irregular porous surfaces in *S. domuncula* primmorphs by downregulating aquaporin-8 gene expression (Müller *et al.*, 2011). Aquaporin has been reported to be involved in dehydration and hardening of bio-silica following the bio-silica polycondensation reaction.

Other mineral elements in the ambient water appear to induce the expression of genes related with structural proteins. Both silicatein and collagen, which appear to be co-involved in spicule formation, were upregulated after silicic acid exposure (Krasko *et al.*, 2000) or ferritin exposure (Krasko *et al.*, 2002). Conversely, myotrophin seems to increase collagen gene expression but not silicatein expression (Krasko *et al.*, 2000).

5.2. Pollutants, stress, and gene expression

One of the most extensively dealt issues related to protein/gene expression in sponges is the assessment the potential effects of pollution. Differential gene expression in sponges submitted to several kinds of pollutants and stressors has been studied trying to detect early effects of noxious substances on sponge populations before they become lethal. Several studies analyzed the up- or downregulation of the expression (cDNA) of functional genes under various levels of metal and PCB pollutants, while others reported on protein expression (e. g. Agell *et al.*, 2001; Cebrian *et al.*, 2006; López-Legentil *et al.*, 2008; Wiens *et al.*, 1998) or, more rarely, protein activity (Saby *et al.* 2009).

Regeneration after partial mortality is at the basis of the sponge ecological success. Urgarković *et al.* (1991) determined, by densitometric scanning of the autoradiograms from dot-blot analyses, that the expression of RAS gene involved in regeneration in *Geodia cydonium* (Jameson) was strongly induced by genotoxic xenobiotics. Reaggregation of dissociated *G. cydonium* cells was induced by an aggregation factor that caused expression of RAS gene in the cells, and this expression was inhibited by detergents at concentrations within a pollution-realistic range.

Polychlorinated biphenyl PCB 118 pollutants induced the expression of two chaperone proteins in the marine sponge *Geodia cydonium* (Wiens *et al.*, 1998): the 14-3-3 protein and the heat shock protein HSP70 (primarily involved in folding of proteins). Using the cDNAs coding for these two proteins and northern blot electrophoresis, the authors demonstrated that none of the two chaperones were detected in the absence of PCB. In contrast, after incubation of sponge tissue with PCB 118 during 12 h, the transcripts of the 2 chaperones were detectable, and the corresponding proteins appeared after 24 h. Due to the broad cross-reactivity of their antibodies throughout the Metazoa, these chaperones were proposed as useful biomarkers for monitoring environmental PCBs. The expression of both HSP proteins and corresponding genes have been widely assessed in sponges thanks to the extremely conserved sequences of these genes, which allowed the use of universal primers for cross-amplification in sponges (Koziol *et al.*, 1996; Koziol *et al.*, 1997; Krasko *et al.*, 1997; Müller *et al.*, 1995; Wiens *et al.*, 1998). The expression of stress

proteins was also quantified in natural populations of the sponge *Crambe crambe*, under both polluted and non-polluted conditions, both at the laboratory and in a field experiment, which demonstrated that HSP70 expression was induced under metal pollution, although only semi-quantitative methods (western blot) were used for protein determination (Agell *et al.*, 2001). In contrast, no differential HSP protein expression was found, either in the laboratory or in a transplant experiment, in *Chondrosia reniformis*, under high copper concentrations (Cebrian *et al.*, 2006) compared to low copper controls.

Episodes of mass diseases and mortalities have been often reported for sponges inhabiting both tropical and temperate Seas (Cebrian *et al.*, 2011; Galstoff, 1942; Webster, 2007). The giant barrel sponge *Xestospongia muta*, which is one of the largest and most conspicuous components of Caribbean coral reef communities, increased expression of the HSP70 gene (as measured with real time qPCR) when it underwent fatal bleaching in the field and in response to thermal and salinity variation in the laboratory, while HSP expression remained constant during the cyclic sublethal bleaching (López-Legentil *et al.*, 2008).

Müller *et al.* (1995) and Bachinski *et al.* (1997) studied the effects of thermal stress on the sponges *Suberites domuncula* and *Ephydatia fluviatilis*, respectively, by analyzing either HSP protein or gene expression, which was upregulated under the high temperature treatment. Krasko *et al.* (1999) reported significant increase of the intracellular Ca^{++} concentration and reduction of the starvation-induced apoptosis in sponge aggregates of the demosponge *Suberites domuncula* submitted to 5 mM ethylene. The expression of two genes, a SDERR encoding for a potential ethylene-responsive protein, termed ERR_SUBDO, and a Ca^{++} /calmodulin-dependent protein kinase, were up-regulated after exposure to ethylene. Database searches revealed that the sponge polypeptide shared high similarity (82% on amino acid level) with the ethylene-inducible protein from plants. No other ethylene-responsive proteins had been identified in Metazoa previously. The expression of both genes in primmorphs of *S. domuncula* increased 5-fold after a 3-day incubation period with ethylene. Ethylene is one major alkene in seawater resulting from oceanic dissolved organic carbon by photochemical reactions. The authors proposed that, because sponges are efficient benthic filter-feeders, they are likely to take up large amounts of ethylene from the surrounding water, and this organic molecule could play a positive role in

calcium homeostasis and apoptosis reduction of the sponge cells.

Krasko *et al.* (2001) identified sponge genes involved in cell protection in front of environmental threats. Besides the previously identified efficient defense systems such as chaperones and the P-170/multidrug resistance pump system (Müller *et al.*, 1996), they reported a further multidrug resistance pathway that is related to the pad one homologue (POHL) mechanism that had also been identified in humans. It is suggested that proteolysis is involved in the inactivation of xenobiotics by the POHL system. Two cDNAs were cloned, one from the demosponge *Geodia cydonium* and a second from the hexactinellid *Aphrocallistes vastus* Schulze. The two sponge cDNAs were highly similar to each other as well as to the known sequences from fungi and other Metazoa. Under controlled laboratory conditions, the expression of the potential multidrug resistance gene POHL was strongly upregulated in response to the natural toxins staurosporin or taxol in *G. cydonium*. The relevance of the expression pattern of the *G. cydonium* gene POHL for the assessment of pollution in the field was determined at differentially polluted environments.

Differential gene expression related with immune reactions in response to the bacterial endotoxine LPS in *Suberites domuncula* was analyzed in populations from a confined, polluted habitat (i.e., Limski Canal in Croatia: Mediterranean Sea) and from the open coast in the same area (a *et al.*, 2005). Most of the differentially expressed transcripts coded for the allograft inflammatory factor-1 (AIF-1), a molecule involved in self/non-self recognition in *S. domuncula*. The level of gene expression of the AIF-1 gene was determined by northern blotting and real-time qPCR, and resulted in much higher values in the sponges living in the open Sea. These results pointed to an immuno-depression in sponges inhabiting contaminated areas. Other potential indicators of sponge immuno-depression, such as the inhibition of the 2-5'-oligoadenylate synthetase (OAS) in sponges exposed to metal pollution, have been reported (Saby *et al.*, 2009). Sponge OAS activity was quantified as the amount of reaction products (2-5A oligomers) after the incubation in a PCR machine of the sponge extracts with ATP, using a C18 reverse-phase column on HPLC. In vitro, activity of OAS from *Geodia cydonium* and *Crella elegans* (Schmidt) was inhibited to a variable extent by Cu, Mn, Zn and Fe ions.

5.3 Symbioses and horizontal gene transfer

Symbiosis with cyanobacteria is widespread in sponges inhabiting shallow waters, in particular in the Indo-Pacific ocean shelf (Lemloh *et al.*, 2009; Wilkinson, 1987; Wilkinson and Fay, 1979) but also in other tropical and temperate seas (Erwin and Thacker, 2008; Vacelet, 1975). Cyanobacteria influence sponge growth and competition abilities (Erwin and Thacker, 2008) and thus greatly account for the sponge ecological success (see Thacker and Freeman, this issue). Steindler *et al.* (2007) analyzed for the first time gene expression in relation to the presence of symbiotic cyanobacteria. The target species was the common Mediterranean sponge *Petrosia ficiformis* (Poiret) that can harbor or not cyanobacteria as a function of the amount of irradiance arriving to its habitat. After suppression subtractive hybridization (SSH) to identify uniquely expressed genes, and separation and cloning of the PCR products resulting from symbiotic and aposymbiotic individuals, the authors isolated two novel genes (named PfSym1 and PfSym2). These genes were screened for differential expression in two fragments of the same individual (clones), which harbored or not the symbiotic cyanobacteria (after a medium term transplantation experiment), via Northern blotting. Both genes were upregulated in sponges harboring the cyanobacterial symbionts.

Despite the recent blooming literature on the identification of sponge-associated microbes by pyrosequencing techniques (e.g., Lee *et al.*, 2011, Turque *et al.*, 2010; Webster *et al.*, 2010) the molecular basis of the sponge-microorganism symbioses and the exchange of metabolites between sponges and their associated microbes remain poorly understood. Gene expression studies may help to understand the interactions between the symbiotic partners. Müller *et al.* (2004) reported that the demosponge *Suberites domuncula* expressed, under optimal aeration conditions, the enzyme tyrosinase, which synthesizes diphenols from monophenolic compounds. The authors assumed that these products serve as carbon source for symbiotic bacteria to grow.

Bacteria and fungi are common symbionts of sponges (Taylor *et al.*, 2007; Erwin *et al.*, 2011). Horizontal gene transfer from bacteria (Jackson *et al.*, 2011) and fungi (Rot *et al.*, 2006) to sponges has been suggested. Horizontal gene transfer is considered to be a major evolutionary force in eukaryotes (Kozo-Polyansky *et al.*, 2010). Jackson *et al.* (2011) have recently identified a new protein (spherulin) from

the spherulites that form the calcareous skeleton of the sponge *Astrosclera willeyana*, and speculated on a horizontally transferred gene from the symbiotic bacteria to the sponge. Rot *et al.* (2006) showed some lines of evidence demonstrating that introns can be found in the mitochondria of sponges (Porifera) based on the sequencing of a 2,349 bp fragment of the mitochondrial COI gene from the sponge *Tetilla* sp. (Spirophorida). They report the presence of an intron with group I intron characteristics as in Cnidarians. The intron encodes a putative homing endonuclease. A phylogenetic analysis of the COI protein sequence and of the intron open reading frame suggests that the intron may have been transmitted horizontally from a fungus donor. The authors suggested that the horizontal gene transfer of a mitochondrial intron was facilitated by a symbiotic relationship between fungus and sponge. Once more, ecological (symbiotic) relationships seem to have implications at the genomic level. To better understand the mode of transmission of mitochondrial introns in sponges, Szitenberg *et al.* (2010) have studied COI intron distribution among representatives of Tetillidae. Out of seventeen COI sequences of Tetillidae examined, only six were found to possess group I introns. These different forms of introns had distinct secondary structures. Since sponges harboring the same intron type did not always form monophyletic groups, the authors suggested that sponge introns might have been transferred horizontally.

5.4. Expression of other sponge genes with ecological implications.

Most sponges are long living organisms, which confers stability to the sponge-dominated assemblages. As a consequence of their permanence across years in the same habitat, many sponges are keystone species in structuring benthic assemblages, where they play a significant role in species interactions such as competition, facilitation, or symbiosis, among others (Sarà and Vacelet, 1973). The sponge longevity has been associated to the reported capacity of sponge cells to proliferate almost indefinitely due to the presence of high telomerase activity (Kozioł *et al.*, 1998). To this regard, Schröder *et al.* (2000) identified genes in the marine sponge *Suberites domuncula*, which were differentially expressed in proliferating and non-proliferating cells. Moreover, they sequenced the complete cDNA corresponding to the transcript of the SLAGL gene, which encoded a polypeptide (named putative

longevity assurance-like polypeptide LAGL_SUBDO), which showed a high sequence similarity to the longevity assurance genes from other Metazoa. This gene was highly expressed in aggregated proliferating cells but not in isolated single cells.

5.5. Conclusions

The publication of the complete genome of *Amphimedon queenslandica* (Srivastava *et al.*, 2010) has accelerated the identification of new sponge genes, shedding light on the sponge development and phylogenetic relationships with other animals. These studies have mainly focused on the identification and expression of genes related to functional aspects of sponges, such as the origin of the animal body plans and the developmental patterning processes (e.g., Adamska *et al.*, 2007; Lapébie *et al.*, 2009; Larroux *et al.*, 2006). Enumeration of these works is out of the scope of this ecologically-oriented review. However, the publication of a sponge genome has been undoubtedly a major breakthrough in sponge science. Further exploitation of this invaluable resource will likely lead in the next years to the characterization of many genes of ecological relevance. Moreover, protein expression studies should be conducted in parallel to gene expression studies to validate the true repercussion of the observed differential gene expression in the cell biology of the sponges.

Coupled with the generation of genomic information, the application of high throughput sequencing technologies to gene expression quantification, the so-called RNA-seq techniques (Marioni *et al.*, 2008; Wang *et al.*, 2009) is expected to boost the number of studies on sponges in the forthcoming years. This technique is being employed to quantify differential expression of stress-related genes of the Mediterranean sponge *Crella elegans* submitted to temperature stress, as well as during its reproductive cycle and the resistance phases posterior to larval release (authors, current research).

6. Forthcoming trends in sponge molecular ecology: hopes and prospects

A simple literature perusal shows that the main topics covered in this review have blossomed in the last two decades, with a markedly increasing trend in number of publications and citations (Fig. 4). We expect that this ongoing trend of fruitful research in the interface between molecular systematics and molecular ecology will continue to progress significantly in the near future. In particular, we foresee that more and more instances of cryptic speciation will be uncovered and will require modification of existing taxonomic arrangements. This field will likely progress along two ways: deciphering the mechanisms of formation of sibling species (vicariance, isolation by distance, founder effects, etc.) and improving the formal incorporation of molecular (genomic, proteomic, and metabolomic) knowledge in species definition (Ivanešević *et al.*, 2011; Cárdenas *et al.*, this volume). The use of functional genes related to reproduction, such as gamete recognition proteins (Swanson and Vacquier, 2002; Turner and Hoekstra, 2008) in parallel with neutral markers and metabolic fingerprinting will greatly aid in the first of these goals.

At the same time, we anticipate that new and better markers will be developed for sponge population genetic studies. Next generation sequencing methods allow faster and more efficient development of microsatellites, as well as identification of new candidate genes for population level studies. These data can ease the incorporation of other kinds of markers requiring extensive genomic knowledge, such as Single Nucleotide Polymorphisms (SNP, Kim and Misra, 2007). SNP have never been used in sponges, but are nevertheless promising tools judging from their results in other groups (reviewed in Clark *et al.*, 2010), and may outcompete microsatellites in the future (Morin *et al.*, 2004).

According to the consistent results reported in previous studies, we can predict that the present trend of high population structure and little gene flow will continue to prove its generality in sponge populations. However, we hope to be able to adequately track the (occasional) long distance dispersal events that have been suggested for some species. Better sampling designs and analyses with more informative markers, coupled with knowledge of regional current patterns and particle dispersal models will undoubtedly contribute to this goal (Siegel *et al.*, 2008).

Patterns of temporal genetic variation are increasingly being studied in benthic invertebrates, and they add another dimension to the picture of the distribution of genetic variability (e.g., Calderón *et al.*, 2009, 2011; Lee *et al.*, 2007). These studies

are more challenging in sponges, as they cannot be reliably aged in general. However, studies on annual species (Guardiola *et al.*, 2011) or monitoring of yearly recruits in populations can shed light on the dynamic mechanisms that result in the spatial distribution of genetic variation that we observe.

We also expect to see more studies that analyze the relative role of sexual and asexual reproduction in the building up of the populations. The studies so far seem to indicate that the importance of asexual reproduction is less than foreseen, but the picture can change as more species and particular habitats are investigated. Furthermore, the studies should move from intra-population processes to the assessment of the importance of asexual propagules in the colonization process and the gene flow among populations. As for the other ecological fields mentioned, we await the development of highly variable markers, which is a pre-requisite for this kind of studies (Arnaud-Haond *et al.*, 2005).

Hoopefully, molecular studies will unearth whether chimerism, only found up to now in natural populations of *Scopalina lophyropoda* (Blanquer and Uriz, 2011), is widespread in other sponge species, which will raise intriguing questions as to its generation, maintenance, and ecological relevance. We also foresee that the genetic basis of self/non-self recognition (Grosberg, 1988b) in sponges will be fully described and the genes implicated will be incorporated to the field of sponge molecular ecology.

At the same time, the range of questions addressed will widen with the application of new techniques of gene expression analyses (Steindler *et al.*, 2007; Marioni *et al.*, 2008; Wang *et al.*, 2009). Differential gene expression will allow us to gain insights into the actual functioning and responses of individuals and populations rather than just assessing demographic parameters and connectivity. Further research likely will also focus on microRNA genes regulating gene expression. Only recently, the first paper reporting the application of interference RNA techniques for gene silencing in sponges has been published (Rivera *et al.*, 2011). These techniques open a new avenue of possibilities for reverse genetics studies on functional aspects of sponges.

Sponges are paradigmatic invertebrates as for their ancestral symbiosis with an array of prokaryotic communities (Taylor *et al.*, 2007). Horizontal gene transfer from their symbiotic prokaryotes, which has been recently reported in a couple of cases

(Jackson *et al.*, 2011; Rot *et al.*, 2006), may prove to be prevalent in sponges, where studied with the appropriated molecular tools.

All together, we urge researchers to turn sponges into a data-rich group in terms of the molecular patterns and mechanisms underlying their ecological distribution and vulnerability (Carvalho *et al.*, 2010). If we succeed, sponges can make a pivotal group for studies on conservation and management of the marine coastal environment. Too often, this research focuses on some “emblematic” groups or “flagship” species, sometimes more “conspicuous” but less relevant ecologically than sponges. Because of their diversity, abundance and roles in the trophic networks, and their plentiful interactions with prokaryotes and eukaryotes, sponges must be in the limelight in all studies aiming at setting up conservation policies (Cowen *et al.*, 2007; Arrieta *et al.*, 2010).

We are at present in the brink of a revolution in molecular ecology (Tautz *et al.*, 2010), and sponge science will follow this lead in parallel with (and hopefully not behind) marine science in general. We can predict that studies will move from genetics (the use of one or a few genes) to genomics (extensive genome or transcriptome sequencing) (Bouck and Vision 2007, Dupont et al 2007, Mittard-Runte *et al.*, 2010, Wilson *et al.*, 2005). Conceptual and methodological developments made in model organisms will be extended to non-model species, and sponges should benefit from this trend. We are moving from a field that was rich in theory and poor in data, to a data-rich world, which should be matched with new theoretical and analytical tools (Hamilton, 2009). Proteomics and metabolomics will join in, and together they will transform many aspects of sponge research, allowing a more complete ecological approach involving the simultaneous study of many components of the interactions of organisms with the environment (Johnson and Browman, 2007). Thus, sponge ecologists will soon have the tools to answer ecological and biological questions that would have been impossible to address a few years ago (Clark *et al.*, 2010).

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Legend to figures

Fig. 1. An example of highly dynamic encrusting sponges: three sequential snapshots showing fissions, fusions, growth and shrinkage of the cryptic Mediterranean species *Scopalina lophyropoda* and *S. blanensis*, along the year (arrows point of a reference debrisymastigma).

Fig. 2. Schematic representation of the potential contribution of the main types of asexual propagules to the connectivity among sponge populations.

Fig. 3. Asexual reproduction in Antarctic sponges with patchy distributions. A) External budding of a *Rossella* (Hecactinellida) species. B) *Rossella* bed. C) Internal gemmules in a Tetillidae. D) Tetillidae bed. Photographs by Thomas Lundälc, UGOT.

Fig. 4. Bibliometric data of the main fields of sponge research covered in this review during the last 20 years (in 5-year intervals). Data were obtained from searches in the Science Citation Index Expanded through the Web of Science (Thomson Reuters©). Search criteria (in the “Topic” field) were as follows: For cryptic speciation: “(sponge or porifera) and (“cryptic speci*”)”; for phylogeography and population genetics: “(sponge or porifera) and (phylogeog* or “population genetic*”)”; for gene expression: “(sponge or porifera) and (“gene expression”)”. Searches were refined manually to meet the criteria of this review (f.i., non-molecular-based studies of cryptic speciation were filtered out). (A). Number of publications since 1990. (B) Number of citations since 1990.

Fig. 1

Fig. 3

Fig. 4

Table 1. Selected cases of sponge species complexes resulting in the determination of cryptic sibling species after a molecular study.

Original species or species complex	Molecular marker	Resulting cryptic species	Reference
<i>Suberites ficus</i>	Allozymes	<i>S. pagurorum</i> <i>S. rubrus</i>	Solé-Cava & Thorpe 1986
<i>Tethya aurantium</i>	Allozymes	<i>T. aurantium</i> <i>T. citrine</i> <i>Tethya sp.</i>	Sarà <i>et al.</i> 1989
<i>Axinella damicornis</i> / <i>A. verrucosa</i>	Allozymes	<i>A. damicornis</i> <i>A. verrucosa</i> <i>Axinella sp.</i>	Solé-Cava <i>et al.</i> 1991b
<i>Clathrina clathrus</i>	Allozymes	<i>C. aurea</i> South-West Atlantic <i>C. clathrus sp.</i> Mediterranean	Solé-Cava <i>et al.</i> 1991a
<i>Clathrina cerebrum</i>	Allozymes	<i>C. cerebrum</i> Mediterranean <i>C. brasiliensis</i> North Atlantic	Solé-Cava <i>et al.</i> 1991a
<i>Oscarella lobularis</i>	Allozymes	<i>O. lobularis</i> Mediterranean <i>O. tuberculata</i> Mediterranean	Boury-Esnault <i>et al.</i> 1992
<i>Petrosia ficiformis</i>	Allozymes	<i>P. ficiformis</i> Mediterranean <i>P. clavata</i> Mediterranean	Bavestrello & Sarà 1992

<i>Plakina trilopha</i>	Allozymes	<i>P. trilopha</i> widespread Mediterranean <i>P. endoumensis</i> cave1 Mediterranean <i>P. jani</i> cave2 Mediterranean <i>Plakina sp3</i> cave3 Mediterranean	Muricy <i>et al.</i> 1996
<i>Latrunculia</i> spp.	Allozymes	8 <i>Latrunculia</i> species	Miller <i>et al.</i> 2001
<i>Astrosclera willeyana</i>	ITS1-5.8S-ITS2 region	<i>Astrosclera</i> sp1 Red Sea <i>Astrosclera</i> sp2 Great Barrier Reef <i>A. willeyana</i> Fiji/Vanuatu	Wörheide <i>et al.</i> 2000
<i>Pachymatisma normani</i>	COI and ITS1-5.8-ITS2	<i>P. normani</i> North Atlantic <i>P. johnstoni</i> North Atlantic	Cárdenas <i>et al.</i> 2007
<i>Chondrosia reniformis</i>	Allozymes	<i>C. reniformis</i> Mediterranean <i>Chondrosia</i> sp. Atlantic (from Bermuda to Brazil)	Lazoski <i>et al.</i> 2001
<i>Chondrilla cf nucula</i>	5'-end COI	<i>C. cf nucula</i> Caribbean mangroves <i>C. cf nucula</i> Caribbean reefs	Duran & Rutzler 2006
<i>Halichondria panicea</i>	5'-end COI	<i>H. panicea</i> sp1, Alaska <i>H. panicea</i> sp2 NE Atlantic	Erpenbeck <i>et al.</i> 2004
<i>Scopalina lophyropoda</i>	5'-end COI, 28S, 16S	<i>S. lophyropoda</i> Mediterranean <i>S. blanensis</i> Mediterranean <i>S. ceutensis</i> Mediterranean,	Blanquer & Uriz 2007, 2008a

		North Africa <i>S. canariensis</i> Atlantic, Canary Islands	
<i>Cliona celata</i>	5'-end COI, Atp8, and 28S	<i>C. celata</i> sp1, North Atlantic <i>C. celata</i> sp2, North Atlantic <i>C. celata</i> sp3, Western Mediterranean <i>C. celata</i> sp4, Western Mediterranean	Xavier <i>et al.</i> 2010a
<i>Hexadella</i> spp	COI, 28S, ATPS- intron	<i>H. detritifera</i> NW Atlantic (Irish, Scottish and Norwegian coasts and Greenland Sea) <i>H. cf detritifera</i> Ionian Sea, Irish coasts, Bay of Biscay <i>Hexadella</i> sp Mediterranean deep sea <i>Hexadella cf pruvoti</i> Mediterranea, shallow sea	Reveillaud <i>et al.</i> 2011a
<i>Plocamionida</i> spp	MIM6 and I3M11partitions of COI & 28S	<i>P. tylotata</i> , North Atlantic <i>P. grandichela</i> North Atlantic <i>P. ambigua (tornata)</i> North Atlantic <i>P. microcionides</i> North Atlantic	Reveillaud <i>et al.</i> 2011b
<i>Halisarca</i> spp	Mitochondrial genome	<i>H. dujardini</i> <i>Atlanto-Mediterranean</i> <i>H. harmelini</i> <i>Mediterranean</i>	Ereskovsky <i>et al.</i> 2007

Table 2. Collation of works reporting values of population differentiation (DIFF, using F_{st} or analogous measures) in sponges. Approximate geographic ranges are listed (taken from Google Earth when not indicated by the authors). The differentiation criterion “YES” or “NO” is not absolute, it is based on the appreciation that most population comparisons did or did not indicate significant differentiation. Results of analyses of isolation by distance (IBD) are also listed when reported (otherwise NA: not applicable). HWE: Hardy-Weinberg Equilibrium

SPECIES	MARKER	RANGE	DIFF	IBD	OBSERVATIONS	REFERENCE
<i>Phyllospongia lamellosa</i>	allozymes	10s to 550 Km	YES	NO	HWE except 2 populations	Benzie <i>et al.</i> 1994
<i>Phyllospongia alcicornis</i>	allozymes	10s to 700 Km	YES	YES	HWE	Benzie <i>et al.</i> 1994
<i>Carterospongia flabellifera</i>	allozymes	10s to 500 Km	YES	YES	HWE	Benzie <i>et al.</i> 1994
<i>Collospongia auris</i>	allozymes	10s to 250 Km	YES	NA	HWE except one population	Benzie <i>et al.</i> 1994
<i>Chondrilla 'nucula' B</i>	allozymes	2-2700 Km	YES	NA	Evidence for cryptic speciation	Klautau <i>et al.</i> 1999
<i>Chondrosia</i> sp.	allozymes	Up to 8600 Km	YES	NO	HWE. High genetic identity but significant F_{st}	Lazoski <i>et al.</i> 2001
<i>Crambe crambe</i>	COI	10s to 2700 Km	YES	NO	Only 2 haplotypes	Duran <i>et al.</i> 2004a
<i>Crambe crambe</i>	ITS	10s to 3000 Km	YES	NA	Intragenomic variation detected	Duran <i>et al.</i> 2004b
<i>Crambe crambe</i>	microsats	10s to 3000	YES	YES	Heterozygote deficiency, clonality	Duran <i>et al.</i> 2004c

		Km			present	
<i>Haliclona</i> sp.	Allozymes	10s m	NO	NA	Heterozygote deficiency	Whalan <i>et al.</i> 2005
		100s m	YES	NA	Heterozygote deficiency	
		100s Km	YES	NA	Heterozygote deficiency	
<i>Chondrilla</i> cf. <i>nucula</i>	COI	4 to 18 Km	YES	NA	Suspected ecological speciation process	Duran and Rützler 2006
		1000 to 1700 Km	YES	NA	Suspected ecological speciation process	
<i>Crambe crambe</i>	microsats	0 to 7 m	YES	NA	Heterozygote deficiency, clonality present	Calderón <i>et al.</i> 2007
<i>Pericharax heteroraphis</i>	ATPS β -III	Up to 2000 Km	YES	NA	HWE in general. Differentiation between pooled regional populations	Bentlage and Wörheide 2007
<i>Hymeniacidon flavia</i>	ITS, nad5	10s to 1000 Km	YES	NA	Only 2 nad5 haplotypes	Hoshino <i>et al.</i> 2008
<i>Hymeniacidon sanguinea</i>	ITS, nad5	10s to 1600 Km	NO	NA	Only 2 nad5 haplotypes	Hoshino <i>et al.</i> 2008
<i>Leucetta chagosensis</i>	ATPS β -III	10s to 4500 Km	YES	YES	SW Pacific subset of populations	Wörheide <i>et al.</i> 2008
<i>Scopalina lophyropoda</i>	microsats	0 to 100 m	YES	NA	Heterozygote excess, little clonality	Blanquer <i>et al.</i> 2009

<i>Rhopaloeides odorabile</i>	allozymes & COI	1 to 10s of Km	NO	NA	No differentiation in general, but some localized divergent populations	Whalan <i>et al.</i> 2008
<i>Xestospongia muta</i>	COI	150 to 1800 Km	YES	NO	I3-M11 COI partition	López-Legentil & Pawlik 2009
<i>Spongia lamella</i>	microsats	7 to 1800 Km	YES	YES	Heterozygote deficiency	Noyer 2010
<i>Phorbas fictitius</i>	COI	10s to 100s Km 100s to 1000s Km	YES YES	NO NO	I3-M11 COI partition	Xavier <i>et al.</i> 2010b
<i>Callyspongia vaginalis</i>	COI	10s to 100s Km	YES	NO	Possible occasional long distance dispersal	DeBiasse <i>et al.</i> 2010
<i>Scopalina lophyropoda</i>	microsats	10s m to 10s Km 10s to 100s Km	YES YES	YES YES	Heterozygote excess. Little clonality Heterozygote excess. Little clonality	Blanquer and Uriz 2010
<i>Paraleucilla magna</i>	microsats	10 to 50 m	YES	YES	Heterozygote deficiency. Introduced species	Guardiola <i>et al.</i> 2011
<i>Spongia officinalis</i>	COI & microsats	7 to 1900 Km	YES	YES (but not within basins)	Heterozygote deficiency.	Dailianis <i>et al.</i> 2011