RECENT CONTRIBUTION OF GENETICS TO THE STUDY OF SPONGE SYSTEMATICS AND BIOLOGY

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ABSTRACT

In this short review we will discuss the recent contributions of genetics to our understanding of the biology and evolution of sponges, particularly on the reappraisal of longstanding beliefs held by sponge taxonomists. The main questions addressed are the following. Are sponges animals? After centuries of controversy, there seems to be a consensus, now, that sponges are metazoans. Phylogenetic studies also indicate that animals are closely related to choanoflagellates. This indicates that choanoflagellate-like structures should not be considered a synapomorphy of the Porifera. Is the phylum Porifera monophyletic? Three main hypotheses are still prevailing: the Porifera are monophyletic; the Porifera are paraphyletic with the Hexactinellida being considered the more basal group of sponges, mostly because of their syncytial nature, or the Demospongiae and the Hexactinellida together, the Calcispongia being a sister-group of the Eumetazoa. Are the currently accepted Classes supported by molecular data? Molecular data confirms the presence of two monophyletic clades within the Calcispongia. On the other hand, the distinction of demosponge classes Tetractinomorpha and Ceractinomorpha, based on an oviparous versus viviparous reproduction, has been rejected by all molecular phylogenies produced so far. Are there true cosmopolitan sponge species? All putative cosmopolitan sponges species have turned out to be, under molecular scrutiny, groups of evolutionary very distinct species. We believe, thus, that the number of true cosmopolitan sponges is likely to be very small. Can sponge populations be homogeneous over large areas? Most sponge species studied to date have shown a rather small capability for long-range dispersal. This indicates that sponge larvae, both from viviparous and oviparous species, do not disperse very much. How important is asexual reproduction in the establishment and maintenance of sponge populations? Molecular markers confirm the presence of extensive asexual reproduction in sponges. The possibility of larval fusion and chimerism has important evolutionary consequences, but has not yet been tested molecularly.

KEY WORDS

Evolution, genetics, cosmopolitanism, molecular markers, clonal reproduction, phylogeny.

INTRODUCTION

The use of a genetic approach has been a valuable contribution to the study of many long-standing problems in sponge taxonomy, from events that happened over 600 million years ago to those that are happening in an ecological or microevolutionary time. But, perhaps more importantly, it has also challenged many

conclusions that were considered by taxonomists to be quite well established. In this short review we will discuss the recent contributions of genetics to our understanding of the biology and evolution of sponges.

The first genetic work on sponges was the one by CONNES *et al.,* (1974), using isozyme electrophoresis to analyse populations of *Suberites massa* from the Thau lagoon (NW-Mediterranean, French coast). The first to use molecular sequences for formulating phylogenetic hypotheses were those of DAMS *et al.,* (1982), who used 5S rRNA sequences for a preliminary investigation on the place of Porifera among Metazoa, and that of KELLY-BORGES *et al.,* (1991) who used 18S sequences to formulate phylogenetic hypotheses for sponges of the order Hadromerida. Twenty years have now passed since those pioneering works, and over 40 nuclear genes have been sequenced in sponges (GenBank data). Although this is still a very small number, considering the developments in genome projects of other animals, it is, nonetheless, a major progress in relation to what we knew of the molecular biology of sponges not long ago. Reviews of genetic approaches to sponge taxonomy and evolution have been published regularly (SOLÉ-CAVA & THORPE, 1987, 1994; SOLÉ-CAVA & BOURY-ESNAULT, 1999; BORCHIELLINI *et al.,* 2000; MÜLLER, 2001; VAN OPPEN *et al.,* 2003).

Are sponges animals?

Whether the sponges are highly specialized protists with no relationships to true Metazoans or constitute a basal metazoan lineage has been a long standing debate among zoologists. During the 4th symposium of the Zoological Society Professor Yves Delage (1899) said that "Undoubtedly their place is among the Metazoa". Nevertheless, that position was never completely settled, so that even a century later it still was necessary to repeat that, based on sequence data, the "Porifera should be placed into the Kingdom Animalia" (MÜLLER, 1998).

Spongologists had been convinced of the metazoan nature of sponges, based on the sexual reproduction, embryology and cell diversity (DELAGE, 1899; BRIEN, 1967), but due to their simple organisation and their plasticity, not all biologists accepted this and, indeed, some textbooks still describe this issue as controversial. Recently the question has received convergent answers through a better knowledge of ultrastructural, biochemical and molecular features of sponges and many synapomorphies currently support the monophyly of Metazoa with the sponges included (*e.g.* MANUEL *et al.,* 2000).

Are the choanoflagellates the sister-group of Metazoa?

In most text-books the Porifera are considered as one phylum which has a basal position within Metazoans. Besides supporting the basal positioning of sponges in the metazoan tree, molecular phylogenies based on 18S rRNA, Hsp 70 and mtDNA (PETERSON & EERNISSE, 2001; SNELL *et al.,* 2001; LANG *et al.,* 2002) have led to the revival of an old idea (JAMES-CLARK, 1866, 1868), according to which the choanoflagellates are the sister-group of the Metazoa. Such a hypothesis suggests a somewhat sponge-like ancestry for the metazoans as a whole (BORCHIELLINI *et al.,* 2001; COLLINS & VALENTINE, 2001; PETERSON & EERNISSE, 2001). If that proves to be true, fundamental sponge features (particularly the presence of choanocytes) classically considered as the few supporting apomorphies for Porifera would be in

fact a plesiomorphy of the Metazoa. This lack of phylogenetic support for the Phylum Porifera could indicate that it is paraphyletic (see below).

Is the phylum Porifera monophyletic?

The phylogenetic relationships among classes, orders or families of sponges still remain too confusing to positively answer this question. Three main clades are presently recognised: Hexactinellida, Demospongiae and Calcispongia.

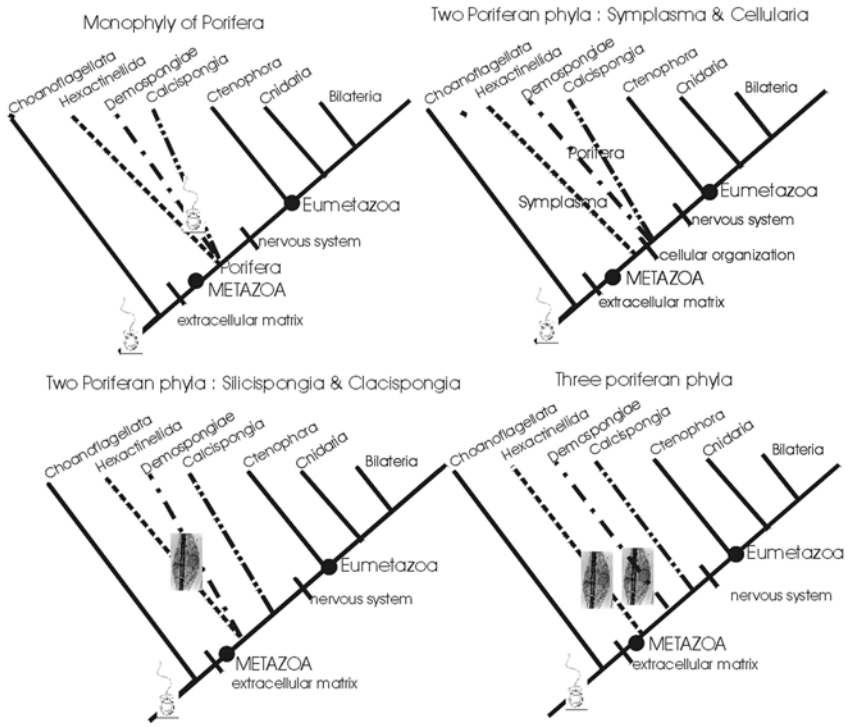
In fact, since the 19th century several authors have proposed at least two phyla within the sponge grade of organisation. There are two main hypotheses:

1.- Hexactinellida constitute a separate phylum from other sponges (BIDDER, 1929; BERGQUIST, 1985)

2.- Hexactinellida and Demospongiae cluster together in a separate phylum, and Calcispongia is the sister group of the Eumetazoa (GRAY, 1867; ZRZAVY *et al.,* 1998; BORCHIELLINI *et al.,* 2001, and others)

In the first hypothesis the syncytial organisation of Hexactinellida is considered as a plesiomorphic character, and the cellular organisation as a synapomorphy of a clade made of Demospongiae, Calcispongia and Eumetazoa. However if the choanoflagellates are the sister group of Metazoa cell organization is a synapomorphy of all Metazoa and syncytial organization becomes, thus, an apomorphy of Hexactinellida.

In the second hypothesis it is considered that the synapomorphies for the Hexactinellida/ Demospongiae clade are the siliceous nature of the skeleton and the intracellular secretion of siliceous spicules around an axial filament. Three independent sets of molecular data: 18S rRNA, 28S rRNA and Protein Kinase C (KRUSE *et al.,* 1998; ZRZAVY *et al.,* 1998; BORCHIELLINI *et al.,* 2001; MEDINA *et al.,* 2001; PETERSON & EERNISSE, 2001) give support to the placing of Calcispongia as the sister-group of the Eumetazoa. However, the position of Hexactinellida, either as forming a monophyletic group with the Demospongiae or still remaining as a separate phylum is still not well resolved, whatever the gene and the reconstruction method used. There is with the sequences of Hexactinellida a problem of long-branch attraction which by the time being is not resolved. Chemical data indicate a closer relationship between Hexactinellida and Demospongiae, at the exclusion of Calcarea (THIEL *et al.,* 2002), but that hypothesis needs to be confirmed by other molecular markers. A diagram comparing the concurring alternatives is presented below:



Are the currently accepted "Classes" supported by molecular data?

Within the Hexactinellida too few sequences are available to allow any conclusions to be drawn about their internal classification.

Within the Calcispongia 18S rDNA sequence data confirm the hypothesis of BIDDER (1898) and MINCHIN (1900) of two monophyletic clades: Calcinea and Calcaronea (MANUEL *et al.,* 2003, MANUEL *et al.,* 2004).

Within the Demospongiae the phylogenetic hypothesis made by LÉVI (1956) based on morphology and embryology has been under dispute for the last 20 years. The distinction of the two sub-classes: Tetractinomorpha and Ceractinomorpha, based on an oviparous versus viviparous strategy of reproduction has been rejected by all molecular phylogenies produced so far. However for the time being the number of sequences available and the number of taxa analysed for the different recognized orders is too small to obtain supported phylogenetic hypotheses for the deep nodes of Demospongiae.

However phylogenetic hypotheses have been recently proposed at different levels from orders to species. For example, it has been shown that Astrophorida and Spirophorida constitute a monophyletic clade which corresponds to Tetractinellida Marshall, 1876. The molecular tree, in this case, is congruent with morphological characters, the synapomorphy for Tetractinellida being the presence of a tetraxon spicule (CHOMBARD *et al.,* 1998). However within Tetractinellida several polyphyletic families have been detected, like the Ancorinidae and Geodiidae, which need a revision from molecular and morphological points of view. For the Haplosclerida,

on the contrary, a very recent work shows the non-congruence between the molecular tree and the currently accepted classification, polyphyly being found within families and genera (MCCORMACK *et al.,* 2002). The polyphyly of Halichondrida, firstly recognized by CHOMBARD & BOURY-ESNAULT (1999), has been confirmed by a recent work on *Spongosorites* (MCCORMACK & KELLY, 2002). The phylogenetic revision of Axinellidae (Halichondrida) (ALVAREZ *et al.,* 2000), has also demonstrated a discrepancy between molecular and morphological trees, not only the Axinellidae but also the genus *Axinella* being polyphyletic in the molecular tree. It is becoming each time more common for taxonomists to rely on molecular phylogenies to give support to studies of new species or redescription of taxa whose affinities are dubious. The relationships of *Thymosiopsis* with *Thymosia* and Chondrillidae were inferred from sequences of 28S rRNA (VACELET *et al.,* 2000) and confirmed the previous assumptions of monophyly of the Chondrosida based only on morphology and cytology. In these proceedings another example is given by the reallocation of the excavating genus *Alectona* to the Tetractinellida instead of Hadromerida based on the molecular tree and a reevaluation of morphological characters (BORCHIELLINI *et al.,* 2004). When morphological and molecular trees are not congruent, we need to choose additional genes but, above all, to reassess very carefully the morphological characters without a preconceived idea.

Species and population level studies

Genetic studies of populations are different from phylogenetic ones not only because of the taxonomic level approached, but also because of their different requirements and assumptions. For molecular phylogenetic studies, one of the most important issues is assuring homology. In this case, intra-group variation is an undesirable source of noise and homoplasy. On the other hand, intra-group variation is the raw material for population genetics. One of the immediate consequences of this difference is that sample sizes at the terminal nodes are usually very small (typically 1 - 3 individuals) in phylogenetic studies, but large (10 - 100 individuals/terminal node) in population studies (AVISE, 1994; SILVA & RUSSO, 2000). Also, the genes selected for population analyses must be very variable (typically with heterozygosities above 0.05 or sequence divergences around 1%).

Mitochondrial genes have been extensively used for population and species level genetics of marine invertebrates (reviews in *e.g.* AVISE *et al.,* 1987; AVISE, 1995). However, to date no complete mitochondrial genome of sponges has been produced (the largest fragment sequenced so far is only 2.6 Kbase long: WATKINS & BECKENBACH, 1999), so the choice of mitochondrial genes to study is still very limited. The few available data, mostly on Cytochrome Oxidase I (DURAN *et al.,* 2002b; ERPENBECK *et al.,* 2002), indicate that the mitochondrial genes of sponges, like those of anthozoans (SHEARER *et al.,* 2002), may evolve extremely slowly for population-level analyses. A complete sequence of mitochondrial DNA of sponges is urgently needed, specially considering the unusual features, like linear molecules and the presence of introns, observed on cnidarian mtDNA (BEAGLEY *et al.,* 1996; PONT-KINGDON *et al.,* 2000; VAN OPPEN *et al.,* 2002). In any case, care must be taken when working on mtDNA of sponges, because of the possible existence of paralogous nuclear copies of mitochondrial genes (see WILLIAMS & KNOWLTON, 2001 for an example on Crustaceans).

The most commonly used nuclear genes in invertebrate population genetics (including sponges) are allozymes (reviewed in THORPE & SOLÉ-Cava, 1994; SOLÉ-CAVA & BOURY-ESNAULT, 1999; VAN OPPEN *et al.,* 2003). Although allozymes are good overall markers for population and species level systematics, they have the major drawback of needing fresh or frozen samples. Alternative nuclear markers are, thus, important to be found. Good candidates are microsatellites (TAUTZ & RENZ, 1984; TAUTZ, 1989; DURAN *et al.,* 2002a; KNOWLTON *et al.,* 2002) and internal transcribed spacers (LOPEZ *et al.,* 2002; WÖRHEIDE *et al.,* 2002, 2003). Potential problems with the use of sponge microsatellites are the difficulties in ascertaining the origin (sponge/symbiont) of the bands observed (*e.g.* BRADLEY & VIGILANT, 2002) and the possibility of homoplasy between alleles of the same size (see *e.g.* ORTI *et al.,* 1997). Problems with internal transcribed spacers are the difficulty in alignment and the possibility of comparing paralogous sequences when gene conversion is not complete (KLINBUNGA *et al.,* 1998; DIEKMANN *et al.,* 2001)

Another source of useful information for population genetics of marine invertebrates has been the EPIC (exon-primed intron crossing; PALUMBI & BAKER, 1994) approach, where PCR primers are designed for conserved regions in exons flanking variable introns (*e.g.* CORTE-REAL *et al.,* 1994; BIERNE *et al.,* 2000; HASSAM *et al.,* 2000; MÜLLER *et al.,* 2002). EPICs have not been used, so far, for population analyses of sponges (VAN OPPEN *et al.,* 2003), but appear as natural choices for future work on their population genetics, since introns are present in sponges, albeit in smaller quantity and size than in the Eumetazoa (MÜLLER *et al.,* 2002). Of the 40 coding nuclear sequences from sponges available in GenBank, 7 appear as potential candidates for EPIC analyses: Calcyphosin (YUASA *et al.,* 2002); Calmodulin (YUASA *et al.,* 2001); Galectin (MÜLLER *et al.,* 2002); BHP1g protein, linked to apoptotic pathways (WIENS *et al.,* unpubl. data); Tyrosine kinase (ROUSSET *et al.,* 1995; GAMULIN *et al.,* 1997) and the stress activated kinases p38 and JNK (MÜLLER *et al.,* 2002). Other intronic loci, whose positions are evolutionary conserved and have been used to study populations of other marine invertebrates are the Mac-1 Actin (OHRESSER *et al.,* 1997; DAGUIN *et al.,* 2001), the Integrin beta subunit (SCHMITT & BROWER, 2001), the Pax C (CATMULL *et al.,* 1998; VAN OPPEN *et al.,* 2000) and the Elongation factor alpha (FRANCE *et al.,* 1999; REGIER & SHULTZ, 2001).

Studies at the population level also include the verification of species boundaries, particularly the detection of cryptic species, and the study of clonal structures, which will be discussed below.

Are there any true cosmopolitan sponge species?

In the end of the XIX century, sponge taxonomists marvelled at the huge diversity of the material deployed to them by the big oceanographic cruises of the time. They interpreted that diversity as resulting from speciation, and named many new species (*e.g.* RIDLEY & DENDY, 1887; SOLLAS, 1888; LENDENFELD, 1889), starting a "splitter" phase of sponge taxonomy. For most of the XX century, however, that high diversity was reinterpreted as intraspecific phenotypic plasticity of species supposedly widely dispersed by their planktonic larvae. Consequently, synonymy lists and accepted species ranges were considerably extended (*e.g.* SARÀ, 1956; BURTON, 1963; KOLTUN, 1970), during what could be described as the "lumper" phase of sponge taxonomy. That position was challenged by the

application of new approaches to sponge systematics, particularly scuba diving and allozyme electrophoresis. Scuba diving allowed taxonomists to have a more intimate view of their subjects, which led to a better understanding of sponge biology. Molecular systematics helped to detect reproductive isolation and estimate levels of genetic differentiation between supposedly conspecific morphotypes. Both approaches indicated that the taxonomists of the XIX century were right: sponges are very diverse and even minute morphological differences can indicate species-level differentiation (KLAUTAU *et al.,* 1999; SOLÉ-CAVA & BOURY-ESNAULT, 1999; BORCHIELLINI *et al.,* 2000; VAN OPPEN *et al.,* 2003).

It is interesting to note that, even when the genetic divergence observed by reproductively isolated morphotypes is small (*e.g.* SOLÉ-CAVA & THORPE, 1986; SOLÉ-CAVA *et al.,* 1991), further, independent, ecological or microbiological work confirmed that they did belong to different species (POND, 1992; MARGOT *et al.,* 2002). In fact, every supposedly cosmopolitan sponge species analysed to date turned out to be, under closer molecular scrutiny, a group of many highly divergent but morphologically similar species (*e.g.* KLAUTAU *et al.,* 1994, 1999; MURICY *et al.,* 1996; WÖRHEIDE *et al.,* 2002, 2003). Hence, we believe that very few sponge species, if any, will be found to truly occur in more than one ocean.

One of the consequences of this recent shift in taxonomic philosophy has been the change in the estimated number of extant sponge species, from around 8,000 in the 1970's (BERGQUIST, 1978) to over 15000 in the 1990's (HOOPER, 1994).

The underestimation of species diversity, particularly in the case of fake cosmopolitan (and common) species has profound consequences. For example, many physiological and chemical studies have been performed with "*Halichondria japonica*" (*e.g.* HAYASHI *et al.,* 1991). However, *H. japonica* turned out to be, in fact, a group of different species (HOSHINO *et al.,* 2004). The same seems to be true for *Halichondria panicea*, arguably the biologically most studied sponge species, and cited all over the world, and which is very likely to be a species complex (KNOWLTON *et al.*, 2002). Artificial lumping of different species can also explain some of the variability observed in pharmacologically important natural products of sponge species (MILLER *et al.,* 2001).

Can sponge populations be homogeneous over large areas?

Most populations of sponge species studied to date have shown to be highly structured, whatever the molecular marker used (SOLÉ-CAVA *et al.,* 1992; BENZIE *et al.,* 1994; DAVIS *et al.,* 1996; BOURY-ESNAULT *et al.,* 1999; KLAUTAU *et al.,* 1999; LAZOSKI *et al.,* 2001; MILLER *et al.,* 2001; WÖRHEIDE *et al.,* 2002, 2003; KNOWLTON *et al.,* 2002. Review in VAN OPPEN *et al.,* 2003). This indicates that sponge larvae, both from viviparous and oviparous species, do not disperse very far, or that some type of strong exclusion of recruits from different areas may occur after microhabitat colonization (DE MEESTER *et al.,* 2002). One exception is the viviparous *Chondrosia* sp. from the Western Atlantic, whose populations show a remarkable homogeneity over 8,000 km [unbiased gene Identity (NEI, 1978) = 0.92; LAZOSKI *et al.,* 2001)].

Although rafting has been suggested as a possible means of dispersal in some species (MALDONADO & URIZ, 1999), its effectiveness for gene flow has never been tested through the use of molecular markers (WÖRHEIDE *et al.,* 2004).

How important is asexual reproduction for the establishment and maintenance of sponge populations?

Like many other marine invertebrates, sponges can reproduce asexually (*e.g.* CORRIERO *et al.,* 1996). However, it is yet not clear how much of a sponge population is made of clone-mates, *i.e.* what is the proportion of genetically unique (= genets; HARPER, 1977) and genetically identical (= ramets) individuals in sponge populations. Graft acceptance/rejection experiments indicate that asexual reproduction can be highly important in the composition of sponge populations (KAYE, 1983; NEIGEL, 1985; ILAN & LOYA, 1990; MARKEZICH & FRANCIS, 1991; reviews in FERNÀNDEZ-BUSQUETS & BURGER, 1999; MÜLLER *et al.,* 1999)

The number of genes involved in self/non-self recognition in sponges is still not known, but it may be small and highly polymorphic (FERNÀNDEZ-BUSQUETS & BURGER, 1997) and the mechanism of historecognition only now starts to be understood (FERNÀNDEZ-BUSQUETS *et al.,* 2002; MÜLLER *et al.,* 2002). This means that, although potentially useful, it is unclear how accurate grafting experiments will be for estimating the extent of asexual reproduction in sponge populations.

Some genetic evidence of clonality, based on compound multi-locus genotyping has been found on *Latrunculia* spp. (MILLER *et al.,* 2001), in *Chondrilla* (KLAUTAU, pers. comm.) and in *Chondrosia* (LAZOSKI *et al.,* 2001). Nonetheless, there are no published studies, to date, where carefully mapped sponge individuals were compared, on different scales, using molecular markers, like done with other sessile marine invertebrates (see *e.g.* COFFROTH & LASKER, 1998; PORTER *et al.,* 2002).

An interesting situation that could be observed in sponges would be the presence of different genets living within one single ramet (SOLÉ-CAVA & THORPE, 1994), as observed, for example, in ascidians (SOMMERFELDT & BISHOP, 1999). One of the possible consequences of fusion, particularly at the larval stage, would be an increased probability of survival in the face of predators, particularly grazers (RINKEVICH & WEISSMAN, 1987; GROSBERG, 1988). However, this hypothesis was found to be false, at least for the sponge *Crambe crambe* (MALDONADO, 1998). Allogeneic fusion in sponges could be more difficult to detect than in bryozoans and colonial ascidians, because, unlike those organisms, in sponges no individual polyps can be identified. This means that allogeneic fusion in sponges could result in a complete cell mixing between the contributing genotypes (SOLÉ-CAVA & THORPE, 1994). A practical consequence of that for genetic studies would be, at least for codominant markers like allozymes, EPICs and microsatellites, the observation of high heterozygote excesses, which have not been reported to date. Recent molecular techniques, like *in situ* PCR, make it, now, possible to determine the fate of the individual cells in a sponge chimera, and highly variable markers have already been used for in vitro cell line identification of *Axinella corrugata* (LOPEZ *et al.,* 2002). Other highly variable markers that could potentially be used for the study of allogeneic fusion would be the immunoglobulin-like genes (PANCER *et al.,* 1998) and the aggregation factor core proteins (MAFp3; FERNÀNDEZ-BUSQUETS & BURGER, 1997).

CONCLUSIONS

Although much progress has been made since the Brisbane Symposium concerning molecular phylogeny and genetics in sponges, it remains necessary to considerably increase the knowledge on these animals because of their key position at the base of the animal tree. The most important challenge for the next years will be to verify the hypothesis of the paraphyly of the Porifera, particularly in relation to the relationships of Hexactinellida and Demospongiae, and to test the monophyly of Demospongiae by comparing sequences of a large and thorough group of species from the currently accepted families and orders. A better knowledge on the number of chromosomes present in species of the different clades would allow making hypotheses on the chromosomal evolution of the group. Given the high incidence of cryptic speciation in sponges, we recommend that taxonomists and ecologists be extremely careful in assigning specimens to species described in a different ocean from the collection site. The study of population structure in sponges is still in its infancy, and more work is necessary, especially with species where independent estimates of larval dispersal could be obtained, to verify the correlation between predicted and realized gene flow in sponge populations.

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