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**Insights into the evolution and development of *Haliclona indistincta* (Porifera, Haplosclerida)**

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A thesis submitted to the Zoology Department, Faculty of Science, National University of Ireland, Galway in fulfilment of the requirements of the degree of Doctor of Philosophy, 2013.

**Supervisor:** Dr. Grace McCormack

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**Declaration:**

This thesis has not been submitted, in whole or in part, to this, or any other University for any degree. This work, except where otherwise stated, is the original work of the author.

Signed.....

Kelly Stephens

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## **Abstract:**

*Haliclona indistincta* (order Haplosclerida, family Chalinidae) is an intertidal, and shallow sub-tidal, dwelling sponge found in Ireland, Britain and elsewhere that possesses a highly variable morphology. It is a member of the *H. rosea* group, proposed as monophyletic by de Weerd (1986; 1989) based on a shared skeletal structure. *Haliclona* is a notoriously problematic genus for systematics because morphological characters are both few in number and highly plastic. This difficulty is noted for the order Haplosclerida as a whole, which is the largest and most diverse group of sponges. In recent years, additional insights into the phylogenetics of the haplosclerids, and *Haliclona*, from molecular data has shown incongruence with the classical classification based on morphology. Whether variable specimens assigned to *H. indistincta* belong to the same species and whether *H. indistincta* is even a haplosclerid has come into question. As with most species of haplosclerids, very little has been investigated with *H. indistincta* beyond the initial description of the species.

This project explored the phylogenetic position of *H. indistincta* utilizing two independent gene loci (28S rRNA and mtCO1), to test if it belonged within the haplosclerids and if the *H. rosea* group is monophyletic. Congruence between morphological and molecular data was found, as both confirmed the phylogenetic position of *H. indistincta* within the haplosclerids although this species was more closely related to members of a different family (i.e. Niphatidae). Morphological variation was found consistent with two species morphotypes, but these were very similar genetically.

The reproductive cycle and developmental biology of *H. indistincta* was also investigated. Utilizing histology, this species was found to be primarily hermaphroditic, however gonochorism was also observed. Data supported the hypothesis that the male gametes (i.e. spermatozoa) were derived from choanocytes and the female gametes (i.e. eggs) were derived from archaeocytes. A fusing behaviour was observed in the larvae of this species and fused larvae settled normally. Typically, the larvae went through three distinct mobile stages (signified by changes in the body shape) prior to settlement.

Cellular rearrangement and differentiation was documented from the first free-swimming stages up to 32 days of settlement. Data was shown that supports the hypothesis that ciliated epithelial cells of the larvae transdifferentiate into two separate cell types of the juvenile (i.e. choanocytes and pinacocytes). Two previously undescribed cell types were documented (cells with inclusions Types 1 and 2). I suggest the cell with inclusions Type 1 have key roles in the developmental process of the species including: canal system formation (which I suggest has started forming prior to settlement), cell fate, and possibly nutrient processing. The basal apparatus of the ciliated epithelial cells were described in detail in hopes that further synapomorphies among haplosclerid species could be found. This work highlights both the need for, and benefits of complementing molecular phylogenetic studies with morphological data.

Chapter 1:

**General Introduction**

This study explores the phylogenetic position and reproductive and developmental biology of the marine intertidal sponge *Haliclona indistincta*. The Porifera (sponges) are widely considered to be the earliest branching metazoans (e.g. Borchiellini et al., 1998; Kruse et al., 1998; Rokas et al., 2005; Adamska et al., 2010; Conaco et al., 2012). Currently phylum Porifera is composed of three taxonomic classes: Calcarea, Hexactinellida, and Demospongiae (van Soest, 2007). Of the 8,553 species described, the class Demospongiae maintains 83% (Van Soest et al., 2012). There is an estimated 7,000 additional extant undescribed species, the estimation is considered conservative (Hooper and Levi, 1994; Hooper and Van Soest, 2002). However, recent ribosomal data (e.g. Collins, 1998; Adams et al., 1999; Borchiellini et al., 2001; Medina et al., 2001; Cavalier-Smith and Chao, 2003; Manuel et al., 2003; Wallberg et al., 2004; Sperling et al., 2007; Gazave et al., 2012) have shown the Porifera to be paraphyletic with members of the Calcarea and Homoscleromorpha (previously a sub-class in Demospongiae) to be more closely related to eumetazoans than to Demospongiae. Recently, the omission of Homoscleromorpha from the demosponges and instating them as the fourth class in the Phylum was suggested by Gazave et al. (2012).

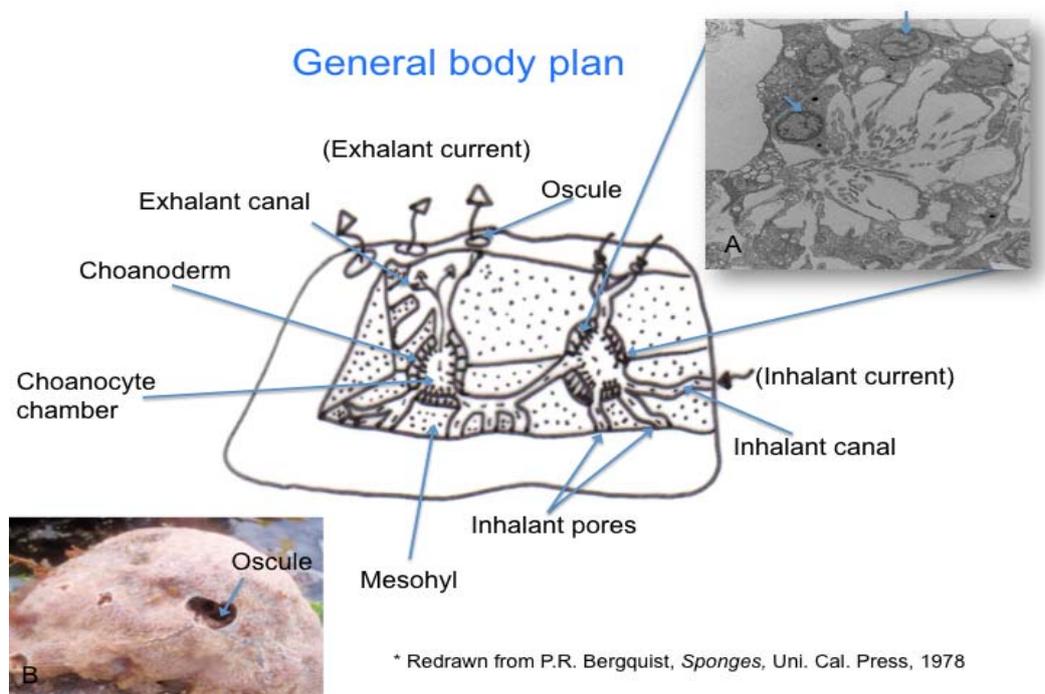
Sponges are relevant to the modern scientific community for ecological, biochemical, and evolutionary studies (e.g. Rao et al., 1998; Avila et al., 2007; van Soest, 2007; Wörheide et al., 2007; Maldonado and Riesgo, 2008). Sponges are key to nutrient cycling processes, and provide stable habitats for multiple species of micro- and macro-flora and fauna (van Soest, 2007). Sponges are sessile animals, and because of their sessile state, bio-chemical compounds, produced by a sponge, and/or by the symbiotic microbes within a sponge, have evolved for chemical defence (Garson et al., 1994; Unson et al., 1994; Bewley et al., 1996; Uriz et al., 1996; Ereskovsky et al., 2005).

These chemical compounds are potentially of great interest across the scientific community to develop pharmaceutical products from biochemical compounds that have anti-fungal, -parasitic, -bacterial, and anti-viral properties (Donia and Hamman, 2003; Ereskovsky et al., 2005; Maldonado and Riesgo, 2008). Compounds from sponges have been studied as part of anti-HIV (e.g. Bokesch et al., 2002) and cancer research (e.g. Proksch et al., 2002) and also have anticoagulant, antidiabetic, anti-inflammatory, and antituberculosis properties amongst others (Mayer et al., 2005).

This ancient and highly successful group can occupy a wide range of aquatic environments, both marine and freshwater (Bergquist, 1978). As sessile animals, sponges are filter-feeders, whose bodies are comprised of three layers; 1) an outer epithelial-like layer, the pinacoderm, that separates the sponge from its environment (composed of pinacocyte cells); 2) an internal layer, the mesohyl, an extracellular matrix composed of proteins, polysaccharides, fibrous collagen fibres, skeletal material and mobile cells and; 3) an internal epithelial-like layer, the choanoderm, that is lined with choanocyte cells (Bergquist, 1978). This general body plan, often referred to as 'simple', has remained veritably unchanged since the Late Cambrian period (~509 million years ago) (Reitner and Wörheide, 2002).

There are three different sponge body arrangements (i.e. asconoid, syconoid and leuconoid) that vary in degrees of complexity (Bergquist, 1978). These categories are based upon the arrangement and anatomy of choanocyte chambers (Bergquist, 1978). The asconoid body type is the most simple in composition and is only found in a few calcareous species (Bergquist, 1968; 2001). These species are tubular in shape with a single osculum at the top containing a single chamber lined with choanocyte cells (Bergquist, 1978; Ereskovsky, 2010). The syconoid body plan is slightly more complex

consisting of canals that are created by an infolded pinacoderm and choanoderm (Bergquist, 1978; Ereskovsky, 2010). The most common, and complex body plan is leuconoid, wherein the choanoderm is folded to the degree where the flagellated surface is completely subdivided into discrete circular chambers (choanocyte chambers) (Bergquist, 1978; Ereskovsky, 2010) (Figure 1). An intricate system of aquiferous canals connects the choanocyte chambers (Bergquist, 1978; Ereskovsky, 2010). Through these canals, ambient water is pumped vigorously in and out of the sponge body by the ciliated choanocytes (Bergquist, 1978; Maldonado and Riesgo, 2008; Ereskovsky, 2010). This facilitates the expulsion of waste and reproductive material into the water column while food (e.g. bacteria, microalgae, and dissolved organic matter), as well as gametes, are extracted from the water by choanocytes, and are then passed to carrier cells. Once passed to carrier cells, in the case of food particulates, phagocytosed by specialized cells, while sperm is carried to waiting oocytes (Bergquist, 1978; Maldonado and Riesgo, 2008; Ereskovsky, 2010).



**Figure 1.** General leuconoid body plan. (A): transmission electron microscope (TEM) micrograph of a choanocyte chamber (arrows) in *H. indistincta* shown in image (*in situ*) (B): external view of *H. indistincta*, a typical demosponge (all of which have the leuconoid type of organisation).

Porocytes are specialized cells that derive from the exo-pinacoderm and are present in the inhalant system, surrounding inhalant pores (ostia) and functioning as a pore canal (Simpson, 1984). Apopylar, or cone cells, (modified choanocytes (Weissenfels, 1981; Langenbruch, 1991), are positioned between apopinacocytes and choanocytes (Ereskovsky, 2010). They are ciliated, have a collar of microvilli, and form a border between the exhalant canals and the opening to the choanocyte chambers (apopyle) (Ereskovsky, 2010). Apopylar cells are commonly found in the Homoscleromorpha, and in demosponge orders: Halisarcida, Dictyoceratida and Dendroceratida (Boury-Esnault et al., 1984; De Vos et al., 1990; Muricy et al., 1996; Ereskovsky, 2006; Ereskovsky and Tokina, 2007; Ereskovsky, 2010), and are also described in haplosclerids such as *H. indistincta* (Langenbruch, 1991), *Petrosia ficiformis* (Langenbruch et al., 1985), and *H. elegans* (Langenbruch and Scalera-Liaci, 1986). The primary function of apopylar cells is suggested to be regulating water flow within the sponge body (Ereskovsky, 2010). A central cell can also be a feature of the choanocyte chamber. Located at the apopyle of a choanocyte chamber (Boury-Esnault and Rützler, 1997; Ereskovsky, 2010), these cells may derive from either choanocytes (Reiswig and Brown, 1977) or endo-pinacocytes (Langenbruch and Scalera-Liaci, 1986) and are ultrastructurally identical to choanocytes (Ereskovsky, 2010). If present in a species (as in *H. elegans*), they are present in only a few of the choanocyte chambers (Langenbruch and Scalera-Liaci, 1986; Ereskovsky, 2010).

Actinocytes, are cells that gather in groups around oscules and major canals (Bergquist, 1978; Simpson, 1984; Boury-Esnault and Rützler, 1997). When present, actinocytes are organized in networks of cells that contact other actinocytes and pinacocytes using filopodia (Bergquist, 1978; Simpson, 1984; Renard et al., 2009). Because of how actinocytes are organized, as well as their spindle-shaped, smooth muscle-like

morphology, the hypothesis that they cause non-muscular contraction and thereby form a rudimentary nervous system in sponges, was widely accepted by sponge biologists (e.g. Pavans de Ceccatty, 1960; Mackie, 1970; Bergquist, 1978; Simpson, 1984). However, recent evidence supports the alternative hypothesis that pinacocytes are responsible for contraction, and actinocytes (myocytes) have little or no role to play in the process of demosponge body contraction (Nickel et al., 2011).

Several types of amoeboid cells are shared among all demosponge species; this includes secretory cells (i.e. collencytes, lophocytes, spongocytes, and sclerocytes (in siliceous sponges)) and archaeocytes (Boury-Esnault, 2006). Collencytes' primary function is to secrete the fibrillar collagen comprising the framework for the sponge matrix (Bergquist, 1978; Simpson, 1984; Boury-Esnault, 2006). They are fusiform to amoeboid in shape. Lophocytes are amoeboid in shape and tend to concentrate their numbers at the base of a sponge and leave a secreted fibrillar collagen trail behind them as they move. Functionally, lophocytes are similar to collencytes, and they have been suggested to be a type of collencyte (Lévi, 1970; Boury-Esnault and Rützler, 1997; Ereskovsky, 2010). Archaeocytes are amoeboid, totipotent cells able to differentiate into any other cell type and are found liberally dispersed throughout the mesohyl (Bergquist, 1978; Simpson, 1984; Boury-Esnault, 2006; Ereskovsky, 2010). They are found clustered under special circumstance, such as during gemmulation, or when tissue needs repair. Through phagocytosis, they consume bacteria in the mesohyl, and accept phagocytosed material from choanocytes (Bergquist, 1978; Simpson, 1984; Boury-Esnault, 2006; Kennedy et al., 2008; Ereskovsky, 2010). Archaeocytes are the "stem cells" for members of Demospongiae and studies employing ficoll, gradient-purified cell fractions (e.g. Buscema et al., 1980) where only aggregates of archaeocytes were present, have shown that, depending on their positioning, archaeocytes take on the necessary roles of

the various sponge cells (Buscema et al., 1980; Gaino and Burlando, 1990; Ereskovsky, 2010).

Polyblasts are archeocyte-like cells that are distinguished from archaeocytes by possessing few to no phagosomes as well as relatively small volume of cytoplasm when compared to nuclear volume (Connes, 1968; Thiney, 1972; Connes et al., 1972; Simpson, 1984; Ereskovsky, 2010). It has been suggested that polyblasts are just an intermediate stage between archeocytes and choanocytes, and Thiney (1972) suggests that they should not be considered a separate cell category. Also thesocytes and histoblasts, which are storage cells involved with gemmule development, are considered a derivative of archaeocytes, as are trophocytes (nutritive cells that aid in oogenesis) (Simpson, 1984; Ereskovsky, 2010).

Mobile cells that have reduced features characteristic of archaeocytes (i.e. fewer mitochondria, and reduced endoplasmic reticulum, generally a non-nucleolated nucleus, and no phagosomes) and can be characterized by the presence of vesicles or granules (or both) that have a particular morphology or chemistry, are grouped under the heading of 'cells with inclusions' (Bergquist, 1978; Simpson, 1984; Ereskovsky, 2010). These include: vacuolar cell or cystocytes, spherulous cells, granular cells (a variety of spherulous cells), microgranular cells, glycocytes, globoferous cells, rhabdiferous cells, spumeuse cells, sacculiferous cells, stylocytes, and chromocytes (Bergquist, 1978; Simpson, 1984; Ereskovsky, 2010). Although distinguishable from archaeocytes, it has been suggested that these cells are directly derived from them, and are the terminal differentiations of the archaeocytes (Bergquist, 1978). Two possible functions have been suggested for the cells with inclusions 1) elaboration of matrix (and mucus) substances, and 2) pigment storage and/or deposition (Simpson, 1984). Many cells with

inclusions appear to be functionally homologous, e.g. rhabdiferous and spherulous cells, which are cells with large inclusions the contents of which are released into the mesohyl for the addition of matrix and mucus substances (Donadey, 1982; Simpson, 1984); and Simpson, (1984) suggested the possibility for the range of cells with inclusions reported was due to chance observations of cells undergoing differentiation.

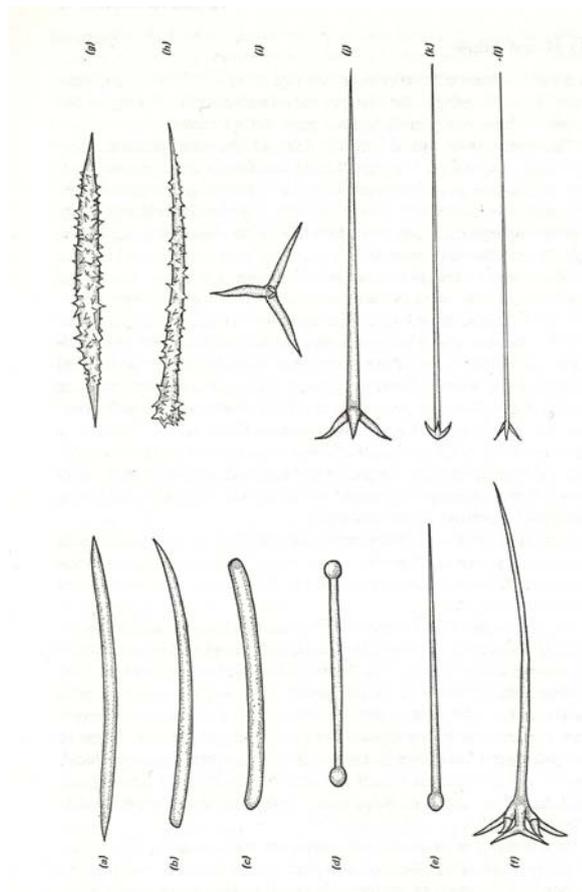
Spherulous cells have been described in marine haplosclerids (e.g. *Amphimedon queenslandica* (Conaco et al., 2012), *Chalinula saudiensis* (Vacelet et al., 2001), and *H. elegans* (Herlant-Meewis, 1948) as have granular cells (e.g. *Siphonodictyon* sp. (Simpson, 1984)), gray cells (glycocyte) (*Siphonodictyon* sp. (Simpson, 1984)), globoferous cells (e.g. *H. tubifera* (Woollacott, 1993), *Callyspongia* sp., *Haliclona* sp. (Simpson, 1984), and spumeuse cells (e.g. *Reniera (Haliclona) mucosa* (Donadey, 1982)).

Cytology of larvae can be very different from the adults, for example, *P. ficiformis* (order Haplosclerida) are solid larvae with no cell differentiation (Maldonado and Riesgo, 2009). Unfortunately, data concerning cell types in sponge larvae and juveniles (particularly for marine haplosclerids) is severely lacking. Studies of metamorphosis in some cases refer to cell types in more general terms such as “amoebocyte” and have not described their specific characteristics (e.g. Woollacott, 1993; Uriz et al., 2008). Unlike cells that are commonly found in all demosponges (e.g. archaeocytes) some cells such as vacuolar cells, have been found to show phylogenetic signal for closely related species (e.g. species within genera *Oscarella* and *Halisarca*) and other cells (i.e. spherulous cells) have been found to show species-specific morphology (Bergquist, 1996; Muricy et al., 1996; Boury-Esnault, 2006; Ereskovsky, 2010). Given the lack of unique skeletal characteristics present in haplosclerids for classification and

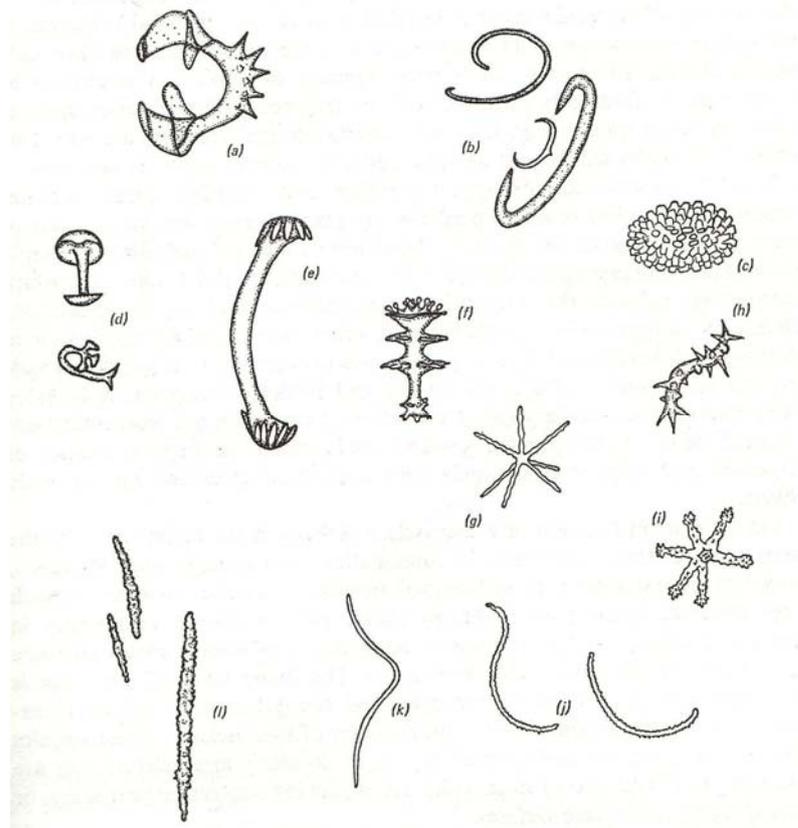
phylogenetic studies, the addition of novel and useful cytological and developmental characters could help reconcile the discrepancies that exist between molecular and morphological data (introduced below). In this study I provide such data for *H. indistincta*.

Symbiotic bacteria are also commonly found within sponge bodies from early on in sponge development. For demosponges, these organisms are often endocytosed by oocytes from the maternal mesohyl, and stored in vesicles to either be digested by the oocyte, or passed to the blastomeres in the developing embryos (Green and Bergquist, 1979; Gaino et al., 1986; Sciscioli et al., 1989; Mariani et al., 2001; Ereskovsky et al., 2005; Mariani et al., 2005; Ereskovsky et al., 2007; Maldonado et al., 2005; Maldonado, 2007; 2009; Riesgo and Maldonado, 2009). Bacteriocytes, which are amoeboid cells occasionally found in demosponges, house prokaryotic symbionts in vacuoles (Ereskovsky, 2010). There are two general types of bacteriocytes described. One type has numerous small vacuoles containing bacteria as described in the carnivorous sponge *Asbestopluma hypogea*, and in *H. tubifera* (Woollacott, 1993; Vacelet and Boury-Esnault, 1996; Vacelet and Dupont, 2004; Ereskovsky, 2010). The second type has one large bacteria-containing vacuole as described in *Aplysina cavernicola*, *P. ficiformis*, *Biemna ehrenbergi* and *Suberites domuncula* (Vacelet, 1970; Vacelet and Donadey, 1977; Bigliardi et al., 1993; Ilan and Abelson, 1995; Böhm et al., 2001; Maldonado, 2007; Ereskovsky, 2010). Vertical transmission of symbiotic microbes is not a mechanism employed by all demosponges, as was shown for *P. ficiformis* and *A. aerophoba*, bacteria may be transferred from ambient water at any stage post-release (Maldonado, 2007; 2009).

Sponges produce either a proteinaceous, or mineral skeleton to support their body growth (Uriz et al., 2003). This skeleton serves various purposes including growth, support, facilitating water exchange as well as defense (Rissgard and Larsen, 1995; Uriz et al., 2003, Uriz, 2006). Most demosponges secrete siliceous skeletons comprised of needle-like structures referred to generally as spicules and more specifically (depending on their size) either micro- or megascleres. There is a small range of different mega- and microscleres present in demosponges and Figures 2 and 3 show the diversity observed. The skeleton of haplosclerids is a particularly simple isodictyal skeletal structure being comprised primarily of megascleres that are either short, fusiform oxeas or, short strongyles, and microscleres (if present) are microoxeas/strongyles, sigmas, toxas, raphides and amphidiscs (van Soest and Hooper, 2002).



**Figure 2.** Representatives of the megascleres present in demosponges. **(a) oxea**; (b) style; **(c) strongyle**; (d) tylote; (e) tylostyle; (f) plagiotriaene; (g) acanthoxea; (h) acanthostyle; (i) calthrops; (j) orthotriaene; (k) anatriaene; (l) prototriaene. Diagram was taken from *Sponges* (Bergquist, 1978). \* Bolded megasclere names correspond to megascleres found in marine haplosclerids (*sensu* Van Soest and Hooper, 2002).



**Figure 3.** Representatives of the microscleres present in demosponges. (a) anchorate isochela; **(b) sigmas** (various sizes); (c) sterraster; (d) bipocilli; (e) arcuate isochela; (f) discorhabd; (g) euaster; (h) spiraster; (i) acanthotylaster; (j) sigmaspirae; **(k) toxas**; (l) acanthose microxeas. Diagram was taken from *Sponges* (Bergquist, 1978). \*Bolded microsclere names correspond to microscleres found in marine haplosclerids (*sensu* Van Soest and Hooper, 2002).

Spiculogenesis follows a consistent pattern (Uriz et al., 2003; Maldonado and Riesgo, 2007). Specialized cells called sclerocytes, secrete and guide the overall formation of spicules which are secreted in layers (Uriz et al., 2003; Uriz, 2006; Madonado and Riesgo, 2007). A layer of proteinaceous material and silicate covers the organic axial filament, which is the core of the spicule (Uriz et al., 2003; Weaver and Morse, 2003; Uriz, 2006; Madonado and Riesgo, 2007). Then, through a process called “polycondensation”, ortho-silicic acid is transferred from the ambient silica-saturated water, and amorphous hydrated silica layers are formed on top of the axial filament (Simpson, 1984; Garrone and Lethias, 1990; Uriz et al., 2000; Uriz et al., 2003; Uriz, 2006). Silica spicule formation mainly occurs within sclerocytes (Garrone, 1969;

Simpson and Vaccaro, 1974; Hartman, 1981; Custódio et al., 2002) but has also been reported to occur extracellularly, however, it is possible that (especially for large spicules) that the process is only begun intracellularly and then completed extracellularly (Simpson, 1968; 1984; Uriz et al., 2000; Uriz, 2006; Maldonado and Riesgo, 2007). It is still uncertain whether or not multiple sclerocytes take part in the development of a single spicule (Uriz et al., 2000). Spongocytes secrete collagen fibers around spicules once they are fully developed and in position forming the characteristic skeletal structure of that species (Uriz et al., 2003).

There are six basic types of skeletal structures found in demosponges (often with intermediate forms) and are usually related to growth anatomy (Boury-Esnault and Rützler, 1997; Uriz et al., 2003). Generally, sponges that encrust substrates in thick sheets tend to have a plumose skeletal type that has primary fibers (or spicule tracts) and the skeletal elements radiate out in a slanting or sloping reticulation, while those that encrust substrates in thin sheets tend to have a hymedesmoid (loosely or jumbled) skeleton (Boury-Esnault and Rützler, 1997; Uriz et al., 2003). Massive, tubular, and more erratic body shapes might be supported by either a reticulated skeleton, as is the case for *H. indistincta*, or one that has no apparent order (Boury-Esnault and Rützler, 1997; Uriz et al., 2003). Globular or sub-spherical shaped sponges tend to have a supporting skeleton composed of radiating spicules. Branching and cylindrically shaped sponges have an axial spicule arrangement (Boury-Esnault and Rützler, 1997; Uriz et al., 2003). Currently the classification of sponges is primarily based on skeletal structure (including the shape, size and arrangement of the spicules and the amount of spongin fibers present) (Boury-Esnault, 2006). However, Boury-Esnault (2006) suggests it is problematical to expect a single approach to sponge systematics (especially for demosponges) to provide all necessary data concerning phylogenies, and stresses the

importance of implementing additional techniques which include: cytology, embryology and DNA sequencing, for pushing the understanding of these animals further. All of these approaches have been employed here to further understand the evolution and development of *H. indistincta* and towards providing additional data on marine haplosclerids in general, a notoriously difficult group.

Sponge morphology can be highly plastic, and because of this, sponge biodiversity can be under-estimated due to the difficulties in identifying species based on morphological characters (Ackers and Moss, 1987; Bell, 2007). Sponges go through continuous remodelling processes, adapting to environmental conditions, which can mean considerable variation, even within a species (Bell, 2000a; 2001). Competing strategies may also be a factor influencing gross sponge morphology where in areas of high biodiversity, thickly encrusting sponges (>2mm in thickness) have been shown to have a competitive advantage over thinly encrusting sponges (<1mm thick) (Bell and Barnes 2003). Plasticity has been particularly problematic for the demosponge order, Haplosclerida which is considered the most diverse sponge order as far as diversity of habitats and species richness (maintaining approximately 12% of the total sponge species (Van Soest et al., 2012)) of are concerned, and is also noted for the paucity of defining morphological characters throughout the group (van Soest and Hooper, 2002; Borchiellini et al., 2004; Erpenbeck et al., 2007).

Sponges can either be oviparous (broadcasting both eggs and sperm into the water column) or viviparous (brooding eggs internally and releasing fully developed larvae) (Bergquist, 1978; Ereskovsky, 2010). Reproductive process (i.e. spermatogenesis, oogenesis, larval production, gemmule formation and hatching) occur at specific times of the year because sponge sexual reproduction can be influenced by environmental

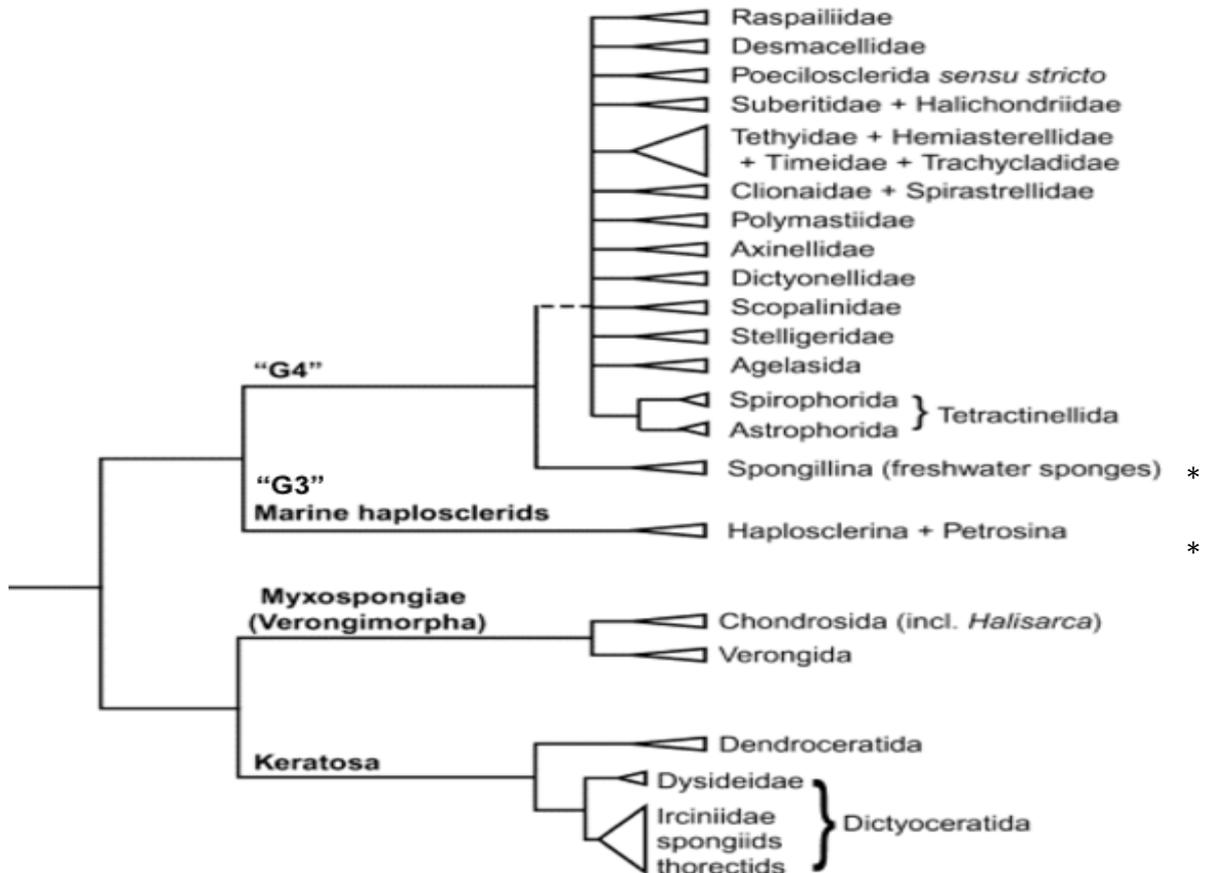
factors (e.g. water temperature) that can dictate when their cycles take place (Simpson and Gilbert, 1973; Fell et al., 1979). Asexual modes, as well as hermaphroditic and gonochoric modes of reproduction are common (Bergquist, 1978). Asexual reproduction includes external budding, fragmentation, or the production of gemmules (gemmulogenesis) (Bergquist, 1978; Ereskovsky, 1999; Ereskovsky, 2003).

For haplosclerids, male gametes have been shown to develop from choanocytes (Maldonado and Riesgo, 2009), and female gametes are suggested to develop from either choanocytes or archaeocytes, depending on the species (e.g. Tuzet, 1932; Meewis, 1936; Leveaux, 1941; Brien, 1967; Saller and Weissenfels 1985; Saller, 1988; Weissenfels, 1989; Ereskovsky, 1999). Most are hermaphroditic, however, gonochorism is also observed (Bergquist, 1978; Ayling, 1980; Ilan and Loya, 1990a; Sarà, 1993; Maldonado and Riesgo, 2009). Haplosclerid reproduction has been shown to generally take place during the warmer summer months (Maldonado and Riesgo, 2009). However, spawning during the winter months, and even year round sperm production, has been reported for some species (Fromont, 1988; Ilan and Loya, 1990a; Maldonado and Riesgo, 2009). Haplosclerids have parenchymella type larvae, which may have oxeas at the posterior end and even choanocyte chambers (although rarely) (Meewis, 1939; Bergquist et al., 1970; Simpson, 1984; Wapstra and van Soest, 1987; Ilan and Loya, 1990a; Woollacott, 1993; Fromont, 1994; Ereskovsky, 1999). Larvae are reported to remain mobile hours to weeks before they settle and develop their sessile body plan (Maldonado et al., 2008). Both viviparous and oviparous reproductive strategies are found in haplosclerids. In this thesis, Chapter 3 focuses on the reproductive cycle and some larval features of *H. indistincta*.

Systematics is a fundamental field of biology concerned with classification and nomenclature (Michener et al., 1970; Schuh et al., 2009). Early sponge taxonomists who considered variability in reproductive modes (i.e. oviparity vs. viviparity) to be phylogenetically significant, used this approach (in part) to divide orders into their various sub-orders, such was the case for the Haplosclerida (Lévi, 1956; van Soest and Hooper, 2002). Further characters employed, as mentioned previously, are skeletal characteristics including the arrangement of the skeleton as well as the type and arrangement of spicules. In some cases cytology and developmental characters have been suggested to be useful, although these later characters have not been widely used. A branch of systematics, known as molecular phylogenetics, is the study of the diversification and the relationships among living organisms over time by analysing hereditary molecular differences in amino acid or nucleotide sequences (Michener et al., 1970; Schuh et al., 2009). The advent of Polymerase Chain Reaction (PCR) (which amplifies millions of copies of a specific DNA gene sequence), and DNA sequencing, has revolutionized this field of research within the last 30 years, since the development of the PCR in 1983 (Bartlett and Stirling, 2003). Because genomes evolve through accumulated mutations in gene sequences, the greater the amount of time, the more mutations that occur, and since every nucleotide comprising a gene sequence is an inherited character, it follows that more closely related organisms would have more similar genomes, and conclusions concerning their most recently shared ancestor can be drawn (Graur and Li, 2000a; 2000b). These relationships are visualized as phylogenetic trees that are drawn using mathematical models to explain the divergences between the genomic sequences (e.g. Felsenstein, 1981; Kimura, 1981; Zharkikh, 1994).

In *Systema Porifera* (Hooper and van Soest, 2002) (the most recent and comprehensive account of sponge classification based on morphology), the order Haplosclerida consists

of three suborders, one freshwater (Spongillina) and two marine groups (Haplosclerina and Petrosina). The marine sub-orders were formed on the basis of members having viviparous or oviparous reproductive modes respectively. Molecular systematics has changed the view of classical sponge taxonomy and congruence between molecular phylogenies (especially within the haplosclerids) and classification based on morphology has been elusive (e.g. McCormack et al., 2002; Borchellini et al., 2004; Erpenbeck et al., 2004; Nichols, 2005; Raleigh et al., 2007; Redmond et al., 2008; 2011). Recent molecular studies utilizing ribosomal genes (e.g. 18S rRNA, 28S rRNA) as well as mitochondrial genes (e.g. mtCO1) have shown that the order Haplosclerida is polyphyletic with the freshwater haplosclerids (suborder Spongillina) more closely related to demosponges outside of the order Haplosclerida (i.e. the G4 clade (*sensu* Borchellini et al., 2004)), than to members of the two marine suborders (G 3 clade) and most families and genera, were also found to be polyphyletic (Figure 4) (e.g. McCormack et al., 2002; Borchellini et al., 2004; Erpenbeck et al., 2004; Nichols, 2005; Raleigh et al., 2007; Redmond et al., 2008; 2011).



**Figure 4.** Phylogenetic tree from Wörheide et al. (2012), reconstruction of the current demosponge phylogeny from ribosomal and mitochondrial data. \* Indicates marine and fresh-water haplosclerid groups.

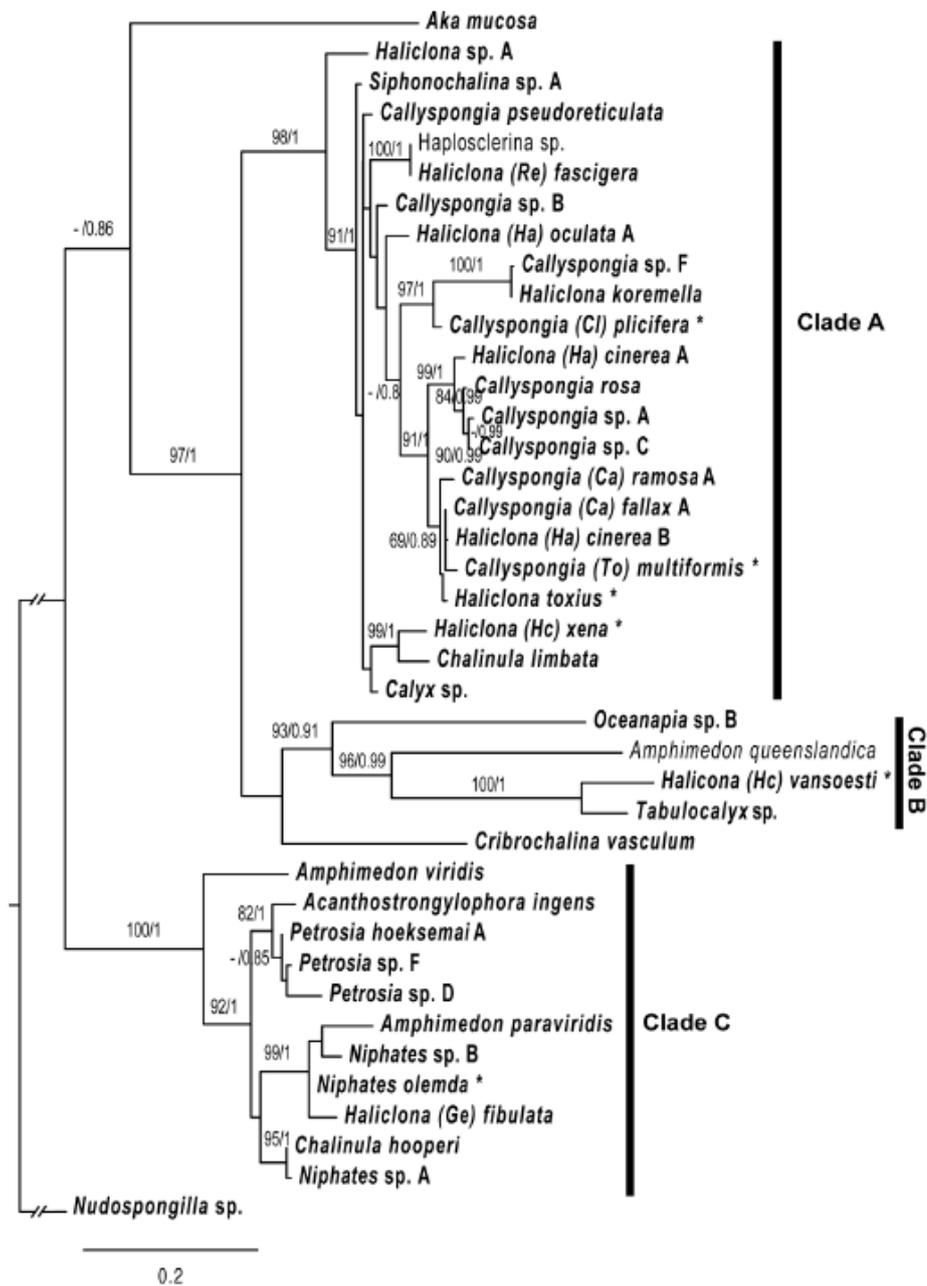
Mitochondria are membrane-bound organelles that are found in eukaryotic cells (Cooper and Hausman, 2004). They have a range of processes including supplying cellular energy, cellular differentiation, and controlling the cell cycle (i.e. cell growth and death). The genetic system of mitochondria is unique and independent from the nuclear genome. Within one mitochondrion are multiple copies of the genome (Cooper and Hausman, 2004) and unlike those of bilaterian animals, the mitochondrial genomes of non-bilaterians (e.g. sponges) are not generally conserved with regards to size and genetic content (Lang et al, 1999). The mitochondrial gene cytochrome oxidase 1 (CO1) is an encoding gene for a key enzyme in aerobic metabolism (Capaldi et al., 1983) and is a widely used marker for sponge phylogenies (e.g. Wörheide et al., 2000; Schröder et al., 2003; Duran et al., 2004; Nichols, 2005; Erpenbeck et al., 2006a; 2006b; Wörheide,

2006; Blanquer and Uriz, 2007; Itskovich et al., 2007; Raleigh et al., 2007; Redmond et al., 2011). However, the mtCO1 gene can be problematic when reconstructing haplosclerid phylogenies, as this gene has been shown to have variable evolution rates for species within the order (Redmond et al., 2011).

Ribosomes are very important organelles that are present in all living cells. They are important for protein synthesis (Mindell and Honeycutt, 1990; Lewin, 2004; Korostelev, 2011). There is a direct relationship between the number of ribosomes present in a cell, and the level of protein-synthesis that the cell is responsible for (Mindell and Honeycutt, 1990). A ribosome is made up of two subunits, termed the large subunit (LSU or 60S (“S” means sedimentation value that is based upon an index of weight and shape)) and small subunit (SSU or 40S), each consists of ribosomal RNA genes (rRNAs) and proteins (Mindell and Honeycutt, 1990). The small subunit (SSU or 40S) has 18S rRNA and 33 proteins, while the large subunit (LSU or 60S) has three rRNAs: 28S, 5.8S and the 5S, and approximately 49 proteins (Lewin, 2004; Ben-Shem et al., 2011; Klinge et al., 2011). rRNAs can be found in mitochondrial (mt), chloroplast, and nuclear genomes (Mindell and Honeycutt, 1990). The 28S rRNA gene varies in length from 2,900 to 4,700 bases, depending on the presence or absence of expansion segments (Hillis and Dixon, 1991; Lewin, 2004). It is located at the 3’ end of the rRNA operon (Mindell and Honeycutt, 1990) and is particularly useful in molecular phylogenetic studies because it contains both slower evolving (conserved) regions as well as faster evolving (variable) regions, which means it can be informative at different phylogenetic levels.

Both the mtCO1 and the 28S rRNA genes were utilized in this study (Chapter 2) to investigate (among other things) the phylogenetic position of *H. indistincta*. The

morphologically-based classification for the Haplosclerida has been disputed by molecular data (e.g. McCormack et al., 2002; Borchiellini et al., 2004; Erpenbeck et al., 2004; Nichols, 2005; Raleigh et al., 2007; Redmond et al., 2008; 2011). In particular, the family Chalinidae and its constituent genera, e.g. *Halicona*, were shown to be polyphyletic (Figure 5) (McCormack et al., 2002; Borchiellini et al., 2004; Erpenbeck et al., 2004; Nichols, 2005; Raleigh et al., 2007; Redmond et al., 2008; 2011).



**Figure 5.** Maximum-likelihood phylogeny reconstructed using the D1–D5 region of the 28S rRNA gene from Redmond et al. (2011) showing polyphyly of the genus *Haliclona* (family Chalinidae, Order Haplosclerida) (with species present in separate clades (i.e. A and B)). Note the *Niphates* species (family Niphatidae) forming a separate clade (i.e. Clade C) from the Chalinidae.

Due to there being a paucity of defining morphological characters, and a high level of morphological plasticity observed in sponges in general, but particularly for members of the genus *Haliclona*, classification based solely on morphology can be problematic (de Weerd, 1986; Maldonado and Young, 1998; de Weerd and van Soest, 2001; van Soest and Hooper, 2002; Borchiellini et al., 2004; Erpenbeck et al., 2007; Raleigh et al.,

2007). De Weerd (1986) systematically revised and re-described the *Haliclona* of the North-eastern Atlantic. In doing so, she examined seventy-two species assigned to the genus *Haliclona*, as well as species that had been placed in other genera, but which she considered to be junior synonyms of *Haliclona*. The junior synonyms included: *Adocia*, *Gellius*, *Orina*, *Reniera*, and *Toxadocia*. De Weerd (1986) concluded that the plethora of species included in the genus *Haliclona* was due to taxonomists describing different growth forms of the same species as being separate species. At the completion of the study, de Weerd (1986) recognized only twelve of the initial seventy-two species as valid, and described a new species. De Weerd (1986) arranged the resulting thirteen species into six groups distinguished on the basis of assumed apomorphic (derived) morphological characters and later she hypothesized the phylogenetic relationships of these six groups. The *Haliclona rosea* group (featured in Chapter 2) contained the species: *H. rosea* (for which the group was named) *H. viscosa* and *H. indistincta*. This group was formed based on their shared skeletal structure. Chapter 2, which focuses on the systematics of *H. indistincta*, looks at the monophyly of the “*H. rosea* group” as a testable group to see if congruence between the relationships suggested by morphology (in this case, skeletal structure), and phylogenies, suggested by molecular data, can be found.

Sponge biologists seek morphological characters that have phylogenetic signal, to not only inform concerning sponge phylogenetics, but also shed light on early multicellular evolution. The cilium, and its accompanying basal apparatus, is a common structure observed throughout the animal kingdom, and is a trait that has been passed on from, what is considered the last common ancestor to all extant animals, choanoflagellate protists (Woollacott and Pinto, 1995; Degnan et al., 2005; Gonobobleva and Maldonado, 2009). Also, it is possible that by understanding the developmental

processes that occur during embryogenesis and metamorphosis of the most basal metazoans, sponges, we can better understand the ontogeny of multicellular animals (Gonobobleva and Ereskovsky, 2004; Degnan et al., 2005; Gonobobleva and Maldonado, 2009). In this study, I hoped to contribute towards improving our knowledge of the evolution and systematics of *Haliclona* by describing structural characters (e.g. basal apparatus) and developmental aspects of a member of this genus.

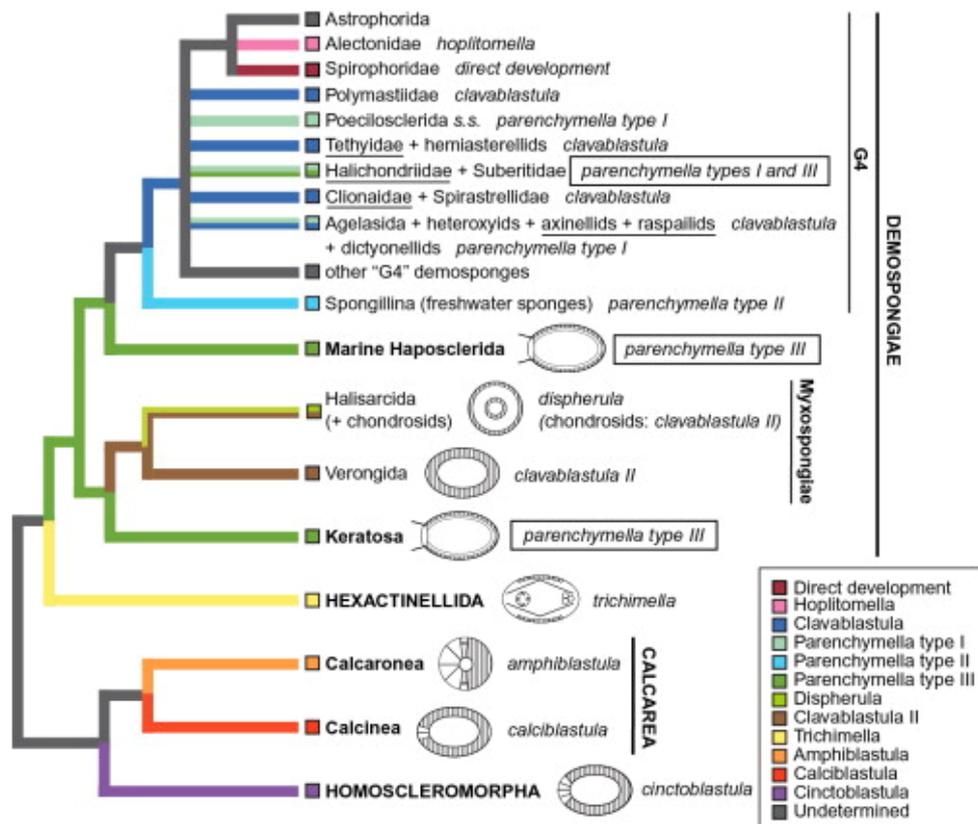
Whether gastrulation occurs in sponges is still a matter of great debate (e.g. Efremova, 1997; Leys, 2004; Ereskovsky and Dondua, 2006). The process of gastrulation has recently been suggested to occur in some sponges during larval metamorphosis and is thought to be represented by the movement of the external epithelial cells inward and their trans-differentiation into the choanocytes (e.g. Gaino and Burlando, 1990; Amano and Hori, 1993; 1998; Leys and Degnan, 2002; Maldonado, 2004; Leys and Eerkes-Medrano, 2005; Ereskovsky et al., 2007b; Ereskovsky et al., 2009). Other studies suggest that the choanocyte cells are derived from internal totipotent archaeocyte cells (or both the external epithelial cells and totipotent archaeocyte cells) contribute to the development of the choanocyte cells (Gonobobleva and Maldonado, 2009). However, the question of whether or not sponges undergo gastrulation does not rest solely upon the fate of the ciliated external cells, or the origins of the choanocyte cells. It has been suggested that during sponge embryological development, six different modes of gastrulation processes that can be equated to gastrulation modes observed in other invertebrates, are observed (i.e. emboly, centrifugal migration, selective centrifugal migration, delamination, multipolar ingression, and epiboly) (Leys, 2004; Maldonado, 2004; Riesgo et al., 2007).

It has been suggested that a distinguishing characteristic between higher metazoans and sponges is lack of body symmetry in sponges (Ereskovsky 2007a). However, while higher animals have definite body symmetry with two body axes, dorsal-ventral and anterior-posterior, sponges display polarity as well, having distinct anterior and posterior ends. This polarity is most apparent during the larval stage, but is also observed during embryonic development and also in adults (denoted by the presence of osculum on the surface) (Leys and Degnan, 2001; 2002; Adamska et al., 2007; Ereskovsky et al., 2009). Whether poles of a sponge are equivalent to the anterior-posterior axes of higher animals is still debated (Ereskovsky 2007a). However, the presence of body axis in sponges have been further supported by recent work that has shown that developmental genes (e.g. Wnt pathways), observed in bilaterians, are involved in cell movement during axial formation in sponges (Windsor and Leys, 2010; Adamska et al., 2011). Whether the seemingly unorganized extracellular matrix, the mesohyl, is “true tissue”, and whether the external and internal epithelial systems of sponges can be equated with the ecto- and endo- dermal layers of members of other Phyla, is still debated (Maldonado and Riesgo, 2008).

Although sponges do not have a nervous system, sponge larvae have a means of obtaining information from their environment and to respond appropriately. Motility of many demosponge larvae is influenced heavily by light (Leys et al., 2001; Maldonado et al., 2003). The posterior ring of columnar monociliated epithelial cells that have pigment-filled protrusions are suggested to function as photoreceptors (i.e. parenchymella larvae of *Amphimedon queenslandica*) (Leys et al., 2001; Wörheide et al., 2012). Cilia also function as directional apparatuses and for locomotion. Once the sponge has settled, ciliated cells disappear from the surface of the larva (Amano and Hori, 1996). Through metamorphosis, larvae undergo dramatic changes, including the

migration and trans-differentiation of specific cell types and the formation of a sessile adult body structure (Larroux et al., 2006). However, responses to environmental conditions are still apparent even in settled sponges as seen in reproduction (i.e. water temperature regulating sexual reproduction) (Fromont, 1993).

Wörheide et al. (2012) defines type 1 parenchymella as being evenly and completely ciliated (but may have a small area at the posterior pole bare). The freshwater parenchymella that have an internal cavity are type 2; and type 3 are defined as having a bare posterior pole that is surrounded long cilia and ring of pigmented cells (e.g. *A. queenslandica* (marine haplosclerid) Leys et al., 2001) (Wörheide et al., 2012).



**Figure 6.** Diagram from Wörheide et al. (2012) showing the types of larvae found in the various Porifera groups.

The larva of *H. indistincta* (Bowerbank, 1866; Lévi, 1956) under Wörheide et al.'s (2012) definition, is a type 1 parenchymella as it is fully and evenly ciliated, however, as shown in Figure 3, Wörheide et al. (2012) does not include type 1 parenchymella as occurring in the marine haplosclerids. In previous studies that included *H. indistincta*, Langenbruch and Jones (1990) suggested that positioning of choanocyte chambers in a sponge species (i.e. directly in contact with the mesohyl or separated from it) has phylogenetic significance. From the haplosclerid species compared (i.e. *H. indistincta*, *H. oculata*, *H. rosea*, *H. simulans*, *H. fistulosa*, *H. elegans*, and *Niphates digitalis*), Langenbruch and Jones (1990) were led to suggest that *H. indistincta* (which was unlike other *Haliclona* species (including *H. oculata*, which is the type species of the genus)), was more closely related to the order Poecilosclerida than to the Haplosclerida. This would also be consistent with larval ciliation patterns as described above.

Given the molecular evidence suggesting that the genus *Haliclona* is polyphyletic and suggestions from cytology and larval ciliation patterns that *H. indistincta* may not be a haplosclerid, the aims of this project include determining the phylogenetic position of *H. indistincta*, using two independent gene loci (i.e. 28S rRNA and mitochondrial cytochrome oxidase subunit 1 (mtCOI)). I also attempt to find reconciliation between morphology-based classification and molecular data by investigating the monophyly of one of the six species groups proposed by de Weerd (1986; 1989) (i.e. *H. rosea* group containing: *H. rosea*, *H. viscosa* and *H. indistincta*). The phylogenetic position of additional *Haliclona* specimens collected in Ireland during the project was also investigated. Detailed descriptions concerning cytology and morphogenesis of sponges are few; therefore, further aims of this project were to describe in detail the reproductive and developmental biology of *H. indistincta*. Questions concerning cell fate, when

cellular differentiation occurs, whether the internal anatomy of the larvae reflect the distinct anterior-posterior polarity of the larvae were investigated.

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Chapter 2:

**The evolutionary relationships of *Haliclona indistincta*  
and other Irish *Haliclona***

This work contributed to the published manuscript:

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## **Introduction:**

Members of the order Haplosclerida are known for both the plasticity as well as the scarcity of defining morphological characteristics (van Soest and Hooper, 2002; Borchiellini et al., 2004; Erpenbeck et al., 2007). The family Chalinidae (order Haplosclerida) is a large group with a worldwide distribution (de Weerd and van Soest, 2001) and contains four recognized genera, *Chalinula*, *Cladocroce*, *Dendroxea* and *Haliclona* (van Soest and Hooper, 2002). Because of the high number of species, as well as the limited number of characters, members of the family Chalinidae (particularly those in the genus *Haliclona*) are considered to be one of the most difficult to identify and classify (de Weerd, 1986; de Weerd and van Soest, 2001).

*Haliclona* is one of the most taxon rich genera within the Porifera with 255 species currently listed (<http://www.marinespecies.org/porifera/>). The external appearance of species in the genus varies from cushion, tube, repent ramose, or thinly encrusting (de Weerd, 1986; 1989; 2000; 2002). As described by de Weerd (*Loc. cit.*), the surface features include oscular mounds or fistules. Textures range from smooth to hispid, as well as slightly to strongly punctate; the colour range is considerable, and even bi-coloration is common. Tissue consistencies range from soft to firm, elastic to brittle or corky (*Loc. cit.*). *Haliclona* megascleres are smooth oxea, diactines or strongyles, and measure usually from 80-250µm in length and 5-10µm in width (*Loc. cit.*). If microscleres are present, they include sigmas, toxas, raphides or microxeas and an ectosomal skeleton may, or may not be present (*Loc. cit.*). Choanosomal skeletons can be regular, ladder-like reticulation of uni-, pauci- or multispicular primary lines, connected by unispicular secondary tracts (*Loc. cit.*). The choanosomal skeleton may also be a dense subisotropic (mesh with two or more spicules) reticulation with many subdermal and choanosomal spaces. Spongin may be present in small amounts, adding

support to the interconnecting spicules, or may be heavily distributed throughout the skeletal structure (*Loc. cit.*).

Using morphological characters, de Weerd (1986; 1989) revised and re-described the North-eastern Atlantic Chalinidae. Of the 72 species, described as *Haliclona* from this region only 12 were recognized as valid by de Weerd (1986) She also included a description of a new species (*Haliclona xena*) and proposed six monophyletic species groups for these 13 species: the “*oculata*” group composed of *H. oculata*, *H. urceolus*, *H. simulans*, and *H. cinerea*, the “*aquaeductus*” group, composed of *H. primitiva*, the “*fistulosa*” group composed of *H. fistulosa*, the “*arenata*” group composed of *H. xena*, the “*angulata*” group composed of *H. anguata*, *H. fibulata*, and *H. rava*, and the “*rosea*” group composed of *H. rosea*, *H. viscosa* and *H. indistincta* (de Weerd, 1986).

*H. indistincta* (Bowerbank, 1866) was placed in the *rosea* group due to it possessing a skeletal structure of wavy, pauci-multispicular primary lines (de Weerd, 1986; 1989). The species is distributed throughout the Atlantic coasts of France and the Amphi-Atlantic, the British Isles including Orkney Islands, Exmouth, Guernsey and the W., S. and E. shores of Ireland (Bowerbank, 1866; de Weerd, 1986). The described habitat for this species is marine intertidal, and shallow, subtidal areas, and it is commonly found under limestone rocks and boulders (de Weerd, 1986). Conflicting descriptions of this species by some leading sponge biologists (i.e. Bowerbank (1866), de Weerd (1986), and Topsent (1888)) suggest the presence of two distinct morphotypes. Bowerbank (1866) described the body as massive, while Topsent (1888) and de Weerd (1986), described the body as encrusting. Bowerbank (1866) did not mention the very visible excurrent canals radiating from oscules that de Weerd (1986) later described. Bowerbank (1866) also described the species as having a smooth surface with a translucent dermal membrane, punctured by small, irregularly positioned pores while

De Weerd (1986) described the surface to be shaggy in appearance with a softly friable consistency. Furthermore, de Weerd (1986) described the spicules as slender and fusiform, and Bowerbank (1866) described them as short and stout. Bowerbank (1866) and de Weerd (1986) agreed on the external colour, the shape of the oscules and its skeletal structure. However, de Weerd (1986) provided more detail in her overall description. The colour was described as greenish-brown. The oscules were described as irregularly scattered and circular with a diameter of 1.5 to 3mm (Bowerbank, 1866; de Weerd, 1986). No ectosomal skeleton or microscleres were present (de Weerd, 1986). The choanosomal skeleton was described as a close mesh with pauci-multispicular primary and unispicular secondary lines, confused with un-attached spicules. Spongin, when present, was confined to the nodes (de Weerd, 1986). The larva of *H. indistincta* are described as 500x250µm in size, pink to violet in colour with a white anterior pole, with a rapid, irregular swimming movement (Lévi, 1956; Wapstra and Van Soest, 1987).

With the advent of molecular techniques, a number of studies have shown incongruence between molecular phylogenies and the morphology-based classification in that the order Haplosclerida, and many of its families and genera (including the Chalinidae, and the chalinid genera) were polyphyletic (e.g. McCormack et al., 2002; Borchellini et al., 2004; Erpenbeck et al., 2004; Nichols, 2005; Raleigh et al., 2007; Redmond et al., 2008; 2011). Data obtained from 18S rRNA, 28S rRNA and mtCO1 genes have shown freshwater haplosclerids (suborder Spongillina) to be more closely related to Demosponges of the G4 clade (comprised of the orders Agelasida, Astrophorida, Hadromerida, Halichondrida, Spirophorida and Poecilosclerida), than to the two marine suborders (Haplosclerina and Petrosina). These latter suborders formed the G3 clade (*sensu* Borchellini et al., 2004) though were both shown to be polyphyletic. While

hypotheses from CO1 data are congruent with those from ribosomal genes (i.e. 18S rRNA and 28S rRNA) (Raleigh et al., 2007), utilizing the mitochondrial gene has limitations. Due to unusual evolutionary patterns of this gene in some species (e.g. high evolution rates observed for *Amphimedon queenslandica*), it has been shown to be unsuitable for reconstructing relationships across an entire order or between orders (Erpenbeck et al., 2007; Redmond et al., 2011). Also, this gene can be problematical at a species level as shown in Redmond et al. (2011), where sequences generated from members of the same species were very different (i.e. *Callyspongia vaginalis*) while different species showed identical sequences patterns (i.e. *Niphates erecta* and *N. alba*; and *Haliclona tubifera* and *H. implexiformis*). However, relationships within the marine haplosclerid subclade were congruent with other data, and this gene displayed strong phylogenetic signal at this level. While keeping the limitations in mind, because it has been informative in the past, it was considered a useful gene within the context of this study, as a gene from a separate locus, to support the ribosomal data.

In the phylogenetic trees reconstructed of the Order Haplosclerida in Redmond et al. (2011), *Haliclona* species were distributed virtually across the entire marine haplosclerid clade indicating the genus is polyphyletic, (also congruent with Raleigh et al., 2007). This raises questions not only concerning the phylogenetic position of *H. indistincta* but also the monophyly of de Weerd's (1986; 1989) *Haliclona* groups. Also, because identifying *Haliclona* species is very difficult, in part due to the low numbers of characters, specimens can be left unidentified in published studies (e.g. Stephens, 1912; Bell, 2007) meaning that the number of species residing in Ireland may be higher than currently documented. During this study, a number of sponges consistent with *Haliclona* were collected and sequenced; these included putative *H. urcelous* and *H. fistulosa* as well as three whose species designations were unclear or whose external

characters were not consistent with the descriptions of the known North-eastern Atlantic Chalinidae described by de Weerd (1986).

Thus the main objectives of this chapter were:

- 1) To determine the phylogenetic position of *H. indistincta* within the marine haplosclerids utilizing two independent gene loci: 28S ribosomal RNA (rRNA) (D1-D5 regions) and mitochondrial cytochrome oxidase subunit 1 (mtCOI).
- 2) To attempt reconciliation between morphological and molecular data by testing the monophyly of the “*rosea*” group (i.e. *H. rosea*, *H. indistincta* and *H. viscosa*) (de Weerd, 1986; 1989).
- 3) To combine morphological and molecular data to resolve the phylogenetic position of additional *Haliclona* species collected in the Irish intertidal regions.

## Materials and methods:

*Material*; Specimens used in this study were photographed and collected from various intertidal locations around Ireland by the author, Dr. Bernard Picton (National Museums Northern Ireland, UK), and Dr. Grace McCormack (National University of Ireland, Galway). Area maps of sampling sites are shown in Figure 1. Specimen information is provided in Table 1. *H. indistincta* specimens were divided into two morphotypes based on appearance: Type 1 was cushion-like in form and Type 2 was more flat, as is indicated in Table 1.



**Figure 1.** Maps showing location of sampling sites for *Haliclona indistincta*. **(A)** Corranroo, Co. Clare. Arrows indicate the general confines of the sampling area. **(B)** New Quay Co. Clare. Arrows indicate the general confines of the sampling area. **(C)** Map showing the Corranroo and New Quay sampling sites in conjunction with one another. NQ= New Quay, C=Corranroo. **(D)** Lough Hyne Co. Cork. Arrow indicates the general area specimens were sampled.

**Table 1.** Specimens collected during this study (except for *H. rosea*, *Amphimedon paraviridis* and *Tabulocalyx pedunculatus* (indicated by \*)). Y=yes; N=no; N/A= not applicable (for specimens that were not used in molecular analysis). For *H. indistincta*, Type 1=1 (cushion form), Type 2=2 (flat form).

Name	Voucher ID	Collection Date	Location	Latitude & Longitude	28S rRNA (D1-D5)	mtCO1
<i>H. indistincta</i> 1A	MIIGO236	18/11/2008	Lough Hyne Co. Cork	Lat: 51° Lng: -9°	Y	N
<i>H. indistincta</i> 1B	MIIG0165	14/10/2008	New Quay Co. Clare	Lat: 53° Lng: -9°	Y	N
<i>H. indistincta</i> 1C	MIIG0156	14/10/2008	New Quay Co. Clare	Lat: 53° Lng: -9°	Y	N
<i>H. indistincta</i> 2D	MIIG0198	16/11/08	Lough Hyne Co. Cork	Lat: 51° Lng: -9°	Y	Y
<i>H. indistincta</i> 2E	MIIG0185	16/11/08	Lough Hyne Co. Cork	Lat: 51° Lng: -9°	Y	Y
<i>H. indistincta</i> 2F	MIIG0186	16/11/08	Lough Hyne Co. Cork	Lat: 51° Lng: -9°	Y	Y
<i>H. indistincta</i> 2G	MIIG0205	16/11/08	Lough Hyne Co. Cork	Lat: 51° Lng: -9°	Y	N
<i>H. indistincta</i> 2H	MIIG0201	16/11/08	Lough Hyne Co. Cork	Lat: 51° Lng: -9°	N	Y
<i>H. indistincta</i> 1I	MIIG0435	10/09/2009	Corranroo Co. Clare	Lat: 53° Lng: -9°	N/A	N/A
<i>H. indistincta</i> 1J	MIIG0389	21/08/09	Corranroo Co. Clare	Lat: 53° Lng: -9°	N/A	N/A
<i>H. indistincta</i> 1K	MIIG0303	24/06/09	Corranroo Co. Clare	Lat: 53° Lng: -9°	N	Y
<i>H. indistincta</i> 2L	MIIG0379	20/08/09	New Quay Co. Clare	Lat: 53° Lng: -9°	N/A	N/A
<i>H. viscosa</i>	CW51	15/09/09	Achillbeg Island Co. Mayo	Lat: 53° Lng: -9°	Y	Y
* <i>H. rosea</i>	POR:16756	20/07/00	Artic Ocean	Lat: 66° Lng: -167°	N	N
<i>H. cinerea</i> A	MIIGO233	18/11/2008	Lough Hyne Co. Cork	Lat: 51° Lng: -9°	Y	N
<i>H. cinerea</i> B	MIIGO232	18/11/2008	Lough Hyne Co. Cork	Lat: 51° Lng: -9°	Y	N
<i>H. cinerea</i> D	MIIG0154	14/10/2008	New Quay Co. Clare	Lat: 53° Lng: -9°	Y	N
<i>Haliclona</i> sp. A	MIIG0256	02/11/2009	Ardfry Point Co. Galway	Lat: 53° Lng: -9°	Y	N
<i>Haliclona</i> sp. B	MIIG0210	16/11/08	Lough Hyne Co. Cork	Lat: 51° Lng: -9°	Y	N
<i>Haliclona</i> sp. C	MIIG0442	18/09/2009	Corranroo Co. Clare	Lat: 53° Lng: -9°	Y	N
<i>H. fistulosa</i>	MIIG0194	16/11/08	Lough Hyne Co. Cork	Lat: 51° Lng: -9°	Y	N
<i>H. urceolus</i>	MIIG0223	17/11/2008	Lough Hyne Co. Cork	Lat: -5° Lng: 120°	Y	N
<i>Chalinula limbata</i>	MIIG0250	02/11/2009	Ardfry Point Co. Galway	Lat: 53° Lng: -9°	Y	N
* <i>Amphimedon paraviridis</i>	146	12/05/2001	Indonesia	Lat: 53° Lng: -9°	Y	N
* <i>Tabulocalyx pedunculatus</i>	185	27/02/2002	Kenya	Lat: 1° Lng: 38°	Y	N
<i>Phorbis plumbosm</i>	MIIG0152	14/10/2008	New Quay Co. Clare	Lat: 53° Lng: -9°	Y	N
<i>Polymastia</i> sp.	MIIG0157	14/10/2008	New Quay Co. Clare	Lat: 53° Lng: -9°	Y	N
<i>Dercites</i> sp.	MIIG0151	14/10/2008	New Quay Co. Clare	Lat: 53° Lng: -9°	Y	N

*Methods; Skeletal mount method 1* (Hooper, 2003); Tissue was stored in 70-90% ethanol. A piece of tissue was sectioned and was covered in clove oil until the skeleton (comprised of spicules), was cleared of tissue (approximately 5-15minutes). Sections were then mounted on microscope slides using Canada Balsam (Sigma), labelled, and left to dry.

*Skeletal mount method 2* (Ereskovsky A. personal communication, 2011); This method was utilised on a single adult specimen of *H. indistincta* (MIIG435, Table 1). Perpendicular tissue sections were cut and allowed to desiccate for two weeks until only the skeletal scaffolding remained visible. The sections were then rehydrated in distilled water for 30 minutes, then placed in 70% ETH for 1 hour (this step was then repeated). Then they were placed in Acetone for 30 minutes (this step was then repeated), followed by placement in 50/50 acetone/resin (using Agar low viscosity resin (R1078 resin kit, Agar Scientific)) for 1 hour. Sections were then placed in 100% of the above resin and left to stand for 1 hour allowing evaporation of the acetone. Following this, sections were embedded in the Agar low viscosity resin, placed in a mould, and left for 48 hours in an oven at 70° C. Once removed from the oven and the moulds, resin blocks were cut into 1mm thin slices using an Isome, low speed saw (Buehler) and secured to microscope slides by applying fresh resin and baking again in an oven at 70° C over night. The blocks were then further thinned and polished to <1mm sections using a sanding turntable (ESC 300 GTL).

*Spicule mount method 1: Sodium hypochlorite approach* (Hooper, 2003); A piece of tissue was sectioned and placed in a test tube containing sodium hypochlorite (NaOCl) (Sigma) (25% concentration). Once the tissue was dissolved, the NaOCl was then pipetted off and the spicules rinsed in distilled water. The spicules were transferred to a

microscope slide and labelled. After all the water had evaporated, Canada Balsam was used to fix the cover-slip to the slide.

*Spicule mount method 2: Nitric Acid approach* (Hooper, 2003); A section of tissue was removed and placed in a test tube. Two to three drops of Nitric Acid was added and placed over a Bunsen-burner flame until the tissue was dissolved. The spicules were rinsed and placed on a microscope slide, the slide was then washed with water, labelled, and a cover slip was fixed to the slide using Canada Balsam.

*DNA Extraction, PCR amplification and sequencing*; Tissue was collected fresh and stored in 6 M guanidinium hydrochloride (Sigma-Aldrich) in labelled tubes. To approximately 600  $\mu$ l of lysis buffer and tissue 10-20  $\mu$ l of (20mg/ml concentration) Proteinase K (Sigma-Aldrich) was added. Each tube was then placed upon a heating block at 56°C for approximately 16 hours. Genomic DNA was extracted from all of the specimens using a standard phenol-chloroform-isoamyl method (Chomczynski and Sacchi, 1987).

The D1, D2 and D3-D5 regions of the 28S ribosomal gene were amplified using the primer sets displayed in Table 2. Each gene fragment was amplified in 50 $\mu$ l reactions. The final volumes and concentrations of reagents are as follows, 10 $\mu$ l 10X PCR Buffer (Promega), MgCl<sub>2</sub> Solution (25mM stock) ranged from 3 to 4mM final concentration, 1.5 $\mu$ l of dNTP (Promega) mix (10mM stock concentration), 2 $\mu$ M primers and 1.25 units of Taq Polymerase (Promega). The following temperature regime was set on a PCR thermocycler (Techne TC 412, model number FTC41F2D); an initial denaturation of 94°C for 8 minutes followed by 30 cycles, beginning with 30 seconds at 94°C, followed by 45 seconds at an annealing temperature between 38°C to 65°C (adjusted depending

on the DNA template and primer combination), an extension step of 1 minutes 30 seconds at 72° C, and the final extension step of 10 minutes at 72° C. Once completed the program moved to a holding step of 12°C until the samples were removed from the thermocycler. The PCR products (5µl) were visualized by running them on a 1% agarose gel (Sigma) stained with SYBR® Safe DNA gel stain (Invitrogen) using a UV lightsource (Alpha Innotech Corporation light source, Olympus C-5060 digital camera and AlphaDigiDoc software). Cloning of PCR products (of the amplified mtCO1 gene) was carried out using a StratClone PCR cloning kit, (Agilent Technologies) following the manufacturer's instructions. PCR products were gel purified through a 1% Seakem agarose gel (GTG Lonza) and sequenced in both directions by Eurofins MWG Operon. A forward sequence only was returned for the cloned specimens of *H. indistincta* (specimens: MIIG0303, MIIG0186, MIIG0198) and *H. viscosa* (CW51) (Table 1).

**Table 2.** Sequence information for PCR primers used in this study to amplify the D1-D5 regions of the 28S rRNA gene and mitochondrial cytochrome oxidase subunit 1 (mtCO1) gene.

<b>Primers</b>	<b>Direction</b>	<b>5'→3' sequence</b>	<b>Melting Temp (°C)</b>	<b>Reference</b>
<b>28S rRNA</b>				
<b><u>D1 region</u></b>				
LSU5	Forward	TAGGTCGACCCGCTGCCYTAAAGC	63.6	Olsen (Pers. Comm.)
300R	Reverse	CAACTTTCCTCACGGTACTT	57.9	“
<b><u>D2 region</u></b>				
300F	Forward	CAAGTACCGTGAGGGAAAGTT	57.9	Olsen (Pers. Comm.)
ECD2	Reverse	CTTGGTCCGTGTTTCAAGACGGG	64.2	“
<b><u>D3 region</u></b>				
RD3A	Forward	GACCCGTCTTGAAACACGA	56.7	McInerney et al., 1999
RD3C	Forward	CCCGAAAGATGGTGAAGTAT	55.3	McCormack et al., 2002
900F	Forward	CCGTCTTGAAACACGGACCAA	54	
1500R	Reverse	GCTATCCTGAGGGAAACTTCG	54	
RD3R	Reverse	AGTCTTTCGCCCTATACCCA	59.8	McCormack et al., 2002
RD5B2	Reverse	ACACACTCCTTAGCGGA	52.8	McInerney et al., 1999
<b>mtCO1</b>				
<b><u>CO 1</u></b>				
coxF3	Forward	GGTCAACAAATCATAAAGATATTGG	56.4	Folmer et al., 1994
coxR3	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	58.5	“

*Analysis of sequences.* Sequences were assembled into contigs using SeqMan II software from the Lasergene package (DNASTAR Inc.). A multiple alignment was assembled in MaClade 4.08 OS X of concatenated D1 and D3-D5 regions of the 28S gene for the 20 consensus sequences generated here, as well as 37 sequences from Redmond et al. (2011). Overall, the alignment contained 53 sequences from marine haplosclerids, 1 freshwater haplosclerid, and 3 other outgroup species from the G4 clade. The hyper variable D2 region was not included in this alignment due to the wide range of taxa involved. The final alignment was 929bp in length (after 198 bases were removed that had caused ambiguities in the alignment). A second D1-D5 alignment containing only the *H. indistincta* (specimens: A-G, Table 1), *H. viscosa* and *Niphates* sp. (used as an outgroup) sequences, was assembled from the concatenated D1, D2, and D3-D5 regions of the 28S gene. The total length of the alignment was 1,421 bases (after 12 bases causing ambiguous areas were removed). The mtCO1 alignment contained all of the sequences from Dataset S5 Redmond et al. (2011) with the addition of 18 sequences in total from four individuals (3 *H. indistincta* and 1 *H. viscosa*) generated as part of this study. The alignment was 577bp in length. Three freshwater sequences were used as out-group. In total, the alignment consisted of 53 sequences from marine haplosclerids and 3 from freshwater sponges. Before inclusion into alignments, all fasta files were entered into a BLAST algorithm search to check for any contamination.

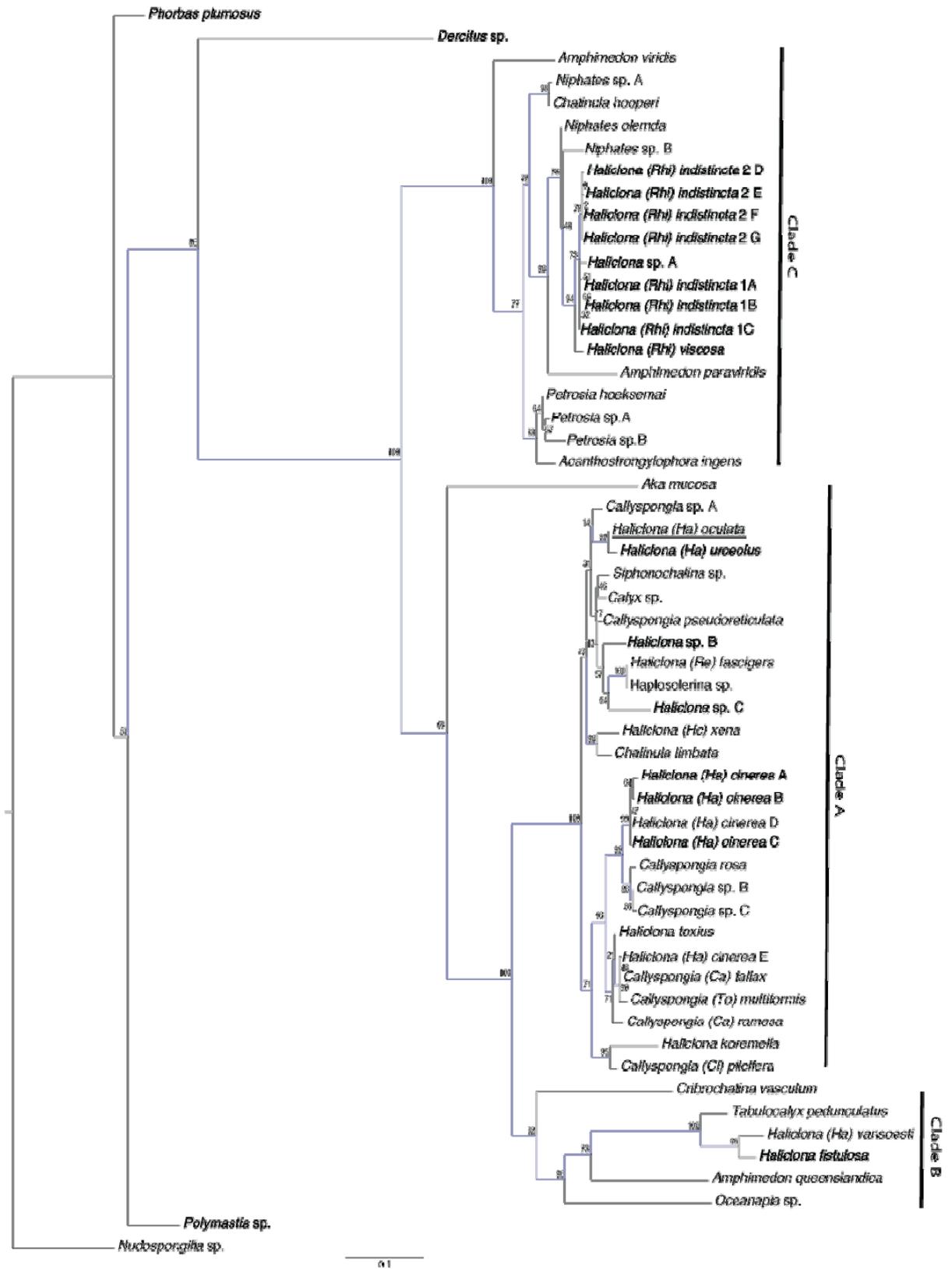
*Phylogenetic analyses;* Phylogenetic analysis was undertaken using RAxML (Randomized Axelerated Maximum Likelihood) 7.0.3 (Stamatakis et al., 2008), using the GTR model of DNA substitution with model parameters optimized in RAxML. The confidence levels were estimated using, bootstrap resampling (1,000 replicates). Trees were visualised in FigTree (v1.3.1).

## Results:

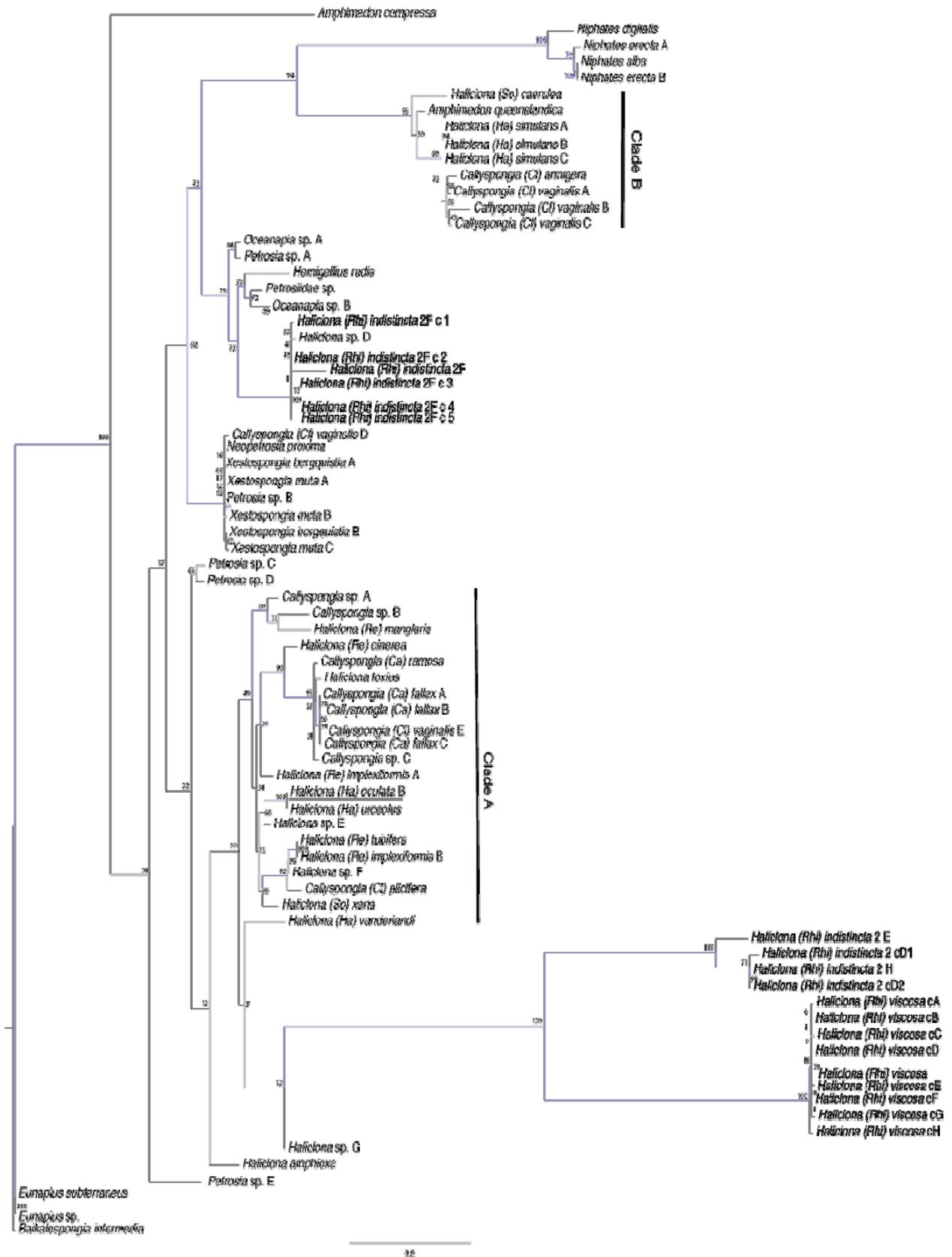
*The phylogenetic position of H. indistincta*; The D1-D5 (excluding the D2 region) 28S rRNA gene tree shows *H. indistincta* placed within the marine haplosclerids, however, the gene tree shows that it is only distantly related to the *Haliclona* type species *H. oculata* (Figure 2). All seven sequences from *H. indistincta* specimens clustered together (73 BP (BP = bootstrap proportions)) with *H. viscosa* (94 BP) in Clade C, (clade names are consistent with Redmond et al. 2011). No sponge data from *H. rosea* could be obtained from the voucher specimen (Table 1). The clade containing *H. indistincta* and *H. viscosa* was most closely related to *Niphates* sp. B and *Niphates olemda*, and together formed a highly supported clade (78 BP) within clade C (Figure 2). Also within clade C were two *Amphimedon* species, *Chalinula hooperi*, *Acanthostrongylophora ingens*, three *Petrosia* species and three *Niphates* species. *Haliclona* sp. A was found to group amongst the *H. indistincta* specimens in clade C. Clade C was a sister group to Clade A, which contained the highest numbers of *Haliclona* (including the type species, *H. oculata*) and all of the *Callyspongia* taxa (Figure 2).

Consensus sequences for *H. indistincta* specimens E, H and the two sequences from PCR clones from specimen D, grouped with 100BP as did all eight clone sequences for *H. viscosa* in the mtCO1 gene tree (Figure 3). These two groups clustered together with 100BP. There was a long branch leading to the *H. indistincta* and *H. viscosa* clades. Some inconsistencies were evident. The two clones from *H. indistincta* D did not group with each other, instead clone 2 was identical to the consensus sequence from *H. indistincta* H. Also, sequences from *H. indistincta* F did not group with other *H. indistincta* sequences but instead clustered with a clade containing *Hemigellius rudis*, *Oceanapia* sp. A, and *Petrosiidae* sp.. The five *H. indistincta* F clones and the

consensus sequence formed a small clade with 100 BP but also contained a consensus sequence from *Haliclona* sp. D.



**Figure 2.** Maximum-likelihood phylogeny reconstructed from the D1-D5 (excluding the D2) region of the 28S rRNA gene. Sequences generated in this study are in bold and the *Haliclona* type species *H. oculata* is underlined. The DNA substitution model parameters estimated by RAxML were: f(A) 0.24, f(C) 0.24, f(G) 0.33, f(T) 0.19; R(AC) 0.62, R(AG) 1.54, R(AAT) 0.76, R(CG) 0.68, R(CT) 5.70, R(GT) 1.00; alpha 0.26. Ca=Callyspongia; Cl=Cladochalina; Ha=Haliclona; Re= Reniera; Rhi=Rhizoniera; To=Toxochalina.



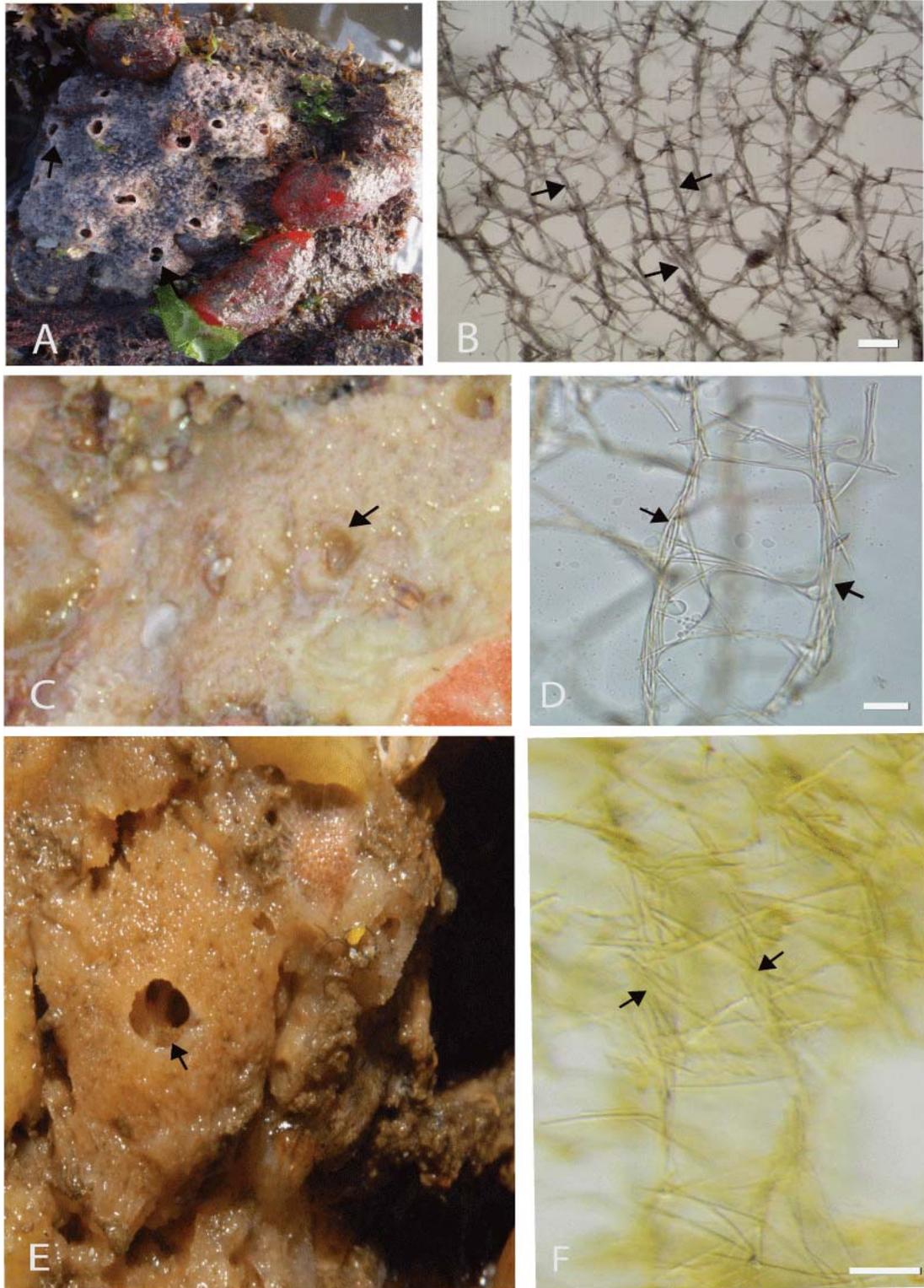
**Figure 3.** Maximum-likelihood phylogeny reconstructed from the Folmer (5') region of the mtCO1 gene. Sequences generated in this study are in bold and the *Haliclona* type species *H. oculata* is underlined. The DNA substitution model parameters estimated by RAxML were: f(A) 0.24, f(C) 0.16, f(G) 0.22, f(T) 0.38; R(AC) 1.59, R(AG) 3.29, R(AAT) 0.87, R(CG) 0.67, R(CT) 4.82, R(GT) 1.00; alpha 0.29. Ca=Callyspongia; Cl=Cladochalina; Ha=Haliclona; Re= Reniera; Rhi=Rhizoniera; So=Soestella; To=Toxochalina; c=Clone colony sequence.

All attempts to sequence the mtCO1 gene from *H. indistincta* Type 1 (cushion form) failed. Consensus sequences, generated from five specimens (MIIG0155, MIIG0156, MIIG0165, MIIG0236, MIIG0303) were of too low quality to be used and showed evidence of mixed templates. Twenty-six clone sequences were generated for the Type 1 *H. indistincta* specimen (MIIG0303, Table 1); however, all BLAST matches were from non-sponge organisms, mostly algae and bacteria but also other animals (i.e. molluscs and arthropods). For Type 2 (flat form): 10 clones were sequenced from MIIG0186, and 3 from MIIG0198; 6 of which were from contaminants (Table 3). For the *H. indistincta* specimens that grouped together in the mtCO1 tree, D, E and H, all sequences were matched via BLAST as being most similar to either haplosclerid or hadromerid taxa (Table 3). The top BLAST hit for the sequence of *H. indistincta* specimen E was *Haliclona* sp., while both clone and consensus sequences generated from *H. indistincta* specimen F (which grouped away from the rest of the *H. indistincta* cluster), received BLAST closest match with *H. aqueductus* (Table 3). Nine sequences were generated from *H. viscosa*, eight of which were clone sequences. The consensus and clone sequences for *H. viscosa* (which grouped with the *H. indistincta* cluster) received highest BLAST matches with hadromerid taxa (Table 3).

**Table 3.** Basic Local Alignment Search Tool (BLAST) results for *H. indistincta* Type 2 (flat form) and *H. viscosa* clone (in bold) and consensus CO1 sequences. Small “c” refers to it being it a clone sequence (as labelled in mtCO1 tree). HAP= order Haplosclerida, HAD= order Hadromerida.

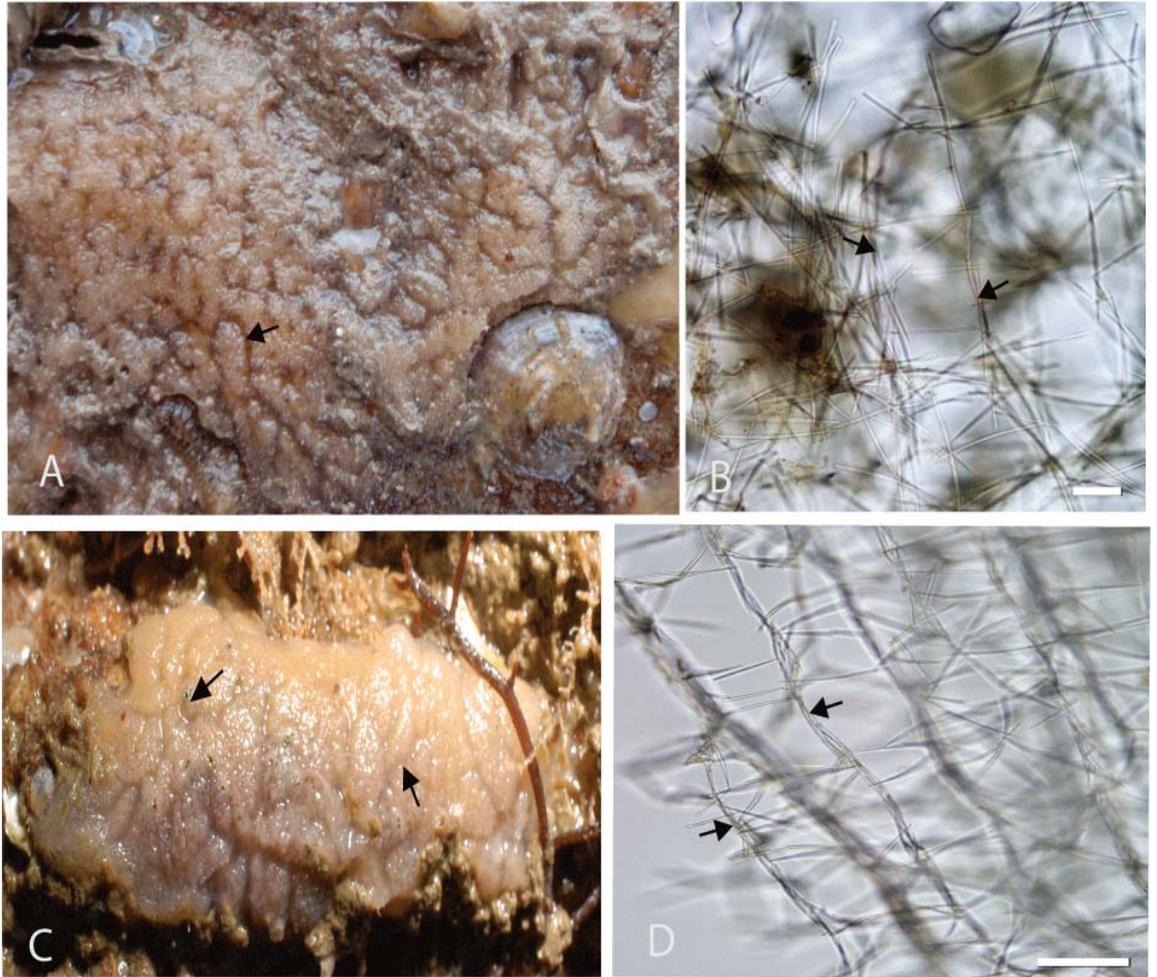
Species name	Voucher ID	Number of sequences	BLAST result	Accession number	Max score	Query coverage	E value	Max identity	Order
<i>H. indistincta</i> 2D	MIIG0198	1	<i>Lentinula edodes</i> (mushroom)	AB306944.1	84.2	18%	5e-13	95%	
<i>H. indistincta</i> 2D c1		1	<i>Trochospongilla pennsylvanica</i>	DO087503.1	353	74%	1e-93	77%	HAP
<i>H. indistincta</i> 2D c2		1	<i>Pione velans</i>	AY561981.1	444	85%	6e-121	78%	HAD
<i>H. indistincta</i> 2E	MIIG0185	1	<i>Haliclona</i> sp.	JN242205.1	313	84%	1e-81	81%	HAP
<i>H. indistincta</i> 2F	MIIG0186	1	<i>Haliclona aqueductus</i>	EF095186.1	510	100%	2e-141	96%	HAP
<i>H. indistincta</i> 2F	MIIG0186	5	<i>Metridium senile</i> (anemone)	AF000023.1	394	90%	4e-106	79%	
			<i>Metridium senile</i> (anemone)	AF000023.1	596	89%	1e-106	79%	
			<i>Metridium senile</i> (anemone)	AF000023.1	390	89%	6e-105	79%	
			<i>Metridium senile</i> (anemone)	AF000023.1	355	88%	2e-94	79%	
			<i>Sepietta oweniana</i> (squid)	AF036913.1	281	78%	4e-72	76%	
<i>H. indistincta</i> 2F c1-5	MIIG0186	5	<i>Haliclona aqueductus</i>	EF095186.1	806	98%	0.0	99%	HAP
			<i>Haliclona aqueductus</i>	EF095186.1	634	100%	1e-178	99%	
			<i>Haliclona aqueductus</i>	EF095186.1	787	99%	0.0	99%	
			<i>Haliclona aqueductus</i>	EF095186.1	625	100%	8e-176	99%	
			<i>Haliclona aqueductus</i>	EF095186.1	652	100%	0.0	99%	
<i>H. indistincta</i> 2H	MIIG0201	1	<i>Adreus fascicularis</i>	HQ379428.1	383	92%	1e-102	78%	HAD
<i>H. viscosa</i>	CW51	1	<i>Rhizaxinella</i> sp.	AY561983.1	320	92%	8e-84	76%	HAD
<i>H. viscosa</i> cA-H	CW51	8	<i>Pione velans</i>	AY561981.1	300	88%	1e-77	76%	HAD
			<i>Pione velans</i>	AY561981.1	289	89%	2e-74	76%	
			<i>Pione velans</i>	AY561981.1	287	89%	8e-74	76%	
			<i>Pione velans</i>	AY561981.1	278	89%	5e-71	75%	
			<i>Pione velans</i>	AY561981.1	261	88%	5e-66	75%	
			<i>Pione velans</i>	AY561981.1	263	88%	1e-66	75%	
			<i>Pione velans</i>	AY561981.1	268	88%	3e-68	76%	
			<i>Pione velans</i>	AY561981.1	278	89%	5e-71	75%	

*Skeletal structure of Haliclona indistincta*; The skeletal preparation method suggested by Dr. Ereskovsky (see Methods) produced a clear view of the reticulation of *H. indistincta*'s skeleton (Figure 4B). The choanosomal skeleton was an anisotropic reticulation with distinct pauci-multispicular primary tracts comprised of 2 to 9 spicules, and pauci-spicular secondary lines that were composed of 1 to 4 overlapping spicules. However, a third pauci-multispicular tract creating a triangular reticulation (Figure 4B) was also observed. These lines lay across the primary tracts both straight across, as well as at an angle. The secondary tracts were either one or two spicules in length. The tracts were parallel to each other from the surface down to the base of the specimen, and slightly curved in tandem. There were several gaps where the choanosomal cavities would occupy.



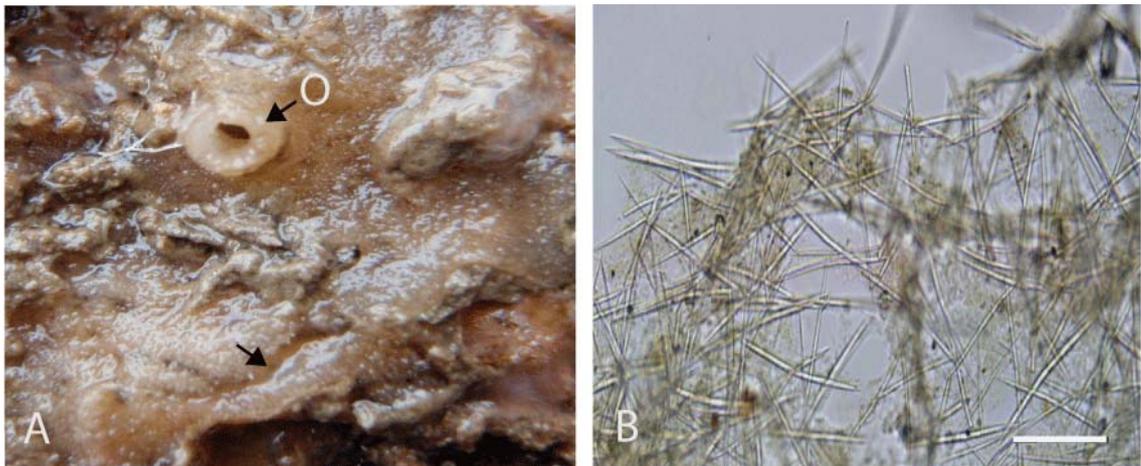
**Figure 4.** (A) *H. indistincta* Morphotype 1 *in situ* (specimen ID: MIIG0435). Arrows indicate large oscules. Purple coloration observed presumed to be due to cyanobacteria. Uneven surface typical of this morphotype apparent. (B) Accompanying perpendicular skeletal section of *H. indistincta* Morphotype 1 *in situ*. Arrows indicating multispicular primary lines and triangular reticulation. (Skeletal sample prepared using Method 2). (C) (Specimen A) *H. indistincta* Morphotype 1 *in situ* (arrows). (D) Accompanying perpendicular skeletal section of *H. indistincta* Morphotype 1 *in situ*. Arrows indicating multispicular primary lines. (E) (Specimen C) *H. indistincta* Morphotype 1 *in situ*. Arrows indicate membrane covering excurrent canal. (F) Accompanying perpendicular skeletal section of *H. indistincta* Morphotype 1 *in situ*. Arrows indicate multispicular primary lines. **Scale bars**=100 $\mu$ m.

*Morphological variation within H. indistincta*; At least two morphotypes were identified amongst *H. indistincta* specimens. Type 1 (Figure 4; Appendix 1 A-B) typically grew in a cushion-like form and had excurrent channels covered by a thin dermal membrane, that radiated from the oscules in tracks. The surface morphology tended to be uneven or bumpy. The colour could be influenced by algae living in the sponge body, which sometimes gave it a purple coloration (as in Figure 4 A). Typically, it tended to be a light to dark tan (Figure 4 C-E). The surface morphologies of *H. indistincta* Type 1 specimens B and C however, (Figure 4 E, Appendix 1A respectively) looked quite different from the other two *H. indistincta* specimens included in Figure 4. *H. indistincta* specimen B (Appendix 1 A-B) had very small and circular oscules and a smooth surface and *H. indistincta* specimen C (Figure 4 E-F) also had a smooth surface, and excurrent canals were not as prevalent as what was normally observed. The surface of Type 2 (Figure 5) had very pronounced excurrent canals and tended to grow as very thin, encrusting forms. Its colour range was usually light cream to dark tan. The two Types (1 and 2) share the same skeletal structure and sticky mucus consistency typical for this species.



**Figure 5.** (A) (Specimen E) *H. indistincta* Morphotype 2 *in situ*. Arrows indicate excurrent canals typically observed in Morphotype 2. (B) Accompanying perpendicular skeletal section of *Haliclona indistincta* Morphotype 2 *in situ*. Arrows indicate multispicular primary lines. (C) (Specimen H) *Haliclona indistincta* Morphotype 2 *in situ*. Arrows indicate pockets or islands typical of Morphotype 2. (D) Accompanying perpendicular skeletal section of *H. indistincta* Morphotype 2 *in situ*. Arrows indicate multispicular primary lines. **Scale bars**=100 $\mu$ m.

The morphology of the Type 2 specimens also varied, especially *H. indistincta* F (Figure 6) which had an unusually pronounced oscule and the skeletal structure was unlike the rest of the specimens of both groups (1 and 2). The spicules were thicker and the skeleton was less regular in the reticulation of the overall structure, with more spicules in confusion instead of the well defined ladder-like reticulation with pronounced multispicular primary tracts (Figure 6).

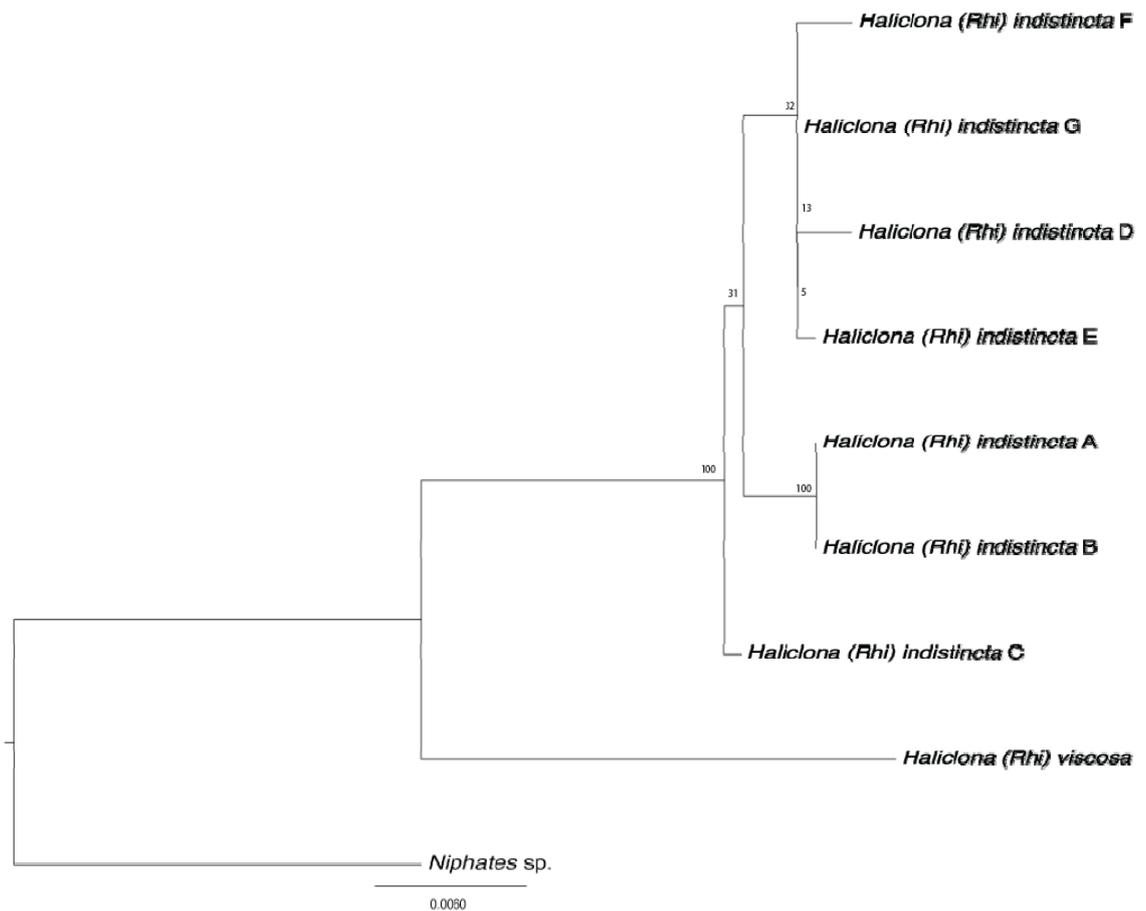


**Figure 6.** (A) (Specimen F) *H. indistincta* Morphotype 2 *in situ*. Arrow indicates slight channel. O=oscule (B) Accompanying perpendicular skeletal section of *H. indistincta* Morphotype 2 *in situ*. Scale bar=100µm.

Though the sequences from *H. indistincta* F clustered together with the rest of Type 2 sequences in the 28S D1-D5 (excluding D2 region) tree (Figure 2), sequences from this specimen grouped away from the other *H. indistincta* Type 2 specimens in the CO1 gene tree (Figure 3).

*Molecular variation within H. indistincta;* Within the 28S Maximum Likelihood (ML) tree (Figure 2), *H. indistincta* specimens formed a single supported clade. A smaller sub-clade of all four *H. indistincta* Morphotype 2 specimens clustered together with high BP value. Two of the three *H. indistincta* Morphotype 1 specimens (A and B) grouped with *Haliclona* sp. A1, while the third *H. indistincta* morphotype 1 specimen (specimen C) grouped on its own (Figure 2) as a sister taxon to all *H. indistincta* specimens. To look for further sequence

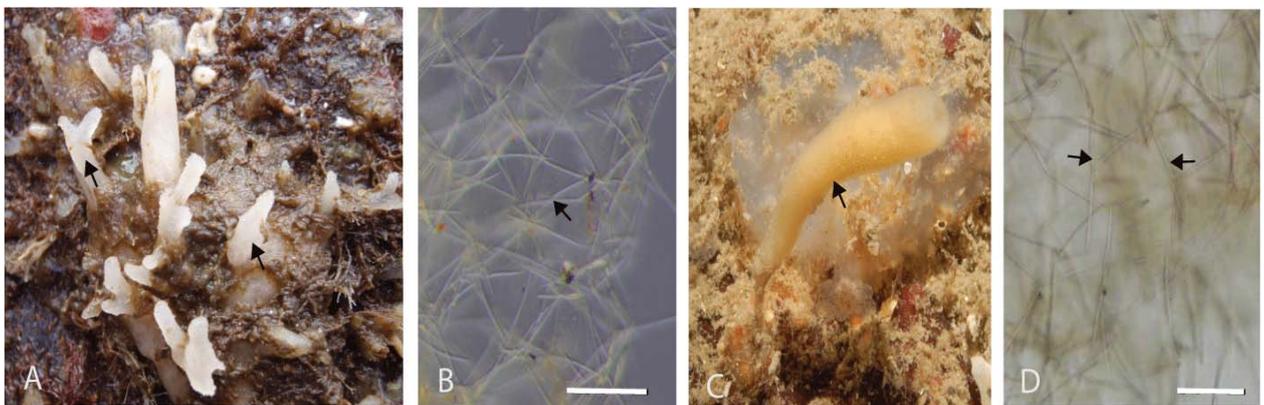
variation between the two morphotypes as well as differences among individuals, the hyper variable D2 region was included in a small alignment that contained only *H. indistincta* specimens, *H. viscosa* and one *Niphates* sp. (acting as out-group). In this ML tree (Figure 7), all *H. indistincta* specimens formed a clade (100 BP) with specimen C as sister group to the rest of the specimens. Type 2 specimens were found to still group together, however this grouping was no longer supported. *H. indistincta* specimens A and B were also found to group together, *Haliclona* sp. A1 was not included in this ML tree as there was no D2 data available.



**Figure 7.** Maximum-likelihood phylogeny reconstructed from the D1-D5 (including the D2) region of the 28S rRNA gene. Sequences generated in this study are in bold. The DNA substitution model parameters estimated by RAxML were: f(A) 0.21, f(C) 0.25, f(G) 0.35, f(T) 0.18; R(AC) 0.71, R(AG) 2.79, R(AAT) 1.17, R(CG) 0.65, R(CT) 4.09, R(GT) 1.00; alpha 0.02. Rhi=Rhizoniera.

*Variation in the genus Haliclona*; Seven additional *Haliclona* specimens had been collected as part of this study. Three specimens putatively identified as *H. cinerea* were confirmed as such due to their 28S rRNA gene sequences clustering with high support of 99 BP, with vouchers of the species.

Also employing 28S rDNA data, the putative *H. fistulosa* specimen clustered in Clade B on the same branch as *H. vansoesti* with (99 BP). The morphology of the putative *H. fistulosa* was consistent with the description of this species described in de Weerd (1986) (i.e. white colouration and fistules protruding from the surface) (Figure 8 A). The skeletal structure of the putative *H. fistulosa* (Figure 8 B) was also consistent with *H. fistulosa* (as described by de Weerd (1986)) as a dense, subisotropic reticulation one or two spicules thick with no distinction between primary and secondary lines, with numerous spaces (not shown in figure provided) (Figure 8 B).



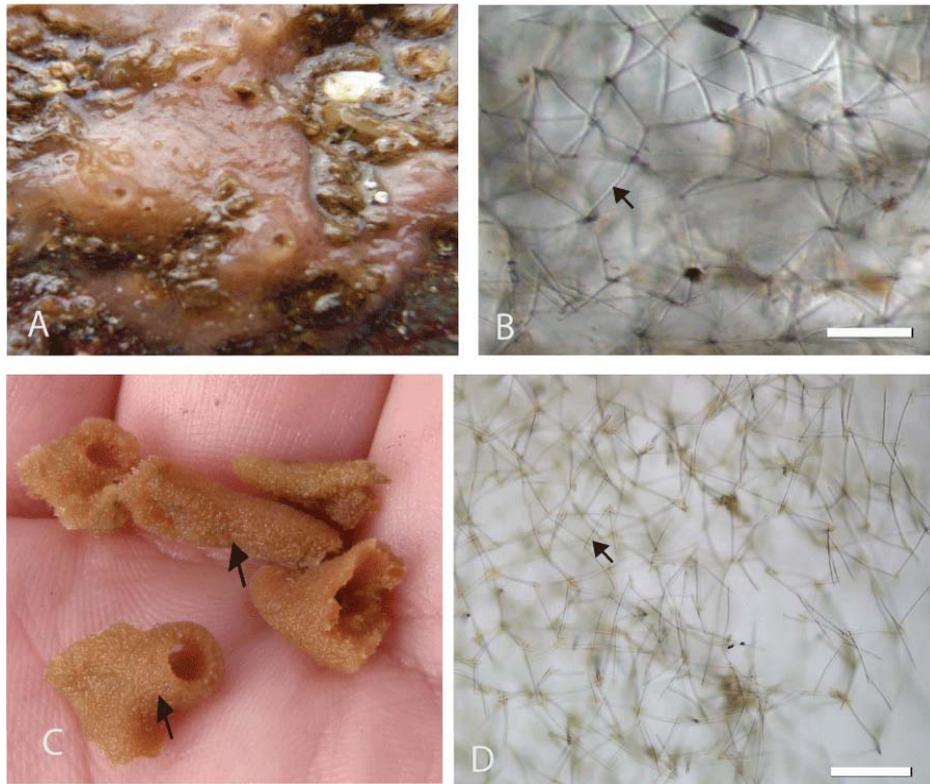
**Figure 8.** (A) *H. fistulosa* in situ. (B) Accompanying perpendicular skeletal section. (C) *H. urceolus* in situ. (D) Accompanying perpendicular skeletal section. Scale bars=100 $\mu$ m.

Also, the architecture of the putative *H. fistulosa* was very similar to *H. vansoesti* and consistent with the results from the molecular data (Figure 2). De Weerd et al., (1999) included *H. vansoesti* in her *H. fistulosa* group as they both share a subisotropic skeleton. De Weerd (1999) describes further similarities between the two species (i.e. bi-coloration

pattern of the body, and spicule lengths), however, the morphology of *H. vansoesti* is different in a number of ways, such as the lack of fistules in *H. vansoesti* as well as a less fragile consistency, denser skeletal structure and thicker spicules.

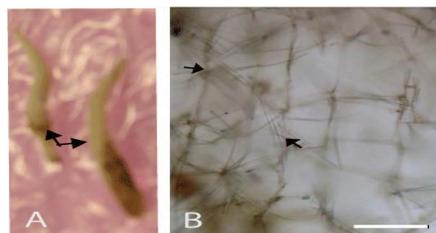
The putative *H. urceolus* grouped with 99 BP with *H. oculata* in Clade A, as might be expected (Figure 2). The putative *H. urceolus* also possessed morphology (i.e. growth form and skeletal structure) (Figure 8 C-D) similar to that of de Weerd's (1986) description of *H. urceolus*, being stalked and shaped like a hollow cylindrical tube, brown colouration with a regularly reticulated skeleton comprised of unispicular primary and secondary lines.

*Haliclona* sp. B1, and *Haliclona* sp. C1, grouped together without support (<70 BP) with *Haliclona fascigera* and Haplosclerina sp. but no species sequenced to date clustered directly with either of these two species/sequences. While the skeletal structure of *Haliclona* spp. B and C (i.e. isotropic reticulated skeleton with few spicules randomly placed) was similar to that described for the "*oculata*" group (described by de Weerd (1986)) which comprised *H. cinerea*, *H. oculata*, *H. urceolus*, and *H. simulans*, the external characteristics of these two undescribed taxa were quite different. *Haliclona* sp. B (Figure 9A) was similar to *H. cinerea* in growth form, but had very small oscules and when broken apart did not have any slime strands (de Weerd, 1986). *Haliclona* sp. C (Figure 9C) was similar to *H. simulans* with large oscules raised on large mounds, however, the consistency was very different being very soft and hispid. *Haliclona* sp. C was also not slimy or sticky in texture.



**Figure 9.** (A) *Haliclona* sp. B *in situ*. (B) Accompanying perpendicular skeletal section. (C) Haplosclerid sp. C. (D) Accompanying perpendicular skeletal section. **Scale bars**=100 $\mu$ m

*Haliclona* sp. A (Figure 10) (that grouped with the *H. indistincta* sequences in the ribosomal tree) is an encrusting sponge with large fistular protrusions is consistent with de Weerd's (1986) description of the external characteristics of *H. fibulata*; however, the skeleton was not. *Haliclona* sp. A had an anisotropic reticulated skeleton with multispicular primary lines (composed of 2-7 spicules) and single secondary tract, 1-2 spicules in width with numerous spicules randomly positioned and there were no sigmata present (Figure 10 B). *H. fibulata* on the other hand, has sigmata and a more irregularly reticulated skeleton (de Weerd, 1986).



**Figure 10.** (A) *Haliclona* sp. A *in situ*. (B) Accompanying perpendicular skeletal section. Arrow indicates multispicular primary tracts. **Scale bar**=100 $\mu$ m.

## Discussion:

Trees drawn from the 28S gene D1-D5 region (D2 not included) and the CO1 were congruent in that they both placed *H. indistincta* firmly within the marine haplosclerids, though only distantly related to the type species *H. oculata*. The hypothesized, “*rosea* group” suggested by de Weerd (1986), based upon their shared skeletal structure, was also supported, although one member of the group was missing from this analysis (i.e. *H. rosea*). All attempts to amplify DNA from available voucher specimen of *H. rosea* failed and only non-target DNA was amplified. Numerous attempts were made to secure fresh *H. rosea* specimens, including sampling trips, as well as personally contacting potential sources for obtaining the species, however, no material was obtained. The closest relatives of the two included members of this group (*H. indistincta* and *H. viscosa*), via the ribosomal data, were *Niphates* species, *N. olemda* and *Niphates* sp. B. The Niphatidae was suggested by de Weerd (1989), to be the “sister-group” to the Chalinidae. *N. olemda*, and other Niphatidae, share with *H. indistincta* and *H. viscosa* a ladder-like reticulated skeleton with multispicular fibers or tracts (Van Soest, 1980). These results show some congruence between molecular and morphological data, and suggests that skeletal structure can be phylogenetically informative, even for the marine haplosclerids. The congruence between skeletal structure and positioning in the phylogenetic tree observed in the ribosomal data also supports the observation by Redmond et al. (2011) concerning a general trend in skeletal structure of spongin becoming less abundant and spicules become more abundant (i.e. from unispicular reticulated skeletons to multispicular tracts) from the Chalinidae to Niphatidae and Petrosiidae. Redmond et al. (2011) suggested that this trend is consistent with molecular data, i.e. as in Clade C, which is dominated by Niphatidae and Petrosiidae (that had skeletal structures with multispicular tracts), which was a sister clade to another clade comprising primarily of Chalinidae (Clade A) (that had more unispicular reticulated skeletons and more spongin present).

Van Soest (1980) established the family Niphatidae, and suggested it include haplosclerids that had a reticulated ectosomal skeleton with paratangential multispicular fibers or tracts, a reticulated choanosomal skeleton comprised of multispicular fibers or tracts, and (when present) microscleres that included only sigmata or microxeas. Van Soest (1980) suggested that chalinid taxa that possessed multispicular fibers or tracts should also be included in this family. For the monophyletic *Haliclona* groups described by de Weerd (1986), the only group whose ground-plan was described as having a reticulated skeleton with paucispicular tracts was the *H. rosea* group. Thus it may not be surprising that *H. indistincta* grouped closely with *Niphates* species, and Van Soest's (1980) suggestion of bringing chalinid species with multispicular tracts into the family Niphatidae may be appropriate, at least for members of the "rosea group" (i.e. *H. indistincta* and *H. viscosa*), although many *Haliclona* species do not have an ectosomal skeleton.

De Weerd (1986) did mention exceptions to the general choanosomal skeletal structure of the "oculata group" where multispicular tracts were occasionally present (de Weerd, 1986). However, it was always in reference to tracts reinforcing an existing skeletal structure in stalked species (i.e. *H. urceolus* and *H. oculata*) (de Weerd, 1986). For *H. urceolus* it was observed that small specimens had uni- to paucispicular primary lines in the skeleton, while a larger specimen had additional reinforcing multispicular tracts (de Weerd, 1986). For *H. oculata*, an individual may have unispicular lines at the periphery and at the top of the body branches, but become multispicular towards the base (de Weerd, 1986). It is possible that the appearance of multispicular tracts observed in other *Haliclona* species (i.e. *H. oculata* and *H. urceolus*) may be why de Weerd (1986) considered the "rosea group" to belong in the genus *Haliclona*.

Of the nine valid genera in the family Niphatidae (i.e. *Amphimedon*, *Cribochalina*, *Dasychalina*, *Gelliodes*, *Haliclonissa*, *Hemigellius*, *Microxina*, *Niphates* and *Pachychalina*) (Desqueyroux-Faúndez and Valentine, 2002), members of the *H. rosea* group were most similar in overall morphology to members of: *Amphimedon*, *Dasychalina*, *Gelliodes* and *Niphates*. Morphological characters are highly variable among the Niphatidae genera (i.e. body forms (e.g. encrusting, massive, tubular), surface texture/features (e.g. smooth, bumpy, spiny), ectosome (present or absent), choanosome (multispicular tracts present or absent), spongin (abundant vs. scarce), and microscleres (present or absent)) (Desqueyroux-Faúndez and Valentine, 2002), and further revision of this group is to be recommended with sequencing more members of this family.

*Haliclona* sp. A grouped amongst *H. indistincta* specimens (Figure 2) on the 28S rRNA gene tree and also had a ladder-like reticulated skeleton with pauci-multispicular tracts. It was unexpected that *Haliclona* sp. A should group with the *H. indistincta* sequences because the external morphology is not at first glance consistent with that of *H. indistincta*. Contamination is a common reason for specimens to cluster in unexpected places, however, it is not a likely the reason for *Haliclona* sp. A to group with the *H. indistincta* cluster, because the *Haliclona* sp. A PCR amplicons were not generated at the same time as the *H. indistincta* amplicons. The other alternative is that *Haliclona* sp. A, is indeed *H. indistincta*. Although the external fistular morphology is similar to *H. fibulata* (de Weerd, 1986), the spicules of the two taxa were very different; the, spicule size in *Haliclona* sp. A being much smaller than in *H. fibulata* and there were no stigmata present. The skeletal structure of *Haliclona* sp. A was similar to *H. indistincta*, however, because this specimen was provided by a second party, an *in situ* image of the entire external morphology of the specimen or a large taxonomic voucher specimen is not currently available. Therefore, until additional

specimens are collected, it is not possible to determine confidently whether the fistules are an additional morphological feature of *H. indistincta* or if contamination took place.

*Haliclona* spp. B and C were both positioned in the same large haplosclerid clade as the type species of the same genus (in Clade A) of the 28S rRNA gene tree. Sequences from these two undescribed species were not identical to each other, or to any of the North-eastern Atlantic *Haliclona* species (described by de Weerdt (1986)) that were present in the 28S rRNA gene tree (i.e. *H. indistincta*, *H. viscosa*, *H. oculata*, *H. urceolus*, *H. xena*, *H. cinerea*, and *H. fistulosa*). Also, of these specimens, the external morphology, skeletal structure, spicule types were not consistent with the remaining species de Weerdt (1986) described (i.e. *H. simulans*, *H. primitiva*, *H. rosea*, *H. anguata*, *H. fibulata*, *H. rava*) which were not represented in the gene tree. Morphological comparisons with other *Haliclona* species, as well as sequences of more *Haliclona* species, is needed to determine the true taxonomic status of these two *Haliclona* species.

The CO1 gene tree showed three *H. indistincta* specimens (D, E and H) forming one group while specimen F grouped away from the others forming a clade with *Haliclona* sp. D with high support (100 BP). The reason for *H. indistincta* F sequences grouping with the *Haliclona* sp. D sequence is not likely due to contamination as the *Haliclona* sp. D sequence was not obtained through this study. Furthermore, the *Haliclona* sp. D sequence is not identical with the *H. indistincta* F sequences. The specimen shown in Figure 6 A was assumed to be a single individual. While the external morphology including the appearance of the excurrent canal, at the lower end of the image, of *H. indistincta* F was typical of *H. indistincta* Type 2, (arrow), the raised oscule, toward the top of the image, was not (Figure 6 A). Given the encrusting nature of the specimen and the surface it was sampled from, we cannot exclude the possibility that the oscule and surrounding tissue, was part of a separate

species that was growing directly beside *H. indistincta* F, which means that tissue from two different specimens may have been placed in the same collection tube. It is interesting that the CO1 and the 28S data show different phylogenetic relationships for this specimen (*H. indistincta* F), and further investigation of this specimen is required, and may also help explain other inconsistencies in the sponge literature where sequences did not group where it may have been expected.

In the CO1 tree, the group of *H. indistincta* specimens D, E and H clustered on a long branch with *H. viscosa*. In general, a long branch may indicate limited sampling of that lineage and/or a high mutation rate among the taxa (Redmond et al., 2011). Interestingly, neither group of *H. indistincta* specimens clustered with *Niphates* spp. as was seen for 28S rRNA gene data. However, this may not be surprising considering the *Niphates* species included in both trees are different; the CO1 gene tree contained *N. digitalis*, *N. erecta*, and *N. alba*, which all originated from the Caribbean Sea; the 28S gene tree contained *Niphates* spp. and *N. olemda* collected from Indonesia and Micronesia. Furthermore, it has been shown that *Niphates* and the Niphatidae, are polyphyletic (Redmond et al., 2011; Erpenbeck et al., 2007), so it is not inconceivable that with additional sequencing of *Niphates* species, CO1 data would support ribosomal data.

The morphological plasticity of *Haliclona* is exemplified by *H. indistincta* through the intraspecific variation observed (e.g. Morphotypes 1 and 2). The possibility of the two *H. indistincta* morphotypes being two separate species was also considered here, however, it was concluded that this is not likely to be the case because the number of base pair differences between the two Morphotypes (1 and 2) were so few. Moreover, it was shown in the 28S rRNA gene trees, that Morphotype 2 is a sub-group within Morphotype 1, rather than a sister clade. The morphology of Morphotype 1 was highly variable. This was

demonstrated by *H. indistincta* specimens B and C (Figure 4 E and Appendix 1 A respectively) which looked very different from the other three *H. indistincta* specimens included in Figure 4. Interestingly, even though *H. indistincta* B was also quite different from *H. indistincta* A in appearance, it grouped closely with *H. indistincta* A, while *H. indistincta* C fell outside of the group. It is possible that Morphotype 1 correlates to the “massive” forms described by Bowerbank (1866) and Morphotype 2 is what was described by Topsent (1888) and de Weerd (1986) to be the “encrusting” form. Although de Weerd’s (1986) report of a “shaggy” looking appearance would be more descriptive of Morphotype 1, the obvious excurrent canals that de Weerd (1986) mentions is more indicative of Morphotype 2 (Figures 5 and Appendix 1 C-F).

It is possible that the two morphotypes are specially suited for specific environments as many of the morphological differences observed within a species are considered to have adaptive significance to specific environmental regimes (Konnecker, 1972; Bell and Barnes, 2000a; 2000b; 2000c). For example, Bell and Barnes (2000b) has shown sponge morphological diversity to be higher in low energy environments (e.g. areas with low water movement and high sedimentation). Konnecker (1972) showed a strong correlation between areas with high levels of sediment and sponge populations that produce heavy mucous (e.g. *Myxilla rosacea*, and *Stelligera stuposa*), which is believed to aid in clearing the surface of the sponge from fouling material. Corranroo, one of the locations used for collecting this species, is highly biodiverse, and has high amounts of sediment as well as fast moving water. New Quay and Lough Hyne sampling areas have lower levels of sediment, but also have strong water currents. Morphotype 1 is commonly found in Corranroo sampling site, Morphotype 2 is also present, but is not common. The more mucous body of Morphotype 1 may mean that it is more suited to the heavily sedimented area of Corranroo than Morphotype 2.

Because large size has been shown to be a competitive advantage in sponges (Bell and Barnes, 2003), it is possible that the larger cushion-form may give Morphotype 1 an advantage over Morphotype 2 in Corranroo, as it allows it to compete with the high abundance and diversity of sponges and other fauna that resides there. Morphotype 1 was also present in New Quay, but was uncommon. When found in New Quay Morphotype 1 was small compared to the specimens in Corranroo. However, because New Quay is located approximately 5km down the shoreline from the sampling site in Corranroo, the presence of Morphotype 1 also in New Quay was not surprising as sponge larvae are distributed by water currents. Also, sampling bias can not be ruled out as a possibility for observing uneven numbers of either Morphotype across the sampling sites. As the primary aims of this study were not to establish population boundaries, the numbers of either morphotype was not carefully documented.

Through this study, I have shown that *H. indistincta* is a marine haplosclerid, although only distantly related to type species, *H. oculata*. Congruence was found between morphological and molecular data. The *H. rosea* group was shown to be monophyletic, and a general consistency between positions of taxa on the 28S gene tree and skeletal structure was observed, which suggests that future molecular-based studies should incorporate patterns observed in morphological data (i.e. skeletal structure) to guide further phylogenetic reconstructions. Additional approaches such as utilizing cytology to investigate sponge reproductive and developmental systems may also shed additional light on phylogenetic relationships.

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Chapter 3:

**Reproductive cycle and larval characteristics of the sponge *Haliclona indistincta*  
(Phylum Porifera, Class Demospongiae)**

This work has been accepted for publication:

\***Stephens K.M.M.**, Galvin J., Lawless A., McCormack G.P. (in press). Reproductive cycle and larval characteristics of the sponge *Haliclona indistincta* (Porifera: Demospongiae). *Journal of the Marine Biological Association of the United Kingdom*.

This work also contributed to the manuscript below that has been accepted with major revisions to *Aquatic Biology*:

Moniz M.B.J., Rindi F., **Stephens K.**, Maggi E., Collins P. and McCormack P. (accepted) Composition and temporal variation of the algal assemblage associated with the haplosclerid sponge *Haliclona indistincta* (Bowerbank). *Aquatic Biology*.

## **Introduction:**

Sponge reproduction has been a topic of study over several decades, (e.g. Delage, 1892; Meewis, 1936; Leveaux, 1941; Lévi, 1956; Bergquist, 1979; Reiswig, 1983; Amano, 1986; Sarà, 1993; Mariani et al., 2000; Ereskovsky, 2005; Mariani et al., 2005; 2006; de Caralt et al., 2007; Maldonado and Riesgo, 2009) but there are still reproductive processes that remain to be investigated, or are unconfirmed (Ereskovsky, 2010). Some sponge species are gonochoristic; however, most sponges are hermaphroditic, and this is also true of the marine haplosclerids (Bergquist, 1978; Ayling, 1980; Ilan and Loya, 1990a; Sarà, 1993; Maldonado and Riesgo, 2009). Viviparous and oviparous reproductive strategies are found in sponges (Bergquist, 1978). These traits were used to separate the marine Haplosclerida into two suborders; Haplosclerina (viviparous) and Petrosina (oviparous) (Lévi, 1956; Maldonado and Bergquist, 2002). Recently, molecular systematic approaches have indicated that both suborders are polyphyletic, suggesting that these traits have been acquired and lost over time in the marine haplosclerids (e.g. Borchelini et al., 2004; Raleigh et al., 2007; Redmond et al., 2007; Redmond et al., 2011). The adult skeleton in haplosclerids forms the basis of the current classification, but is particularly simple leading to many problems in taxonomy and classification in the group. Molecular data suggests that some species are misplaced in the current classification (Raleigh et al., 2007; Redmond et al., 2011) and it is also likely that species have been misidentified and assigned to the wrong family or genus. A review of reproductive patterns across the group is now highly recommended in parallel with classical morphological and molecular approaches given the new indications of phylogenetic relationships in this group.

Haplosclerid reproduction generally takes place during the warmer summer months. However, spawning often takes place during the winter months for oviparous

haplosclerids such as *Petrosia ficiformis* (Maldonado and Riesgo, 2009); and some viviparous haplosclerids such as *Xestospongia testudinaria* and *Chalinula* sp. are reported to produce sperm year round (Fromont, 1988; Ilan and Loya, 1990a). Haplosclerids have parenchymella larvae (Ereskovsky, 1999), which in *Haliclona* as well as freshwater sponges at least, commonly possess a fringe of longer flagella that encircle a strongly pigmented posterior pole that is otherwise devoid of flagellated cells. Haplosclerid larvae have been described as possessing a dense bundle of oxeads located at the posterior end (Bergquist et al., 1970; Simpson, 1984; Wapstra and van Soest, 1987; Woollacott, 1993; Fromont, 1994; Ereskovsky, 1999). The presence of choanocyte chambers inside larvae is uncommon, but has been documented in two marine, haplosclerid species, *H. limbata* and *Chalinula* sp. (Meewis, 1939; Ilan and Loya, 1990a). Once released, free-swimming larvae can remain mobile hours to weeks before they settle and develop into sessile juveniles (Maldonado et al., 2008).

The origin of spermatogonia of freshwater haplosclerids is from flagellated choanocyte chambers, with the choanocytes transdifferentiating into spermatogonia, which then form spermatocysts (Efremova and Papkovskaya, 1980; Sukhodolskaya and Papkovskaya, 1985; Paulus and Weissenfels, 1986; Paulus, 1989; Weissenfels, 1989). However, whether the freshwater haplosclerids (suborder Spongillina) belong in the order Haplosclerida has yet to be conclusively determined; therefore, aspects of their reproduction may not be informative for marine haplosclerids (Redmond et al. 2007; Sperling et al., 2009). However, male gametes are also reported to develop from choanocytes in *P. ficiformis*, a marine haplosclerid (Maldonado and Riesgo, 2009). Female gametes of haplosclerids are thought to develop from choanocytes, or archaeocytes (e.g. Tuzet, 1932; Meewis, 1936; Leveaux, 1941; Brien, 1967; Saller and Weissenfels 1985; Saller, 1988; Weissenfels, 1989; Ereskovsky, 1999), and are

associated with nurse cells; this is a greater specialization compared to other demosponge orders (Ereskovsky, 1999).

While reproductive biology and/or the form and behaviour of larvae have been described for quite a number of marine haplosclerid species e.g. *H. oculata* and *H. xena* (Wapstra and van Soest, 1987), *H. cinerea* (Meewis, 1941), *H. amboinensis* and *H. cymiformis* (Fromont, 1994), *Haliclona* spp., (Fromont, 1999; Whalan et al., 2008), *H. loosanoffi* (Fell, 1976), *Callyspongia paralia*, *Niphates rowi*, *P. elephantotus* and *Amphimedon chloros* (Ilan et al., 2004; Ilan and Loya, 1990a; 1990b), *H. permollis* (Elvin, 1976), *Xestospongia testudinaria*, *X. bergquistia*, and *X. exigua* (Fromont & Bergquist, 1994), *P. ficiformis* (Maldonado and Riesgo, 2009), *A. queenslandica* (Leys and Ereskovsky, 2006), some of these studies provide excellent detail on the morphology throughout development, while others are limited in scope, or old and/or brief, providing considerably less insight, including the publications on *H. indistincta* (Bowerbank, 1866; Lévi, 1956). Furthermore, reports on the reproductive biology and form of the larva differ for some species (reviewed in Wapstra and van Soest, 1987) suggesting the possibility of species misidentification in some cases. This is especially likely in species of the order Haplosclerida due to their notoriously simple skeleton and morphological plasticity.

Here I describe the reproductive cycle and characteristics of the larva of *H. indistincta*, a species commonly found in the intertidal regions around Ireland and elsewhere (deWeerd, 1986). The species name is derived from its variable form and the scarcity of defining characters (Bowerbank, 1866). Its growth form includes thin sheets or cushions, and the colour ranges from light tan to bright purple. Its most defining characteristic is its sticky mucous consistency (Lévi, 1956; deWeerd, 1986). Lévi's

(1956) description of reproduction in *H. indistincta*, is focused upon the embryonic and larval stages and, though informative, is very brief. My aim was to carry out a more detailed investigation of reproduction and development in this species and here I present data based upon histology and light microscopy.

## Materials and Methods:

*Material*; To study the annual reproductive cycle of this species, *H. indistincta* specimens Type 1 morphotype (Chapter 2) (Figure 1A), identified via comparison of morphological characters with type material (DeWeerd, 1986) provided by the Zoological Museum University of Amsterdam), were collected from Corranroo, Co. Clare, Ireland (latitude 53° and longitude 9°). The sampling area was approximately 2,000m<sup>2</sup>. The tidal amplitude was a maximum of 5m during spring tides and approximately 2.5 m at neap tides. In 2009-2010 the average surface water temperature (Malin Head station, 55°N and 7° W) ranged from 6.4°C in February to 15°C in August ([http://www.met.ie/marine/marine\\_climatology.asp](http://www.met.ie/marine/marine_climatology.asp)). The most prevalent rock types found in the area are granite and limestone, with limestone and sheets of algae as the preferred substrates of this species at this site. It can be an opportunistic settler as multiple specimens were also discovered growing upon the shells of bivalves.



**Figure 1.** *H. indistincta*; (A) adult specimen *in situ*. (B) adult specimen with developing larvae (arrows) throughout the mesohyl. Scale bar= 5mm.

Ten complete specimens ranging from 19cm<sup>3</sup> to 30cm<sup>3</sup> were collected monthly from September 2009 to September 2010 with an additional twenty specimens collected between April and July. Specimens were randomly sampled from just below the waterline during the low spring tides. The distance between sampled individuals was not measured but varied from approximately 30cm (individuals on the same rock) to 300m (individuals separated distantly on the same region of shoreline). From April onwards, specimens were dissected to identify whether larvae were present and to observe the distribution of larvae throughout the parent sponge.

*Histological staining;* Given that obvious mature embryos/pre-released larvae were found distributed throughout the sponge bodies (Figure 1B), with a higher concentration seen in the mid-section; subsamples (5mm<sup>3</sup>) were taken from the middle area of each specimen and fixed in Bouins solution, and then transferred to 70% ethanol, with the belief that the other, less obvious gamete (i.e. spermatogonia contained within spermatocysts), would also be present in the middle area of the sponge bodies. The excised sections were washed with 70% ethanol until the Bouins solution had been completely removed, and placed in histology cassettes in a histological tissue processor that moved the sections through a series of alcohol and histoclear baths. The tissue was impregnated and blocked in paraffin wax. Successive serial sections (4 and 7µm) were made with a microtome from each subsample, mounted on microscope slides, and stained with hematoxylin and eosin. Slides were examined using light microscopy (Olympus BX51). Each specimen slide was examined for reproductive elements (oocytes, eggs, spermatocysts, embryos, and pre-released larvae). Ten representatives of each of the reproductive elements present (per slide) were measured where possible. All measurements were taken from the longest diameter of each structure using imaging software Cell<sup>^</sup>D (Olympus). The average size and number of the various reproductive elements of all the

specimens per month was then obtained. Summary statistics and graphs were generated in Microsoft Excel.

*Larvae*; Collection of larvae took place in the middle of the day during the low spring tides through the months May-August. Two methodologies were successfully employed to secure free-swimming larvae. Eight mature sponges were housed *in situ* by placing pyramid-shaped larval traps (made from nylon mesh with two collection tubes for larvae to swim into) over the rock with the sponge attached (Figure 2). The larval collection tubes were checked at midday, five days a week during the last week of June and the first week of July, and once a week from the second week of July until the end of August. Collection tubes were replaced, and removed tubes were taken back to the lab where the contents were placed in 50ml petri dishes, and examined under a dissection microscope. We also induced release of larvae directly from parent sponges by cutting them open, allowing larvae to swim out into a container of water. Initially, larvae were held in varying densities in 25ml petri dishes in seawater. Subsequently, 69 larvae were individually maintained in 25ml petri dishes filled with filtered seawater. Specimens were allowed to settle and develop further. All stages were observed and photographed using a dissecting microscope (Olympus SZX16) and video recorded using a Panasonic 3CCD attached to a light microscope (Nikon SMZ-U).



**Figure 2.** Larval trap *in situ*.

**Results:**

*Reproductive cycle;* There was no evidence of reproductive elements in specimens collected in September, December and January, (Figure 3A; Table 1). All individuals collected between February and May contained reproductive material, this number dropped to 70% of individuals collected in July but was 100% in August (Figure 3A).

The first sign of oogenesis was seen in October when one amoeboid oocyte (29 $\mu$ m in maximum diameter), without the presence of nurse cells, was observed in one specimen. Early oocytes were also seen in low numbers in November (one in each of two specimens, 28 and 32 $\mu$ m). There were no early oocytes present in December and January. Of the 58 early amoeboid oocytes observed (and measured) in specimens from October through August, the highest numbers were observed in March (14), April (18) and May (9). Early oocytes were still present in June-August, but were very few in number with three being present in June (sections from three specimens had one early oocyte each), one each in two July specimens, and four in August in three separate specimens.

Reproductive elements increased in size from May through to August, in parallel to the rising water temperature (Figure 3B and C). The diameter of oocytes (Figure 4A) ranged from 29 $\mu$ m to 78 $\mu$ m (n=1,013 mean 58 $\mu$ m  $\pm$  27 $\mu$ m (SE)). Choanocyte chambers filled with cells and surrounded by a ring of choanocyte cells, suggestive of early spermatozoa forming early spermatid cysts (Appendix 1A), were seen from February through to the end of August. Fully developed spermatozoa in distinct spermatid cysts were only seen during the months of May and June (Table 1) in one sixth of the specimens surveyed.

Of a total of 200 mature *H. indistincta* specimens examined throughout the year, 55 were hermaphrodites, 84 had female gametes only, while four had male gametes only (Table 1). A number of sponges contained developing oocytes and what we presume were early spermatozoa (i.e. transdifferentiating choanocytes) from February, and female-only sponges were also present at this time. By May, four sponges contained both fully developed spermatocysts and late oocytes, while others still contained early spermatogonia along with early and late oocytes. Mature male-only sponges (i.e. with fully developed spermatocysts, Appendix 1B) were seen first in June.

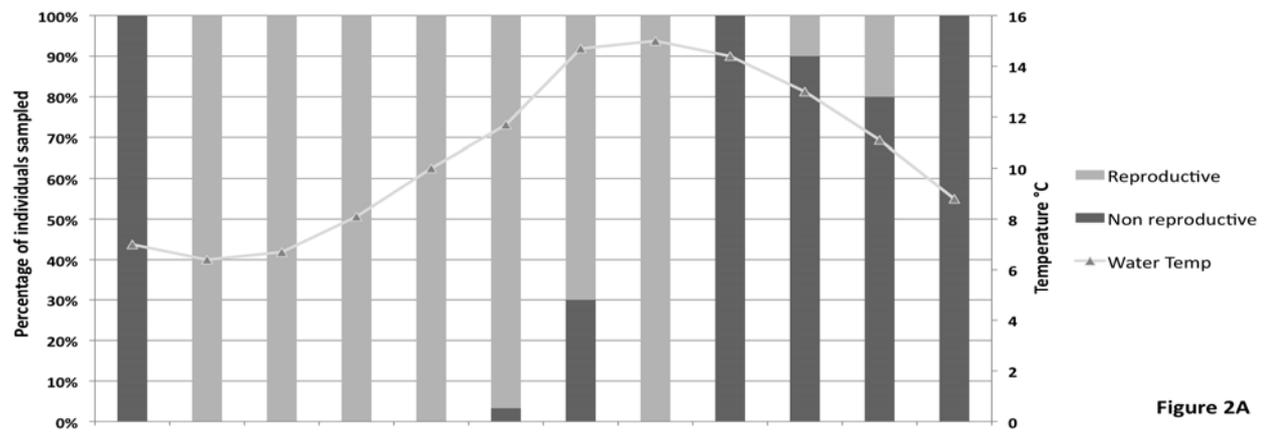


Figure 2A

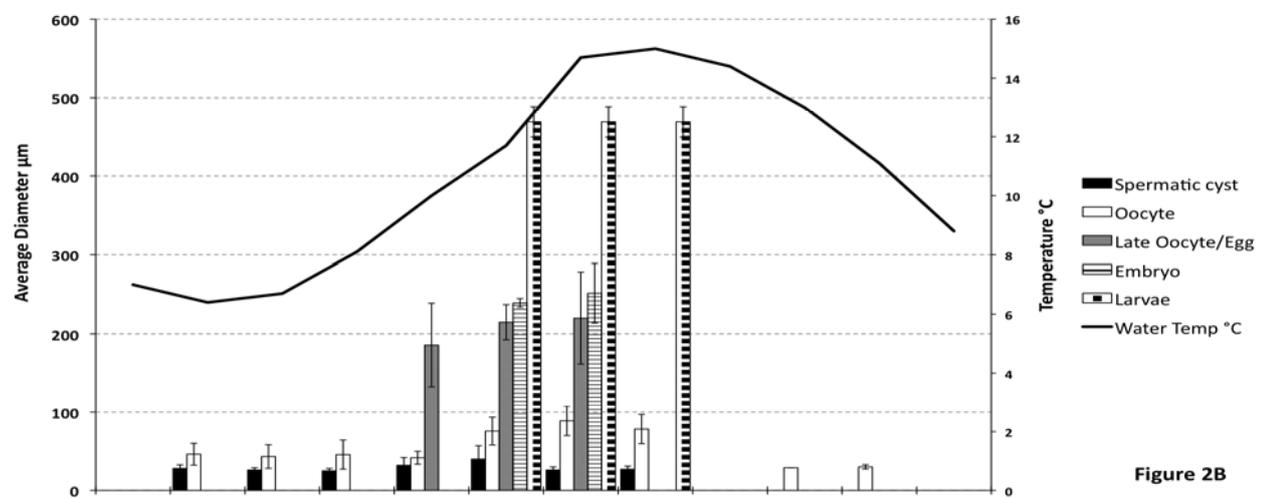


Figure 2B

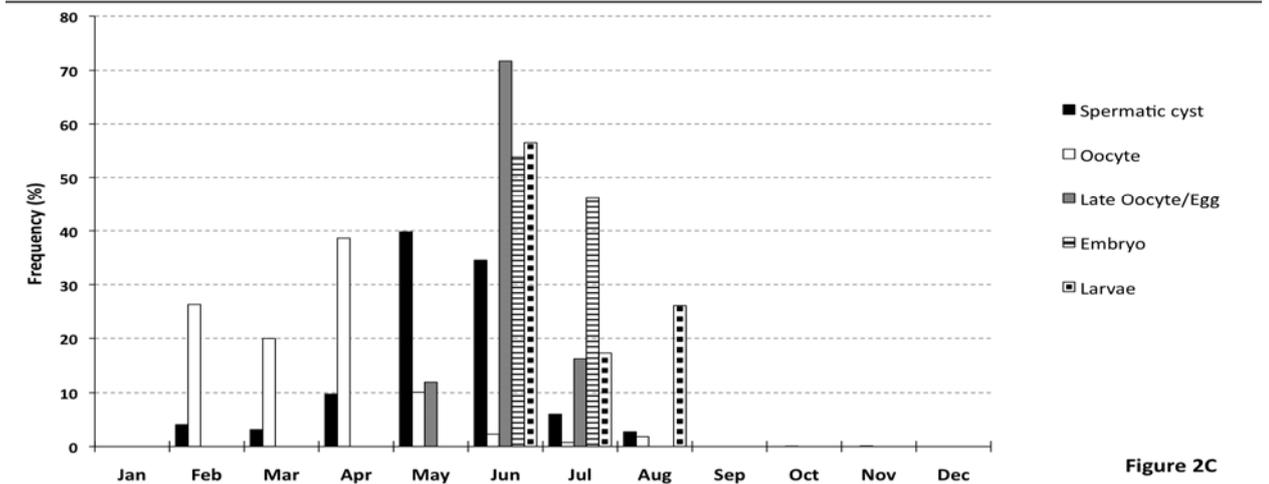
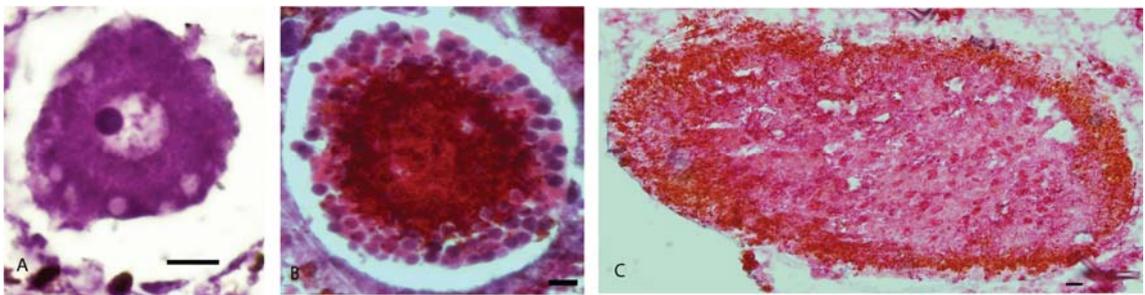


Figure 2C

**Figure 3.** (A) Graph showing (per month) the percentage of individuals sampled that were reproductive and non-reproductive. Also showing a plot of the water temperature over the same time period. (B) Graph showing the average diameter of reproductive elements (standard deviation (error bars) included). Also showing a plot of the water temperature over the same time period. (C) Graph showing the percentage frequency of each reproductive element of *Haliclona indistincta* over a twelve-month period, using 10 replicate individuals per month, e.g. 71% of all late oocytes/eggs were seen in June. The numbers of each reproductive element counted were; Spermatic cysts N = 2166; Oocytes N = 2133; Late Oocyte/Egg N = 92; Embryo N = 13; Larvae (i.e. pre released larvae) N = 23. For “oocyte” both early and mid stages were included, and for “spermatic cyst” both immature (i.e. choanocyte chambers filled with transdifferentiating choanocytes) and mature (i.e. distinct cysts filled with ciliated spermatozoa) stages were included.

By the end of May, late oocytes (eggs) were seen in four out of the thirty specimens sampled and no embryos or larvae were seen. Eggs, (Figure 4B) were circular and had an outer layer composed of yolk granules with an average size of  $9\mu\text{m}$  ( $n=10 \pm 1.7\mu\text{m}$  (SE)) with nurse cells contained within the cytoplasm, as is typical of haplosclerids, with an average maximum diameter of  $3\mu\text{m}$  ( $n=10 \pm 1\mu\text{m}$  (SE)), and a large amount of material that stained bright pink. The diameter of the eggs ranged from  $186\text{-}220\mu\text{m}$  ( $n=261$ , mean  $206\mu\text{m} \pm 45\mu\text{m}$  (SE)). Embryos were similar to the eggs in shape as they were also spherical but ranged in size from  $239\text{-}252\mu\text{m}$  ( $n=64$ , mean  $245\mu\text{m} \pm 22$  (SE)). They did not possess the prominent layer of yolk granules on the exterior, and the internal cavity showed significantly less pink-stained material, being filled instead with dense clusters of cells. As the embryos matured inside the adult body, they attained a more elongated oval shape and, internally, the pink-stained material present formed a layer that separated an outer layer of epithelial cells and an internal area that contained a number of cell types (Figure 4C and Appendix 2).



**Figure 4.** (A) Early oocyte. (B) Egg. (C) developed embryo, longitudinal—section. **Scale bars:** A= $10\mu\text{m}$ ; B= $20\mu\text{m}$ ; C= $10\mu\text{m}$ .

No spicules were present. The highest number of specimens containing mature embryos was in July, and by August, mature embryos were found in only one individual (Table 1). Mature embryos/pre-released larvae occurred in groups of varying numbers and were not held in defined brood chambers but were heavily surrounded by mucus. The size of free-swimming larvae ranged from  $414\mu\text{m}$  to  $477\mu\text{m}$ , ( $n=41$ , mean  $469\mu\text{m} \pm 20\mu\text{m}$  (SE)).

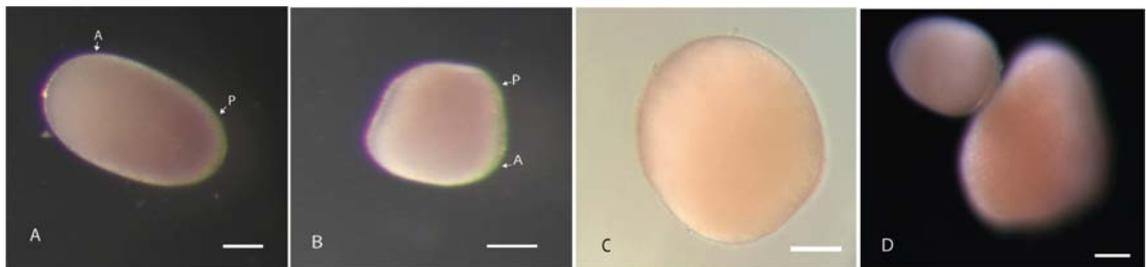
**Table 1.** Numbers of *Haliclona indistincta* specimens per month showing those that were found to be hermaphrodites (H), male (M), female (F) and those which had embryos and larvae of different stages. 0:10 = no specimen out of a total of ten specimens surveyed, 10:30 = ten specimens out of a total of thirty specimens surveyed.

Month	H	M	F	Late oocyte/Eggs	Early embryos	Late embryos	Pre-released larvae
Sept	0:10	0:10	0:10	0:10	0:10	0:10	0:10
Oct	0:10	0:10	1:10	0:10	0:10	0:10	0:10
Nov	0:10	0:10	2:10	0:10	0:10	0:10	0:10
Dec	0:10	0:10	0:10	0:10	0:10	0:10	0:10
Jan	0:10	0:10	0:10	0:10	0:10	0:10	0:10
Feb	5:10	0:10	5:10	0:10	0:10	0:10	0:10
Mar	3:10	0:10	7:10	0:10	0:10	0:10	0:10
Apr	11:30	0:30	19:30	0:30	0:30	0:30	0:30
May	10:30	0:30	20:30	4:30	0:30	0:30	0:30
June	14:30	3:30	12:30	13:30	3:30	2:30	1:30
July	7:30	1:30	13:30	14:30	10:30	6:30	4:30
Aug	5:10	0:10	5:10	0:10	0:10	1:10	1:10

*Larvae*; Six larvae were retrieved from one larval trap on June 29<sup>th</sup> and over the six days between July 1<sup>st</sup> and 8<sup>th</sup> a total of 67 larvae were collected from eight traps (Figure 2) (i.e. eight parent sponges). The numbers of larvae collected in this way varied per day ranging from 0 larvae present in the traps to a maximum of 26 larvae present in a single trap. Only three sponges, out of the eight, released larvae over this time period. No larvae were present in the traps on August 10<sup>th</sup>. Most of the larvae used for observations were obtained through induced release in the laboratory. Three distinct free-swimming larval stages were observed. Figure 5A shows the initial free-swimming, mobile stage, which was oval and showed anterior-posterior orientation. The anterior end pointed upward when stationary, and at a slight angle when in motion (in the direction of movement). Larvae were found to swim in an anticlockwise, spiral or corkscrew pattern. The larvae were uniformly ciliated and the pink pigment associated with this species showed a higher concentration at the posterior pole. Figure 5B shows a later stage where the shape of the larvae had changed to being compact and angular. The pale anterior end diminished in size, but the anterior-posterior orientation was still apparent,

especially when swimming. In a third stage (Figure 5C), the body was nearly perfectly circular, rotating on its axis, the pink colour of the free-swimming stage still apparent.

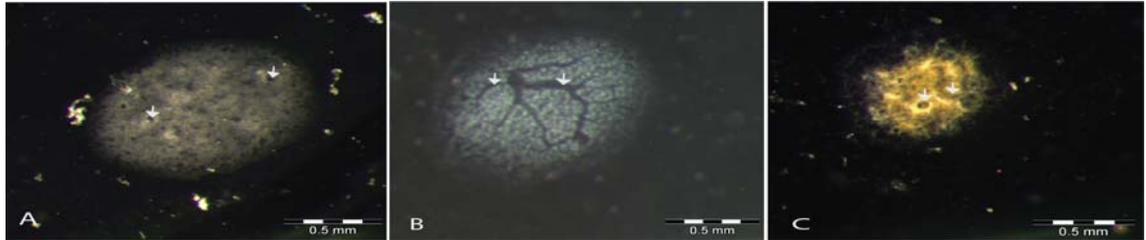
A fusing behaviour among the larvae was observed multiple times throughout this study; the highest number of events taking place in two separate (25ml) petri dishes holding 29 and 16 larvae, respectively. Of the dish containing 29 larvae, four separate fusing events were observed, with one fusion comprising three larvae (Figure 5D), and the remaining three events involving two larvae. In the dish containing 16 larvae, two separate fusion events each involving two larvae occurred. A third distinct fusing event was observed between two larvae when they were placed together in a drop of seawater (10 $\mu$ l) for ten minutes. In all cases, larvae were observed to fuse and continue to develop to settlement stage.



**Figure 5.** *H. indistincta* larvae (A) Free-swimming stage. (B) Compact angular stage showing a diminishing anterior end. (C) Semi-spherical ball stage. (D) Free-swimming larvae fusing with two other already combined larvae. Scale bar=100  $\mu$ m.

Variability was seen in the amount of time it took for each larva to progress through the mobile stages. The shortest time from release to settlement was 25 hours (11/69 larvae), and longest time was 285 hours (3/69 larvae), with an average time of 161 hours (7 days). When about to settle, larvae ceased their horizontal forward corkscrew movement, though still spinning anti-clockwise, and eventually remained stationary, attached via the posterior pole and settled. Two hours after settlement, specimens began to flatten onto the substrate. After 24 hours, specimens were almost completely flattened. Subsequent days produced ostia formation (Figure 6A), the development of

excurrent channels (Figure 6B) and oscular opening formation (Figure 6C). Sponge larvae, and newly settled sponges, were observed to be the dietary target of a water mite that could not be removed by filtering the water. No sponge survived beyond 39 days after release.



**Figure 6.** Post settlement stages of *H. indistincta*. **(A)** Juvenile 37 hrs post settlement showing surface becoming punctated (arrows). **(B)** Juvenile 39 hours post settlement showing excurrent canals (arrows). **(C)** Juvenile 115 hrs post settlement, formation of oscules and excurrent canals (arrows), colour change associated with addition of *Phaeodactylum tricornerutum* (diatom used in feeding juvenile sponges).

## **Discussion:**

I have added to the current knowledge of the reproductive cycle and early development of *H. indistincta* from that presented by Lévi (1956). I show the species to be primarily hermaphroditic, with some gonochoric individuals present in the population. However, as individual adult sponges were not sampled throughout the season, and also given the difficulty in locating male gametes (i.e. sperm cysts) within adult sponges, it is likely that the presence of gonochorism in this species is an artefact due to sampling bias. Female-only sponges appeared first and male-only sponges appeared later (in June), while hermaphrodites were present between February and August. Males were very few in number (4/200).

The first appearance of identifiable oocytes showed amoeboid ((i.e. resembling ameba in form or movement) cells, which were very similar to archaeocytes though larger. Their form, and given that they were not found associated with choanocyte chambers, leads me to suggest an archaeocyte origin for oocytes, which is also consistent with other Haplosclerida (Leys and Ereskovsky, 2006). Although the first early oocyte was reportedly observed in October (Figure 3), given that they were so few in number, it is possible that they were archaeocytes and not early oocytes. The identification of male reproductive elements was difficult, however, I suggest that spermatogenesis in this species is consistent with other haplosclerids. While the identity of mature sperm in the spermatid cysts were confirmed via TEM (Appendix 2C), it was not possible to confirm the identity of developing spermatozoa in the same way. However, the presence of choanocyte-filled chambers (Appendix 2A) is consistent with the manner in which spermatid cysts develop in other sponges including the marine haplosclerid *Petrosia ficiformis* (Maldonado and Riesgo, 2009), and other haplosclerid species, as previously described (e.g. Efremova and Papkovskaya, 1980; Sukhodolskaya and Papkovskaya,

1985; Weissenfels, 1989; Ilan, et al., 2004), as well as other orders (Halicondrida, (Barthel and Detmer, 1990) and Dictyoceratida (Kaye and Reiswig, 1991)).

I observed fewer embryos in the months June-July than oocytes or larvae. It is possible that oocytes were reabsorbed into the sponge body and did not contribute to the reproductive effort. It is also possible that through the sampling and sectioning approach adopted, some embryos were missed. In a similar manner to the embryos described by Lévi (1956), the embryos seen during this work showed the presence of material that stained a bright pink that was associated with the oocytes, embryos and larvae from an early stage (February). This material made it difficult to document cleavage and early development using the approaches employed here. Unidentified, dense, granular material has been found to be associated with developing oocytes of a number of sponge species including the marine haplosclerid *Haliclona cinerea* (Tuzet, 1947), and *Stelletta grubii* (Liaci and Sciscioli, 1967), *Suberites massa* (Diaz et al., 1975), *Aplysina cavernicola* (Gallissian and Vacelet, 1976), *Hippospongia lachne*, *Spongia barbara*, *S. graminea* and *S. cheiris* (Kaye, 1991) and, though its function and composition is still unknown, it has been suggested to be important to oocyte development (Simpson, 1984; Kaye, 1991). I made initial attempts to identify the material, (Masson's Trichrome with Gomori's Aldehyde Fuchsin stain to identify collagen and P.A.S. (periodic acid-Schiff) to identify carbohydrate) with no success.

Increase in the numbers of reproductive individuals, as well as the diameter of reproductive elements (e.g. eggs and embryos) corresponded to an increase in water temperature, a particularly important dynamic for initiating reproductive processes as shown by other authors (e.g. Fromont and Bergquist 1994; Witte et al., 1994; Ereskovsky, 2000; Whalan et al., 2007; Maldonado and Riesgo, 2008). The timing of

larval release described here is shorter than described in Lévi (1956), as I did not find any mature larvae until the end of June while he suggested a period from May to July. It is possible that the geographical difference between the two study sites (Clare, Ireland versus Brittany, France) may have contributed to this difference. The size of the reproductive elements also differed between the studies; Lévi (1956) recorded an egg size of 300-350 $\mu\text{m}$ , which is larger than the eggs recorded in this study and the largest embryo I measured was 252 $\mu\text{m}$ . Lévi (1956) recorded a maximum length of larvae in Brittany to be 500 $\mu\text{m}$ , while the corresponding value for the Clare population is 477 $\mu\text{m}$ . The geographical difference could also contribute to this discrepancy in the size of reproductive elements. However, I also observed two morphotypes currently ascribed to *H. indistincta*. Details of the morphology of the specimens used in Lévi's study are not provided (we investigated the cushion form (Type 1)), and it is possible that the larvae from the two studies are from different morphotypes. Further work on the two morphotypes of this species, and on specimens from different populations would shed light on this matter.

I also observed the fusion of larvae in this species as has been described for other sponges (e.g. *Ophlitaspongia seriata* (Fry, 1971), *Crambe crambe* (van der Vyver, 1970) *Halichondria panicea*, *Hymeniacidon perlevis*, *Pachymatisma johnstonia*, *Clathrina (Leucosolenia) coriacea*, *Iophon hyndmani*, and *Dercitus bucklandi* (Fell, 1974), including haplosclerids (e.g. *Chalinula* sp. Ilan and Loya (1990b), *P. ficiformis*, Maldonado and Riesgo (2009), *Haliclona* sp. (McGhee, 2006) but is still relatively little studied. All fusing larvae from *H. indistincta* seen in this study were from the same parent sponge. Only one sixth of adult specimens collected in May and June possessed both spermatocysts and oocytes, therefore, while self-fertilization may be possible in these individuals (and yield more genetically similar offspring) it is unlikely to be the

case for the entire population. Without genotyping adults and larvae from such fusion events it is difficult to predict whether they are chimeras of siblings that are more genetically similar than expected from sexual reproduction, or not. Ilan and Loya (1990b), and McGhee, (2006) have shown that larvae fuse indiscriminately with other genetically distinct larvae (and juveniles), but that adults will only fuse with fragments from genetically identical individuals, suggesting that the mechanism allowing distinction of self from non-self develops sometime after settlement (Ilan and Loya, 1990b). Fusion of sponge larvae and early juveniles may be an advantage as a higher rate of survivorship has been demonstrated for juvenile sponges that had previously formed fused groups (e.g. Fry, 1971; Connell, 1973; Highsmith, 1982; Ilan and Loya, 1990b). Also, a larger body size gained through fusion may allow an individual to sexually mature sooner (Connell, 1973; Highsmith, 1982; Ilan and Loya, 1990b). However, Maldonado (1998) found evidence suggesting the above, hypothesized selective advantages are not valid explanations for the occurrence of fusing in sponge larvae. However, because fusing appears to be more likely to occur if larvae are under some sort of stress, e.g. forcefully moved, overcrowded or restricted (Ilan and Loya, 1990b; McGhee, 2006) as was also seen during this study for *H. indistincta*. If close proximity of larvae induces the fusion of individuals, which my findings would suggest to be the case, then it could also be a competitive mechanism in environments where resources are limited (McGhee, 2006).

Ereskovsky (2010) described the early presence of a dense bunch of spicules at the posterior pole of haplosclerid larvae as a common feature of this group, and reported this in a number of *Haliclona* species e.g. *H. aqueductus*, *H. cinerea*, *H. ecbasis*, and some fresh water sponges, (Ereskovsky, 1999; 2010). However, spicules were not evident in the larvae of *H. indistincta* and were also not mentioned by Lévi (1956) in his

work on this species. Ereskovsky (2010) also indicated that *Haliclona* larvae possess a ring of longer cilia at an otherwise bare posterior pole, however *H. indistincta* were uniformly ciliated (also described by Lévi, 1956). The external characteristics of *H. indistincta* larvae were also not reminiscent of the *Haliclona* sp., *Chalinula* sp. or *C. limbata* larvae illustrated by Mariani et al. (2005), lacking the longer posterior cilia and/or the pointed anterior pole. Because we observed this uniform ciliation both in larvae whose release from the parent sponge was induced, and from larvae collected in larval traps, the external morphology observed is not likely to be due to immaturity. Further, Maldonado and Bergquist (2002) also describe the larva of *H. petrosioides* as being uniformly ciliated.

Bergquist (1979) reported two larval types in the Haplosclerida. While she described *Callyspongia*, *Adocia* and *Haliclona* as having a ring of long cilia and a bare posterior pole, *Reniera*, which is now a synonym of *Haliclona*, and *Chalinula* larvae both lacked this ring of longer cilia. While Wapstra and van Soest (1987) predicted that Bergquist's *Reniera* would belong to Halichondrida and *Chalinula* to the Poecilosclerida this was never subsequently determined, to my knowledge. In any case, recent molecular phylogenies suggest that the family Chalinidae and the genus *Haliclona* are polyphyletic and that species of what were *Reniera* (e.g. *H. fulva*) are not closely related to the type species *H. oculata* (Redmond et al., 2011).

Another unusual aspect of *H. indistincta* larvae is that they settled on the posterior pole rather than the anterior pole, which was also described by Wilson (1935) for the poecilosclerid *Mycale syrinx* but appears to be otherwise uncommon in sponges. Langenbruch and Jones (1990) suggested that adult *H. indistincta* had a different morphological structure from other *Haliclona* species after histological examination of

the structure of the choanocyte chambers in particular, suggesting that it may be a poecilosclerid; Chapter 2 showed that *H. indistincta* is a marine haplosclerid that is not closely related to the type species (*H. oculata*) but is instead more closely related to *Niphates* and *Amphimedon* taxa (see 28S rRNA gene tree). More research on larval development of sponges in these genera is warranted.

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Chapter 4:

**Ultrastructure of the ciliated cells of the free-swimming larvae and sessile stages of the marine sponge *Haliclona indistincta* (Demospongiae: Haplosclerida)**

This work is submitted for publication:

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Ultrastructure of the ciliated cells of the free-swimming larvae and sessile stages of the marine sponge *Haliclona indistincta* (Demospongiae: Haplosclerida) *Journal of Morphology*.

**Introduction:**

The typical demosponge body plan is comprised of three layers: an external and internal epithelial-like layer, made up of exo-, endo-, and baso-pinacocyte cells, and an extracellular matrix, or mesohyl, which is composed of proteins, polysaccharides, and fibrous collagen and contains cells (Bergquist, 1978; Ereskovsky, 2010). It is generally accepted that sponges are the most basal of the metazoans (Nielsen, 2012). Thus, information concerning the evolution of multicellular animals from unicellular protists could be inferred through genetic, morphological, and developmental investigations in this group of animals (Fahey and Degnan, 2010; Ereskovsky, 2010; Ruiz-Trillo et al., 2008).

Flagellum and cilia are characteristic organelles of eukaryotic cells (Nielsen, 1987). Consistent differences in structure or function between organelles possessing either label (i.e. cilium or flagellum) have not been found (Mitchell, 2007). These terms have also been used interchangeably throughout sponge literature; I therefore, employ the term cilia for those described here. Cilia are present in several different sponge cell types: ciliated epithelial cells of the larvae, endopinacocytes, apopendacocytes, spermatozoa and choanocytes (De Vos et al., 1991; Ereskovsky, 2010). Choanocytes are ciliated collard cells that utilize their cilium to facilitate ambient water in, and throughout, the body in a complex canal system. Gonobobleva and Maldonado (2009) suggest they are a key cell type for understanding the evolutionary transition from a unicellular to a multicellular state. Choanocytes are unique to sponges (Simpson, 1984; Barnes and Harrison, 1991; Maldonado, 2004; Nielsen, 2012). However, the cilium is utilized by nearly all metazoan phyla for a variety of functions, including locomotion and feeding (Nielsen, 1987; 2012). The basic ultrastructure of this organelle and its basal apparatus is almost constant in ciliated cell types of sponges but is more complex

in the larvae (Woollacott and Pinto, 1995; Nielsen, 2012). In an effort to investigate the utility of this organelle for phylogenetic applications, Woollacott and Pinto (1995) reviewed six components of the basal apparatus of the ciliated cells in sponge larvae: basal body, basal foot, accessory centriole, transverse cytoskeletal system, longitudinal cytoskeletal system, and association with Golgi body, from a number of taxonomic groups and found consistent morphologies between the basal apparatus of closely related taxa.

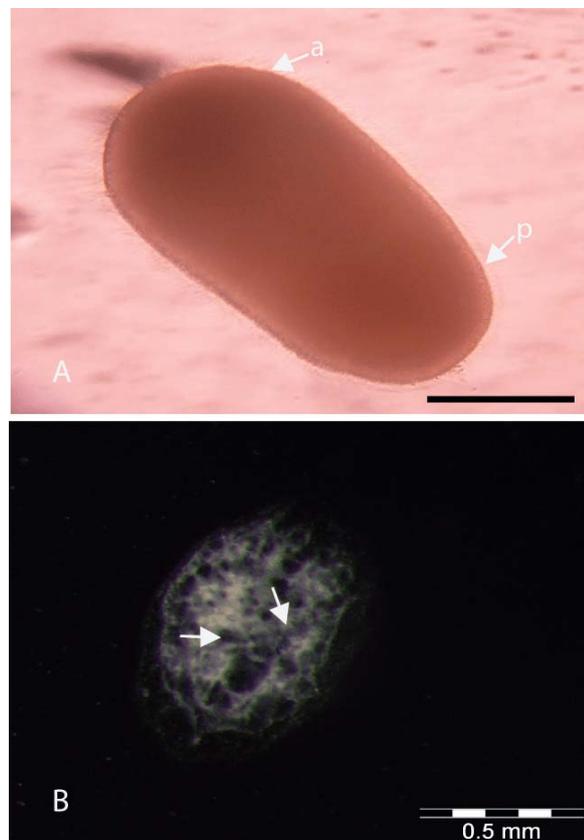
Sponges use cilia for locomotion while at the larval stage, and for feeding during the sessile stage (Maldonado, 2004). Certain demosponge and calcareous sponge larvae use the ciliated cells for both functions. When they metamorphose, some larvae undergo “layer inversion” (Delage, 1892) during which the epithelial ciliated cells migrate inward and transdifferentiate into choanocytes (for review see: Maldonado, 2004; Leys and Eerkes-Medrano, 2005; Ereskovsky et al. 2007 ; Ereskovsky, 2010). Central to studies investigating the trans-differentiation of larval ciliated cells into choanocytes during metamorphosis, was the close examination of the ciliated epithelial cells of the larvae (in particular, the cilia and accompanying basal apparatus of the cell), in comparison to the choanocyte cells of the settled sponge (Gonobobleva and Ereskovsky, 2004; Ereskovsky et al., 2007; 2009; Maldonado, 2009; Ereskovsky, 2010). There have been a number of studies concerning the basal apparatus of either the ciliated larval epithelial cells, or the choanocytes in different sponges species (e.g. Woollacott and Pinto, 1995; Karpov and Efremova, 1994; Leys and Degnan, 2001; Boury-Esnault et al., 2003; Maldonado et al., 2003; Ereskovsky and Tokina, 2004; Gonobobleva and Ereskovsky, 2004; Usher and Ereskovsky, 2005; Ereskovsky and Willenz, 2008). However, studies comparing both cell types in the same species are not common, but exist (e.g. Efremova et al., 1988; Gonobobleva and Maldonado, 2009).

Here I describe and compare the ciliated epithelial cells of the larvae and the choanocytes of the post-settled stage of a marine haplosclerid demosponge *Haliclona indistincta* (Bowerbank, 1866).

In previous work concerning this species (i.e. Chapter 3; Stephens et al., in press) the external morphology of the larva was described as well as the ciliation pattern. The larvae were described as being oval in shape (approximately 477 $\mu$ m in length and approximately 200 $\mu$ m in width) when first released from the parent sponge, and having a uniform ciliation pattern. The larvae were also described as having distinct anterior and posterior ends that would diminish as they progressed through two other mobile stages, becoming more compact and angular and subsequently becoming circular just prior to settlement. However, what was occurring internally during this change in the larval morphology was not investigated.

## Materials and Methods:

*Material;* Larvae of *Haliclona indistincta* Morphotype 1 were collected from Corranroo, Co. Clare Ireland (53° 8'28.50"N and 9° 0'33.70"). Two larvae from each of four developmental points (oval stage, immediately post release (Figure 1A), the compact angular stage, the semi-spherical ball stage, and early recruitment stage (i.e. 32 days post-settlement) and one additional specimen from eight days post-settlement (Figure 1B) were sectioned for electron microscopy.



**Figure 1.** *H. indistincta* **A** First free-swimming stage (colour slightly distorted). **B** Juvenile 192 hrs (8 days), oscules and excurrent canals forming (arrows). a, anterior; p, posterior. Scale bar: A=100µm.

*Electron microscopy;* To describe and compare the ultrastructure of the ciliated epithelial cells of the free-swimming larvae and the choanocytes of the juvenile sponge, transmission electron microscopy (TEM) was employed. For ultrastructural observations, larvae were fixed in a primary fixation solution composed of 2.5%

glutaraldehyde, 1% paraformaldehyde in 0.2M sodium cacodylate/HCL buffer (pH=7.2) (1,400 milliosmole (mOsm)) for 24 hours. Specimens were then postfixed in 1% osmium tetroxide in 0.2M sodium cacodylate/HCL buffer (pH =7.2) (420 mOsm) for approximately two hours.

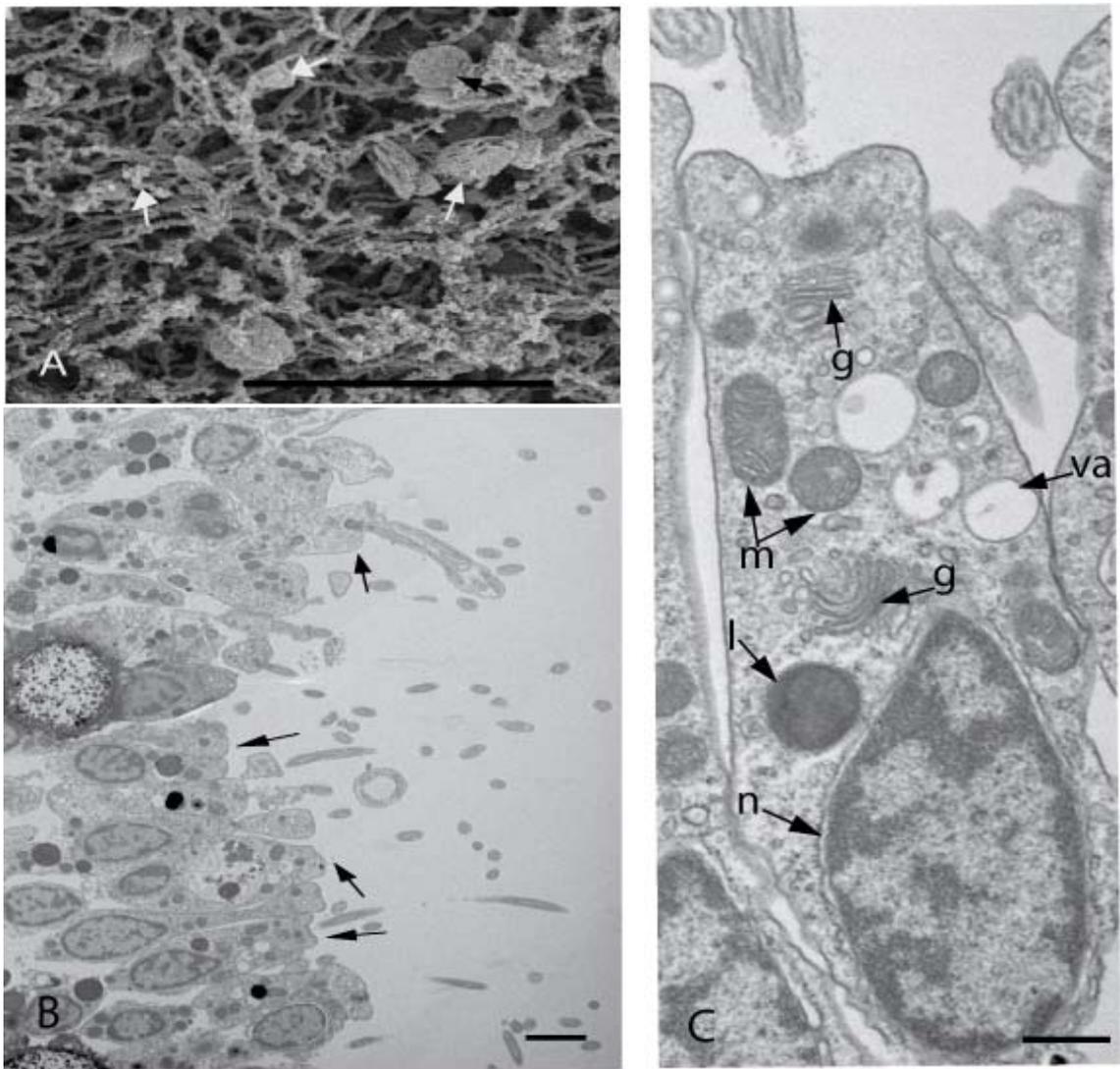
For larvae, fixed specimens were dehydrated in a graded ethanol series and embedded in agar low viscosity resin (R1078 resin kit, Agar Scientific). To ensure detailed observations of the cells, serial ultra and semi-thin sections were obtained with a Reichert-Jung Z00M Stereo-Star Ultra-cut ultra-microtome. The sections were mounted on copper (200µm) mesh grids, and stained with Ultrastain 2 for 36 min via Leica EM (AC20). Hitachi H7000 TEM, operating at 75kV, was used to conduct the observations of the grids. All measurements of organelles and cells were taken at the longest diameter.

*SEM*; To observe the arrangement of the ciliated epithelial cells as well as the general external surface of the free-swimming larva scanning electron microscopy (SEM) was used. Larva (originally fixed in Bouin solution) were dehydrated in a series of graded ethanol baths, critical-point-dried and scatter coated with palladium and gold via emscope SC500. Larvae were then mounted on stubs and visualized using Hitachi S-4700 electron microscope and EDX spectra (Oxford Instruments, Inca Systems, High Wycombe, U.K.).

## Results:

### *Ciliated cells of the larval epithelial layer:*

*General cytology;* The surface of the larva of *H. indistincta* was densely covered by cilia (Figures 1A) many of which were found to have artifacts attached to them (Figure 2A). The ciliated epithelial cells of larvae were pseudo-cylindrical in shape, ranging from 5.4 to 8 $\mu$ m at the longest axis and 1 to 2 $\mu$ m in width (n=5 average 6.3  $\pm$  1.0 (SE) and 1.5  $\pm$  0.4 $\mu$ m (SE) respectively). There were no visible junctions between these epithelial cells; however, they were usually in close contact with neighbouring ciliated epithelial cells (Figures 2B-C).

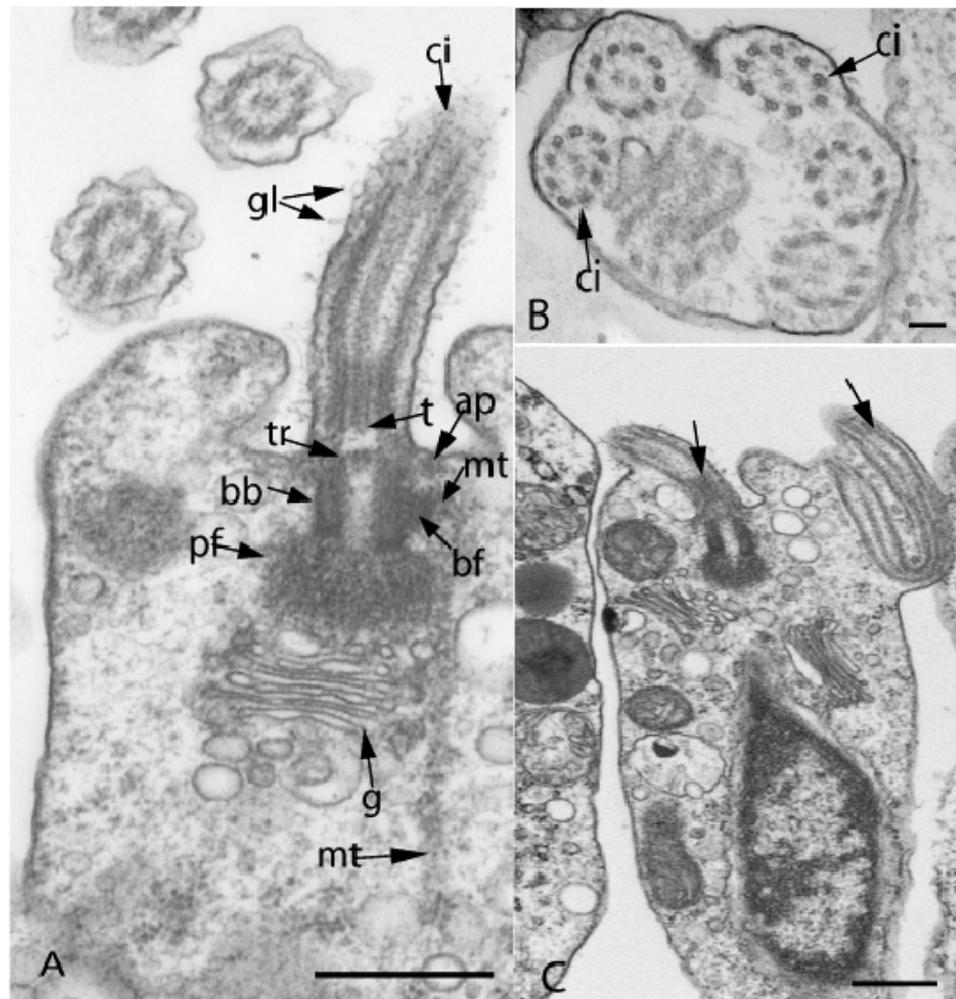


**Figure 2:** **A** SEM image of surface of *H. indistincta* larva showing cilia with some non-sponge material attached to them (arrows). **B** Longitudinal section of epithelial layer of cells. Arrows indicate the depth differences among the cells. **C** Typical example of a ciliated epithelial cell of the larvae (longitudinal section). g, Golgi apparatus; l, lipid; m, mitochondria; n, nucleus; va, vacuole. Scale bars: **A**=12 $\mu$ m, **B**=2 $\mu$ m, **C**=500nm.

Distinct apical-basal polarization was evident by localization of the cilium and its associated basal apparatus at the apical end and the large teardrop-shaped nucleus was at the other end (Figure 2C). Lipid droplets and electron-clear vacuoles were present throughout the cytoplasm. Also present were mitochondria that were spherical in shape that ranged in size from 325-475nm (n=5 average 371nm  $\pm$  60nm (SE)) or ovoid and ranged from 585-760nm in length (n=5 average 711nm  $\pm$ 72nm (SE)) and 350-490nm in width (412nm  $\pm$ 51nm (SE)) in shape (Figure 2C). From the longest axis, the nuclei varied from 1.3 to 3 $\mu$ m (n=5 average 2.4  $\pm$ 0.7 (SE)). The Golgi bodies were generally located near the base of the basal apparatus (Figure, 2C and 3A) but were often also seen in other locations in the cytoplasm.

*The cilium and its basal apparatus;* A cilium was situated at the central part of the apical surface of each epithelial cell and each was ringed by a small depression with an average depth of 324nm (n=5  $\pm$ 18nm (SE)) (Figure 3A). The ciliar axoneme was composed of the typical 9+2 microtubule organization (Figure 3A, B), surrounded by the cell cytoplasm and covered in glycocalyx (Figure 3A). Although mostly monociliated, multiple axonemes were seen inside the apical parts of one cytoplasm (Figures 3B-C) a feature that has been reported in several other sponge larval studies (e.g. Levi, 1964; Boury-Esnault and Vacelet, 1994; Boury-Esnault et al. 1999; Maldonado et al., 2003; Maldonado, 2004). The central microtubules of an axoneme terminated just above the apical surface of the cell's cytoplasm (approximately 50nm) and in the area of the terminating microtubules, there was an electron-dense area, of approximately 150nm in length, ascending up the shaft of the cilium (Figure 3A). Directly below the terminated central microtubules, there was an electron-clear space approximately 41-81nm in length and 97-114nm in diameter between the external microtubule doublets that remain

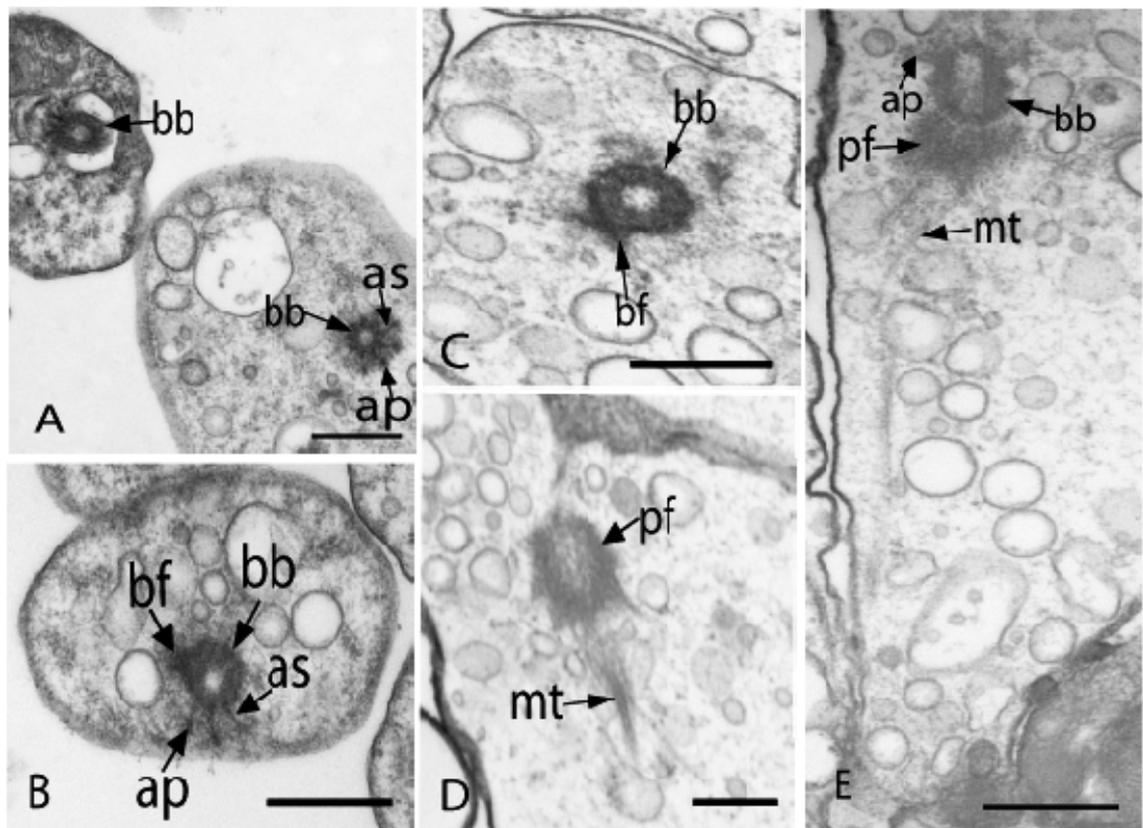
(Figure 3A). The basal body, was located directly below this electron-clear zone. The outer cilia microtubule doublets contacted the basal body (Figure 3A).



**Figure 3:** **A** Longitudinal section of the basal apparatus of an epithelial cell of an *H. indistincta* larva. **B** Cross-section of cilia showing the typical 9+2 configuration of the microtubules of the cilia. Also showing more than one cilium inhabiting one cytoplasm. **C** Longitudinal sections of epithelial cells showing more than one cilia present in one cytoplasm. Ap, anchoring point; bb, basal body; bf, basal foot; ci, cilia; pf, parallel fibers; t, terminating area; tr, transitional region; g, Golgi apparatus; gl, glycocalyx; mt, microtubules. **A**=500nm, **B**=100nm; **C**=500nm.

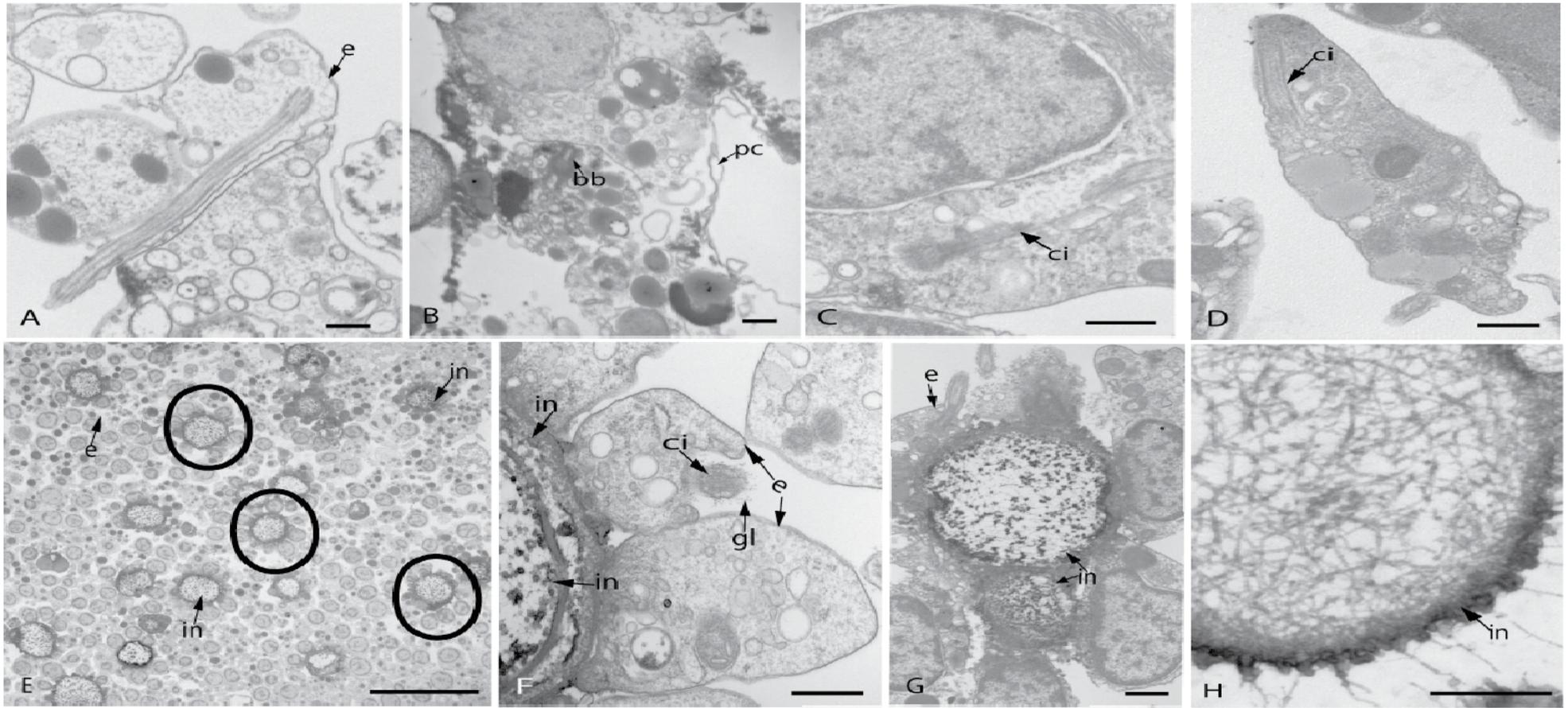
Overall, the basal body was approximately 286nm at the longest axis, and approximately 244nm in width (Figure 3A). A basal foot (approximately 94nm long) emerged from the side of the basal body and was essentially trapezoid in shape, being approximately 110nm in diameter at the base and approximately 104nm at its apical end (Figure 3A). An accessory centriole was not identified. Figure 4A-D shows cross-

sections of the transitional region between the proximal end of the cilium shaft and the basal body. Alar sheets radiated approximately 80nm from the basal body and connected to anchoring points. The anchor points were electron dense spheres, just under the surface of the apical region of the cytoplasm, and were 75nm in width on average ( $n=5 \pm 13.5$  (SE)) (Figures 4A-B). The longitudinal cytoskeleton of the cilium and basal apparatus consisted of a single microtubule that descended from the tip of the basal foot and ran parallel to the basal body deep into the cytoplasm of the cell towards the nucleus (Figure 3A), as well as a skirt of parallel fibers (also described in *H. tubifera* in Wollacott and Pinto, 1995) which were coupled with microtubule strands that were attached to the base of the basal body (Figures 4D-E).



**Figure 4:** A-B Cross-sections of epithelial cells of an *H. indistincta* larvae showing the transitional region between the proximal end of the cilia shaft and the basal body. C Cross-section showing just beneath the alar sheets, basal foot and basal body only seen. D Cross section showing parallel fibers and microtubules at an angle. E Longitudinal section of epithelial cell showing the basal apparatus and microtubules descending from the basal body. ap, anchoring point; as, alar sheet; bb, basal body; bf, basal foot; mt, microtubules; pf, parallel fibers. Scale bars: A-D=500nm, E=100nm.

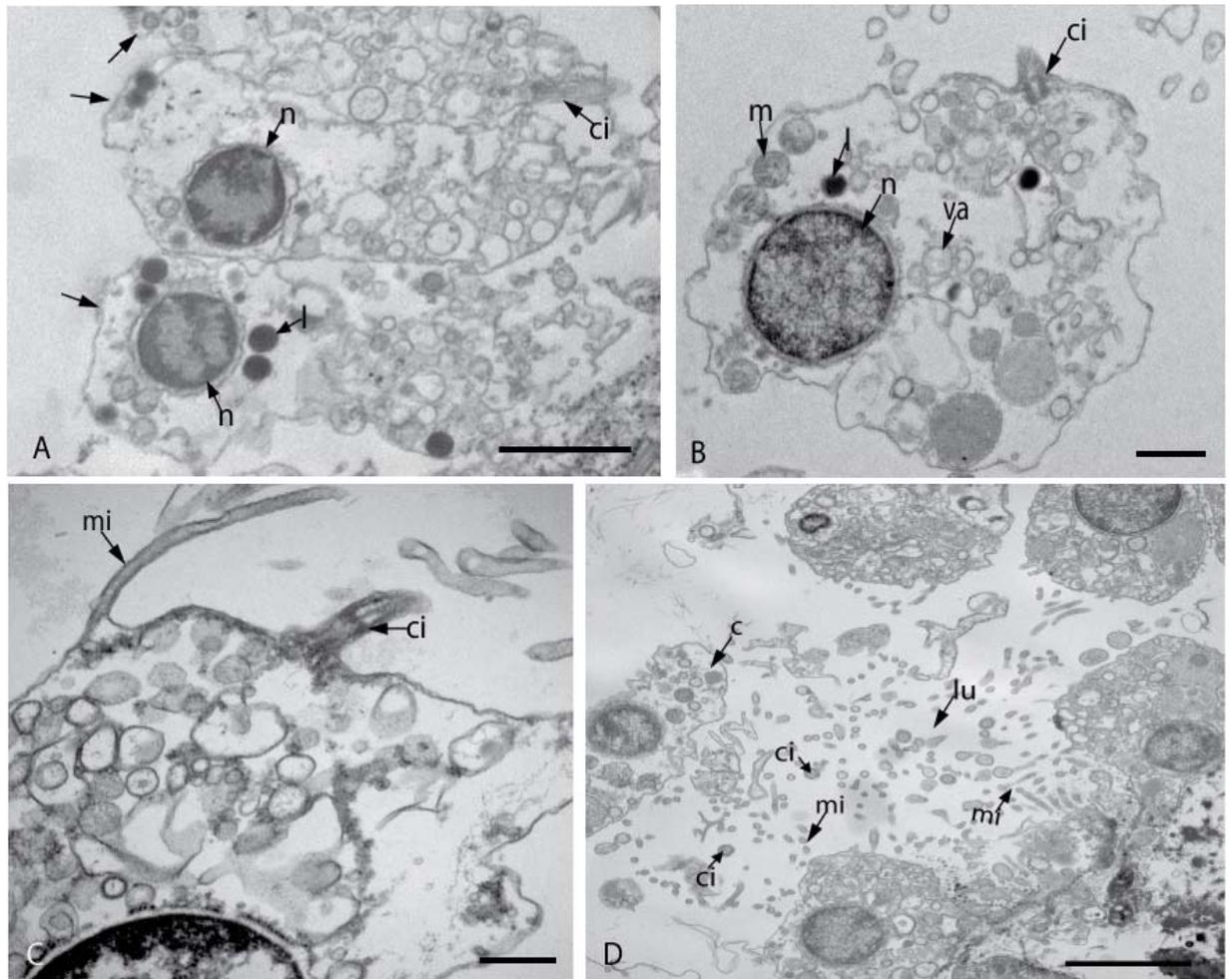
No differences were observed in the anatomy of the ciliated epithelial cells that occupied both the anterior and the posterior poles of the larvae. Some epithelial ciliated cells were found within the inner cell mass (ICM) of the larvae at both larval poles from the first free-swimming larval stage onwards (Figure 5 A). Remnants of ciliated cells were also present within the ICM of the larvae, e.g. the basal apparatus of cilia were contained within phagosomes of amoeboid cells (Figure 5B). Furthermore, remnants of cilia were observed in cells (not within phagosomes) which were reminiscent of modified epithelial cells (e.g. Figure 5 C-D). Within the ICM, numerous larval ciliated cells were attached to cells with large inclusions, which were very prevalent within the larvae (Figures 5E-G). The origin and chemical composition of these inclusions are so far unknown but appeared to contain a fibrous material (Figure 5H). These structures were variable in size and density. Expanded inclusions were much less dense than inclusions that were smaller and not expanded (Figure 5G).



**Figure 5:** **A** Epithelial cell seen within the ICM during the first free-swimming larval stage. **B** Longitudinal section of a cell within ICM of a free-swimming stage larva with the basal apparatus of a ciliated epithelial cell within a phagosome. **C** Longitudinal section showing epithelial cell of the first free-swimming larval stage of an *H. indistincta* larvae with a cilium and basal apparatus inside the cytoplasm. **D** Longitudinal section of epithelial cell within the ICM during the third of the free-swimming stages of this species (note the cilia enveloped by the cytoplasm). **E** Longitudinal section of an epithelial layer showing cells with inclusions associated with some epithelial cells. **F-G** Longitudinal sections of epithelial layer with cells with inclusions associated with some epithelial cells. **H** Close-up of inclusions. bb, basal body; ci, cilia; e, epithelial cell; gl, glycocalyx; in, inclusion; pc, phagocytosing cell. Scale bars: **A-D**=500nm, **E**=10 $\mu$ m, **F-H**=500nm.

*Ciliated cells of the juvenile H. indistincta:*

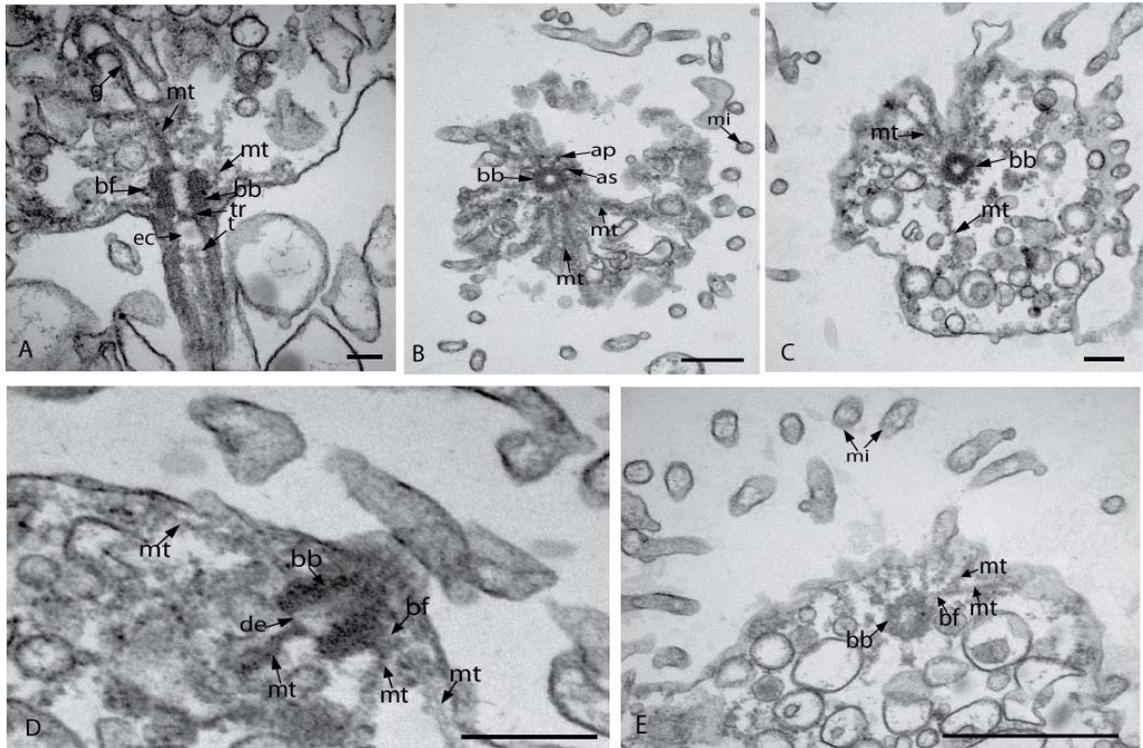
*General cytology;* Choanocytes of the juvenile *H. indistincta* were irregular in shape, and are reportedly highly plastic in morphology (Maldonado pers. comm.), however, two general forms existed; 1) pseudocylindrical with a length of 4-6 $\mu$ m (n=5 average 4.5 $\pm$ 0.85  $\mu$ m (SE)) and width of 1.6-3 $\mu$ m (n=5 average 2.4 $\pm$ 0.7 $\mu$ m (SE)) and 2) ovate to circular, 2.8-3.1 $\mu$ m in length (n=5 average 2.9 $\pm$ 0.1 $\mu$ m (SE)) and 3-4.5 $\mu$ m in width (n=5 average 3.5 $\pm$ 0.6 $\mu$ m (SE)) (Figures 6A, B). A spherical nucleus that ranged from 1.1 to 2  $\mu$ m in diameter (n=5 average 1.5 $\pm$ 0.2 $\mu$ m (SE)) was located at the basal part of the cell (Figures 6A-B) and occupied comparatively less space than the nuclei of the ciliated epithelial cells of the larva. Mitochondria that ranged from 335-440nm (n=5 average 380nm  $\pm$  37nm (SE)) were also seen within the cytoplasm (Figure 6B) and were more consistent in shape (i.e. circular) than those observed in the larval cells (which could be spherical or ovoid). As in the larval ciliated cells, numerous lipid droplets and electron clear and vacuoles were seen throughout the cytoplasm, but they were much more plentiful in the choanocytes (Figure 6A and B). Glycocalyx was present on the apical area of the cell's surface, as well as on the cilia. No specialized junction between the choanocytes was evident. Choanocytes were apical-basally polarized with a cilium encircled by a collar of microvilli (approximately 24-30) protruding from the apical surface (Figure 6C). The apical part of the cells were orientated towards the lumen of the choanocyte chamber (Figure 6D).



**Figure 6:** Details of the ciliated cells of *H. indistincta* juveniles. **A** Longitudinal section of a pseudocylindrically shaped choanocyte cell. (Section from juvenile settled 8 days). **B** Longitudinal section of an ovate shaped choanocyte cell. (Section from juvenile settled 32 days). **C** Longitudinal section of a choanocyte cell showing the microvilli protruding from the surface of the apical end of the cell. **D** View of a choanocyte chamber showing apical ends of the choanocyte cells facing toward the lumen. c, choanocyte cell; ci, cilium; l, lipid; lu, lumen; m, mitochondria; mi, microvilli; n, nucleus; va, vacuoles. **Scale bars:** A=2 $\mu$ m, B-C=500nm, D=2 $\mu$ m.

*The cilia and the basal apparatus;* The microtubules that composed the cilia axoneme were arranged in the typical 9+2 configuration. The central microtubules terminated further from the apical surface of the choanocytes (Figure 7A) than in the larval ciliated cells (150nm compared to 50nm). As in the larval cells there was an electron dense area that extended from the terminating end of the microtubules to approximately 120nm up the cilia shaft (Figure 7A). Below the terminated central microtubules, there was a larger electron-clear space that ranged approximately from 102-173nm in length and 61-123nm

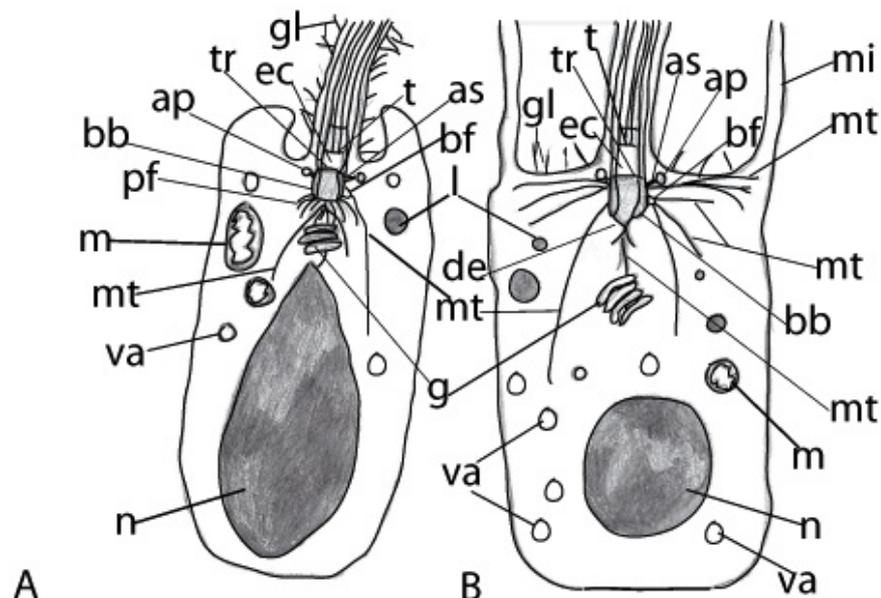
in width between the external microtubule doublets that remained (Figure 7A). The basal body of the choanocyte cilium, located directly below this electron-clear zone, had a transitional region between the proximal end of the cilia shaft and the basal body. From measurements taken along the longest axis, the basal body was approximately 265nm in length and approximately 240nm in width. Alar sheets, observed in Figure 7B, radiated from the basal body (approximately 100nm in length) and connected to electron-dense spheres, anchoring points, 61nm in diameter on average, ( $n=5 \pm 8.2\text{nm (SE)}$ ), and were positioned immediately under the surface of the apical region of the cell membrane (Figure 7C). Below the alar sheets, extended a basal foot approximately 95nm from the side of the basal body. The basal foot was roughly pyramidal in shape, being approximately 60nm in diameter at the apical end and approximately 105nm at the base (Figure 7A,D). Directly below the alar sheets, microtubules radiated in a spiral-like fashion, parallel to the surface (Figure 7C), which may be due to the angle at which the cells were cut. These microtubules, and a forked microtubule that extended from the tip of the basal foot, comprised the transverse cytoskeleton (Figure 7E). The microtubules were more extensive in these cells when compared to the larval cells. There were three microtubules attached above the base of the basal body instead of only one as in the larval epithelial cells (which was attached to the basal foot). Two extended from the basal foot (Figure 7A,D), one parallel to the basal body and one parallel to the surface of the apical end of the choanocyte (as mentioned above) (Figures 7E). The third microtubule was attached to the basal body opposite to the basal foot, and extended downward, toward the nucleus, also parallel to the basal body (Figure 7E). An accessory centriole was also not identified in the choanocytes. The basal body terminated in a  $90^\circ$  angle (Figure 7A). Attached at the tip of the basal body were a bundle of microtubules that extended to a Golgi apparatus, which along with the two longitudinally running microtubules mentioned previously, comprised the longitudinal cytoskeleton (Figure 7A,D).



**Figure 7.** **A** Longitudinal section including microtubule protruding from basal body. **B-C** Cross-sections showing the transition from the alar sheets attached to microtubules that radiate from the basal body in a spiral-like configuration. **D** Longitudinal section showing the basal apparatus of the cilia including microtubules protruding from tip of the basal foot. Also showing the distal end of the basal body and a typical example of the transitional cylinder, electron clear area and terminating area of the cilia seen. **E** Cross-sections showing branched microtubules. ap, anchoring point; as, alar sheet; bb, basal body; bf, basal foot; de, distal end; ec, electron clear zone; g, Golgi apparatus; mi, microvilli; mt, microtubules; t, terminating area; tr, transitional region. **Scale bars:** **A**=100nm, **B-E**=500nm.

*Comparison of ciliated cells of larva and juvenile;* Although the ciliated epithelial cell of the larva was similar in shape to the pseudo-cylindrically shaped choanocyte cells of the juvenile, otherwise, the gross morphology of the two cell types (e.g. cell size, shape and size of nuclei) was very different (Figure 8; Table 1). Also, although there were two general shapes of choanocyte cells (i.e. pseudo-cylindrical and ovate to circular), the only observable difference between the two was the shape of the cells. Thus, the cell drawn in Figure 8B although drawn as pseudo-cylindrically shaped, the basal apparatus drawn is representative of what was found in both choanocyte cell shapes. Vacuoles were much more numerous in the choanocyte cell, and the nucleus of the choanocyte cell was more compact than the nucleus of the ciliated epithelial cell. Also, the mitochondria were more

regular in shape in the choanocyte cells. While both cell types unsurprisingly shared the same cilium shaft configuration (9+2) and shape of the basal foot, the basal apparatus of the choanocyte had a noticeably more elaborate microtubular cytoskeleton than that of the larvae (Figure 8; Table 1). The choanocyte cell had a transverse cytoskeleton comprised of a collar of microtubules that extended from the basal body in a spiral formation (parallel to the apical end), as well as a forked microtubule that extended from the tip of the basal foot that also ran parallel to the apical surface of the cell, while the ciliated epithelial cell had only a longitudinal cytoskeleton. While the ciliated epithelial cell of the larva had a single microtubule extending from the tip of the basal foot running longitudinally towards the nucleus of the cell, a microtubule extending from the tip of the basal foot, running parallel to the basal body, was also present in the choanocyte cell, as well as a third microtubule extending from the basal body on the opposite side of the basal foot that also ran parallel to the basal body. However, the remaining features of the longitudinal cytoskeleton of the larval epithelial cell was more elaborate than the choanocyte cell of the juvenile, with a skirt of parallel fibers as well as microtubule strands, while only a bundle of microtubules were attached to the base of the basal apparatus of the choanocyte (Figure 8; Table 1).



**Figure 8:** A Schematic drawing showing the relevant organelles and structures observed in the longitudinal sections of the ciliated epithelial cell of the larvae (A) and choanocyte cell of the juvenile sponge (B) of *H. indistincta*. ap, anchoring point; as, alar sheet; bb, basal body; bf, basal foot; de, distal end; ec, electron clear zone; g, Golgi apparatus; gl, glycocalyx; l, lipid; m, mitochondria; mi, microvilli; mt, microtubules; n, nucleus; pf, parallel fibers; r, root; t, terminating area; tr, transitional region; va, vacuoles.

**Table 1.** Comparison of ciliated cells of larvae and the choanocytes of the juvenile of *Haliclona indistincta*: gross morphology of cell types, cilium and basal apparatus. **mt**=microtubules.

	<b>Cell characteristics</b>	<b>Larval ciliated epithelial cell</b>	<b>Juvenile choanocyte cell</b>
<b>Gross morphology of cell</b>	<b>Cell shape</b>	Pseudo-cylindrical	Pseudo-cylindrical Ovate to circular
	<b>Cell length and width</b>	5.4-8µm x 1-2µm (n=5)	4-6µm x 1.6-3µm (n=5)
			2.8-3.1µm x 3-4.5µm (n=5)
	<b>Glycocalyx</b>	Abundant	Sparse
	<b>Shape of nuclei</b>	Teardrop	Circular
	<b>Mitochondria length and width</b>	Circular: 325-475nm (n=5) Ovate: 585-760nm x 350-490nm (n=5)	Circular: 335-440nm (n=5)
	<b>Golgi Apparatus</b>	Numerous	Few
<b>Vacuoles</b>	Few	Numerous	
<b>Cilium and basal apparatus</b>	<b>Cilia shaft configuration</b>	9+2	9+2
	<b>Electron-free area at base of cilium</b>	41-81nm x 97-114nm	102-173nm x 61-123nm
	<b>Basal foot shape</b>	Trapezoid	Trapezoid
	<b>Transverse cytoskeleton: microtubules (mt)</b>	None	Two components: 1) collar of mt above basal foot; 2) single mt (forked) from tip of basal foot running parallel to the apical surface of cell
	<b>Longitudinal cytoskeleton</b>	Two components: 1) single mt attached to tip of basal foot parallel to basal body; 2) skirt of parallel fibers with few mt strands	Three components: 1) mt attached to tip of basal foot which ran parallel to basal body; 2) single mt located opposite side of basal foot; 3) grouping of mt attached to basal end of basal body (lacking fine parallel fibers)

## **Discussion:**

Through comparison of the cells that formed the ciliated epithelial layer of the larvae of the haplosclerid sponge *H. indistincta* and the cells that formed the choanoderm of the juvenile, I hoped to show a relationship between the two cell types and thus shed light on the origin of the choanocytes. The cell types were sufficiently different to prevent a direct link between the cells of the two developmental stages; however, all evidence suggests that the origin of the choanocytes is from the ciliated epithelial cells. Changes in the positioning of larval ciliated epithelial cells throughout metamorphosis, suggested two possible fates for the ciliated cells. Larval ciliated cells were observed intact in an internal position throughout the three pre-settlement stages. Migration of the larval epithelial cells inward has been described in some *Calcinea* species (Borojevic, 1969), and in the demosponge *Halisarca dujardini* (Gonobobleva and Ereskovsky, 2004). The first fate of these migrating cells is suggested by the reabsorption of cilia, which is evident in the cytoplasm of some cells. This fate I suggest, facilitates the cell's transformation into a choanocyte, a process that has been described in other haplosclerids, such as *Chalinula* sp. (Ilan and Loya, 1990), *Haliclona permolis* (Amano and Hori, 1996), *Amphimedon queeslandica* (Leys and Degnan, 2002) and the freshwater sponges *Eunapius fragilis*, *Ephydatia muelleri*, and *Spongilla lacustris* (Ivanova, 1997a; 1997b) as well as other demosponges (Borojevic and Lévi, 1965; Gonobobleva and Ereskovsky, 2004). Secondly, phagosomes with remnants of cilia and basal apparatus were observed within amoeboid cells suggesting that some cells are destroyed.

No previous studies concerning the metamorphosis of sponge larvae have mentioned an association of the larval cells with the cells with inclusions as observed in this study (Figure 5). Not all ciliated epithelial cells were associated with these cells with inclusions. When it occurred, the association involved the attachment of the epithelial cells to the cells

with inclusions by the basal end of the attaching cell. The cells with inclusions may have a role in the positioning and fate of the cells. Whole cells, and fragments of cells, were observed attached to these cells with inclusions. The cells with inclusions were concentrated underneath the layer of epithelial cells. This is the same location as the layer of an unidentified material described in Chapter 3 that separated the epithelial layer of the embryos from the ICM. However, throughout the pre- and post-settlement stages, cells with inclusions (and expanded inclusions apparently ejected from the cells), were also visible deep within the ICM of the larvae that were not observed in the embryos examined using histology perhaps due to the methods used. The larvae of *H. indistincta* were sticky. This study suggests that the sticky nature of the larvae may be used as a distinguishing taxonomic feature as also described by Lévi (1956) for the adult of this species. The larvae being sticky may be an adaptation to assist the larvae in settling in the intertidal zone (e.g. anchoring larvae and preventing them from being easily washed out to sea).

*H. indistincta* larvae differ from the larvae of other haplosclerid species described (e.g. *H. tubifera*) by lacking spicules, a trait which is shared with homoscleromorphs as well as some species of the demosponge orders: Halisarcida, Chondrosida, Dictyoceratida, Dendroceratida and Halichondrida (Woollacott and Hadfield, 1989; Woollacott and Pinto, 1995; Boury-Esnault et al., 2003; Maldonado et al., 2003; Ereskovsky and Tokina 2004; Usher and Ereskovsky, 2005; Gonobobleva, 2007; Gonobobleva and Maldonado, 2009; Ereskovsky, 2010). However, given the variation in the appearance of spicules across sponges in the literature (e.g. Ivanova, 1997a; 1997b), the timing of spicule production is likely to be variable and not of phylogenetic importance.

*H. indistincta* larvae also differ from other haplosclerid species described, in the ciliation pattern, being uniformly ciliated and lacking longer cilia at the posterior pole. (Appendix

4). Also unlike other haplosclerid species, but similar to poecilosclerid larvae (i.e. *Mycale syrinx*), *H. indistincta* larval settlement occurs via the posterior pole (Wilson, 1935; Bergquist and Sinclair, 1968; Ereskovsky, 2010; Stephens et al., in press). The positioning of the choanocyte chambers in *H. indistincta* adults is also more similar to poecilosclerids than to other haplosclerid species (including: *H. oculata*, *H. rosea*, *H. simulans*, *H. fistulosa*, *H. elegans*, and *Niphates digitalis*) a feature which led Langenbruch and Jones (1990) to suggest that *H. indistincta* is of poecilosclerid, and not haplosclerid, origin.

Furthermore, the basal body configuration of the ciliated larval epithelial cells of *H. indistincta* and *H. tubifera* (as described in Woollacott and Pinto (1995)) were very different. The most apparent distinction was the more simple microtubule configuration of *H. indistincta*'s ciliated epithelial cells which lacked the lateral arms observed in *H. tubifera* (Appendix 5 and 6) (Figure 8) (Woollacott and Pinto, 1995). This evidence may support the suggestion by Lagenbruch and Jones (1990), that *H. indistincta* is not a member of *Haliclona* and may not be a member of the Haplosclerida. However, phylogenetic reconstructions from two independent gene loci, 28S ribosomal RNA and the mitochondrial cytochrome oxidase subunit 1 (mtCOI) genes confirm *H. indistincta*'s place within the marine haplosclerids (Chapter 2). Although *H. indistincta* (along with *H. viscosa*) grouped away from the clade containing the type species (*H. oculata*), grouping more closely with species of *Niphates*, while *H. tubifera* grouped closely with *H. oculata*, and other species of that genus (Chapter 2; Redmond et al., 2011).

Bergquist et al. (1979) suggested larval ciliation pattern to be an informative character for sponge taxonomy; however, finding congruence between classification and ciliation patterns within the order Haplosclerida has been difficult. The ciliation pattern of *H. tubifera* larvae is considered typical of *Haliclona* species. The larva are uniformly ciliated

with a ring of longer cilia encircling a bare posterior pole (Woollacott, 1993; Woollacott and Pinto, 1995). *H. oculata* larvae also share this ciliation pattern (Wapstra and Van Soest, 1987), however, *H. indistincta* does not. The ciliation pattern observed for *H. indistincta* is present in freshwater haplosclerids (i.e. *Ephydatia fragilis*, *E. muelleri* and *S. lacustris*) (suborder Spongillina) which have recently been shown to be more closely related to Demosponges outside of the order Haplosclerida, (Appendix 4) via ribosomal data (e.g. Borchellini et al., 2004; Nichols, 2005; Itskovich et al., 2007; Raleigh et al., 2007; Redmond et al., 2007; Lavrov et al., 2008; Redmond and McCormack, 2008; Redmond et al., 2011). The uniform ciliation pattern present in *H. indistincta* is also observed in homoscleromorph species *Oscarella imperialis* and *O. tuberculata* (Appendix 4) (Ivanova, 1997a; 1997b; Maldonado and Bergquist, 2002; Boury-Esnault et al., 2003; Ereskovsky et al., 2007; Ereskovsky, 2010); while Ilan and Loya (1988), described a *Niphates* sp. (later referred to as *Niphates rowi* in Ilan et al. (2004)), as a parenchymella type larvae, covered in cilia with longer cilia forming a ring around the posterior pole. Until more larvae of different species are investigated, we will not be able to determine how plastic the ciliation pattern is among larvae, and whether or not it has phylogenetic relevance within certain groups.

Woollacott and Pinto (1995) showed that basal apparatus characters can be useful in identifying closely related species as congruence was found among the poecilosclerid species studied (i.e. *M. cecilia*, *M. contarenii* and *Hamigera hamigera*) in the basal foot morphology, and within the halichondrids, (i.e. *Halichondria melanadocia*, *H. coerulea*, and *H. heliophila*), using basal body morphology (Woollacott and Pinto, 1995). Woollacott and Pinto (1995) also included three haplosclerid species, *H. tubifera* (as mentioned above) which they compared with two *Haliclona* spp.. However, there was data lacking for the *Haliclona* spp., and they were unable to make complete observations concerning the fine

detail of the basal apparatus. However, unlike *H. tubifera*, a transverse cytoskeleton was absent, as was also observed in *H. indistincta*.

Unfortunately, the level of detail included in this work and Woollacott and Pinto's (1995) study, is not yet available for many species so it is not possible to accurately determine the phylogenetic signal associated with the six characters included in Woollacott and Pinto (1995) (i.e. basal body, basal foot, accessory centriole, transverse and longitudinal cytoskeletal system, and the association of the cytoskeleton (i.e. rootlet system) with Golgi body). However, some general comparisons can be made. Despite the apparent lack of similarities between the larval and juvenile ciliated cells in *H. indistincta*, the overall shape of the cells which was similar to that of other sponge orders and freshwater haplosclerids (i.e. *E. fragilis*, *E. muelleri* and *S. lacustris* (Ivanova, 1997a; 1997b)). Gonobobleva and Maldonado (2009) suggest that microtubules, like those that projected from the basal body in a spiral-like arrangement parallel to the apical end of the of the choanocytes of *H. indistincta* are a typical feature of choanocyte cells and have been observed in various other species e.g. *H. rosea* (Garrone, 1969), *Corticium candelabrum* (Boury-Esnault et al., 1984), and *Halisarca dujardini* (Gonobobleva and Maldonado, 2009). Interestingly, *H. indistincta* also possesses a bundle of microtubules attached to the base of the basal body (Table 1; Figure 7A, D, and Figure 8) that is uncommon in other sponge choanocyte cells (Woollacott and Pinto, 1995; Gonobobleva and Maldonado, 2009).

A common structure present in ciliated eukaryote epithelial cells is the accessory centriole (Nielsen, 1987). The accessory centriole is involved during cell division as microtubule organizing centres (Gonobobleva, 2007). However, accessory centrioles were not observed in either the larval ciliated epithelial cells, or the choanocyte cells of *H. indistincta* (Table 1; Appendix 5). The absence of accessory centrioles was also recorded in *H. tubifera*, and

*M. cecilia* (Woollacott and Pinto, 1995), but these are otherwise commonly found in sponge larvae (e.g. Homosclerophorida, Dictyoceratida and Halisarcida) (Maldonado et al., 2003; Ereskovsky and Tokina 2004; Ereskovsky et al., 2007; Gonobobleva, 2007; Gonobobleva and Maldonado, 2009). However, that is not to say that other features of the basal apparatus of the cilia are not informative. As Woollacott and Pinto's (1995) comparative study of eleven species (across five demosponge orders) found, basal apparatus characters can be useful in identifying closely related species. Therefore, additional comparative studies targeting the basal apparatus of putatively closely related species (e.g. *H. indistincta* and *H. viscosa*) are necessary for determining the level at which these characters are informative.

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Chapter 5:

**Cell types and their dynamics in *Haliclona indistincta*  
from pre- to post-settlement.**

**Introduction:**

Sponges are considered to have a very simple body plan with three defined layers: an external epithelial-like layer that separates the animal from its environment, an internal epithelial-like layer, and a glycoprotein and collagen-rich matrix, the mesohyl, that fills the space between the two layers (Pavans De Ceccatty, 1979; Maldonado and Riesgo, 2007; Riesgo et al., 2007; Maldonado and Riesgo, 2008). As simple as their general body plan is, the cells that form a sponge body function in complex ways (Gaino and Burlando, 1990). The ability for almost all adult sponge cells (excluding gametes) to undergo cellular differentiation (and dedifferentiation) into other cell types, gives poriferans a level of morphological plasticity that is unique amongst invertebrates (Gaino and Burlando, 1990; Maldonado and Riesgo, 2008; Ereskovsky et al., 2009). This plasticity is most apparent during the early development of a sponge when a free-swimming larva will settle upon a substrate and metamorphose, forming the sessile body plan of a juvenile sponge (Ereskovsky et al., 2007, Leys and Degnan, 2002). After settlement, the exo-, endo- and baso-pinacodermal layers are first to form, followed by the aquiferous system, choanocyte chambers, and the skeletal structure (Ereskovsky et al., 2007). To serve new functions for the juvenile sponge, cells differentiate and transdifferentiate into specialized cells (e.g. larval ciliated epithelial cells needed for swimming, later transdifferentiate into choanocyte cells needed for feeding) (Borojevic, 1966; Boury-Esnault, 1976; Simpson, 1984; Amano & Hori, 1998; Maldonado, 2004; Leys and Eerkes-Medrano, 2005; Ereskovsky et al., 2007; Chapter 4). However, because of the dynamic nature of sponge cells, even adult sponges are not considered fully developed as their body is in a constant state of change (Diaz et al., 1974; Pavans De Ceccatty, 1978; Gaino and Burlando, 1990; Gaino et al., 1995; Korotkova, 1997; Ereskovsky et al., 2009).

Sponge metamorphosis has been the subject of several studies (e.g. Brien and Meewis, 1938; Levi, 1956; Ilan and Loya, 1990; Amano and Hori, 1993, 2001; Leys and Degnan, 2002; Gonobobleva and Ereskovsky, 2004; Maldonado, 2006; Ereskovsky et al., 2007). Because sponges are considered the most basal of the metazoans, by observing and understanding their cellular organization and the processes concerning gametogenesis, embryogenesis and metamorphosis, in this group, it may be possible to infer information concerning the early evolution of multicellular animals (Bagby, 1970; Kobayashi et al., 1993; Gonobobleva and Ereskovsky, 2004; Philippe et al., 2009; Ereskovsky, 2010). Without even a rudimentary nervous system, sponge larvae often show complex response behaviour towards external stimuli such as light (Ereskovsky and Tokina, 2004); and indeed, must respond to various stimuli that initiate settlement and metamorphosis.

There are five categories of cell types in demosponges: cells that line surfaces (i.e. pinacocytes, porocytes and choanocytes), contractile cells (i.e. actinocytes), secretory cells (i.e. collencytes, lophocytes, spongocytes, and sclerocytes), amoeboid cells (e.g. archaeocytes), and cells with inclusions (e.g. vacuolar, spherulous, microgranular, gray, globoferous, rhabdiferous, spumeuse, sacculiferous cells, and stylocyte, and chromocytes) (Bergquist, 1978; Boury-Esnault, 2006; Ereskovsky, 2010). Cells that line surfaces are present in all species (Simpson, 1984). The term ‘amoebocyte’ is attributed to any sponge cell that has no precise activity that can be determined (Simpson, 1984; Ereskovsky, 2010). The location of a cell in a sponge can denote function, which can be important for identifying cell types (Bergquist, 1978; Simpson, 1984). For example, the three different types of pinacocytes correlate to their specific location in the sponge body; exo-pinacocytes comprise the external surface while, endo-pinacocytes line canals, and the baso-pinacocytes line the base of the sponge forming the attachment epithelium (Bergquist, 1978; Simpson, 1984). Endo-pinacocytes are divided into two categories: proso-

pinacocytes which line the inhalant canals, and apo-pinacocytes that line the exhalant canals (Ereskovsky, 2010).

There are multiple types of larval morphology described for sponges, and larvae have highly variable free-swimming stages, in both the time it takes for a larva to settle (e.g. Bergquist et al., 1970; Maldonado, 2006), as well as any morphological changes that may occur (Mariani et al., 2006). As described in Stephens et al. (in press) (Chapter 3), *H. indistincta* generally undergoes three morphologically distinct mobile stages before settlement. Cell types within these three free-swimming stages have not been described.

Ereskovsky (2010) compiled data on the metamorphosis of marine haplosclerids and suggested a scheme showing the general trends in cell fate for this group. At the larval stage, ciliated cells, sclerocytes, collencytes and archaeocytes are present, deriving from peripheral and internal blastomeres; which in turn (during metamorphosis) differentiate into juvenile cells (e.g. choanocytes, sclerocytes, pinacocytes, secretory cells). According to Ereskovsky (2010), ciliated cells may trans-differentiate into choanocytes or sclerocytes, juvenile sclerocytes and exo-pinacocytes may be derived from larval collencytes; and larval archaeocytes may remain archaeocytes, differentiate into secretory cells, baso-pinacocytes, or differentiate into “other” (un-specified) cell types.

Eleven (or more) pluripotent and differentiated cell types were described in the larvae of *Amphimedon queenslandica*, a marine haplosclerid (previously *Reniera* sp., Leys and Degnan, 2001; Leys et al., 2002; Degnan et al., 2005; Renard et al., 2009). Spherulous cells and sclerocytes were present in the above species while the larvae were developing (Leys and Degnan, 2002), while Uriz et al. (2001) showed that cells with inclusions (i.e. spherulous cells) may not be differentiated at the larval stage in all demosponges, as shown

in *Crambe crambe* (Uriz et al., 1996). Spherulous cells were also recorded in other demosponge larvae (e.g. *Halisarca dujardini*) (Ereskovsky and Gonobobleva, 2000; Mukhina et al., 2006; Ereskovsky and Boury-Esnault, 2002; Ereskovsky, 2010) as have vacuolar cells (e.g. *Hamigera* sp. Simpson, 1984). However, in some cases, specialized cells with inclusions observed in larvae (i.e. spherulous and microgranular cells), have been suggested to be cells passed to the larvae from the mother, as suggested to be the case for verongid sponge (*Aplysina aerophoba*) by Maldonado (2009).

Unlike archaeocytes and other cells found in all demosponges, cells such as vacuolar cells, have been found to show phylogenetic signal for closely related species (e.g. species within genera *Oscarella* and *Halisarca*) while other cells such as spherulous cells have been found to be species specific in their morphology (Bergquist, 1996; Muricy et al., 1996; Boury-Esnault, 2006; Ereskovsky, 2006; Ereskovsky, 2010). Unfortunately, for studies whose focus is not on describing the various cell types present in larvae and juveniles after settlement (e.g. Woollacott, 1993; Uriz et al., 2008) often amoeboid cells are referred to in general terms (e.g. archeocyte-like cells) without giving much detail about the ultrastructure of the cell types or attempting to draw parallels with known cell types, which means there maybe additional cell types that remain undescribed, and possible synapomorphies between sponge species that remain undocumented.

The primary aims of this study were to describe: how the body of the larva of *H. indistincta* changes through the free-swimming stages and metamorphosis (including cell fate); the various cell types observed in each developmental time point/stage and their location; the non-cellular structures (e.g. spicules) and at what stage they are produced. Specifically relating to the first two free-swimming stages, I wanted to discover what of the internal anatomy of the larva reflected the distinct anterior-posterior orientation

observed (i.e. any specialized cells or structures at the posterior/and or anterior end). The overall aim of this project was to further contribute to knowledge of development to inform evolutionary pathways in haplosclerids.

## **Materials and Methods:**

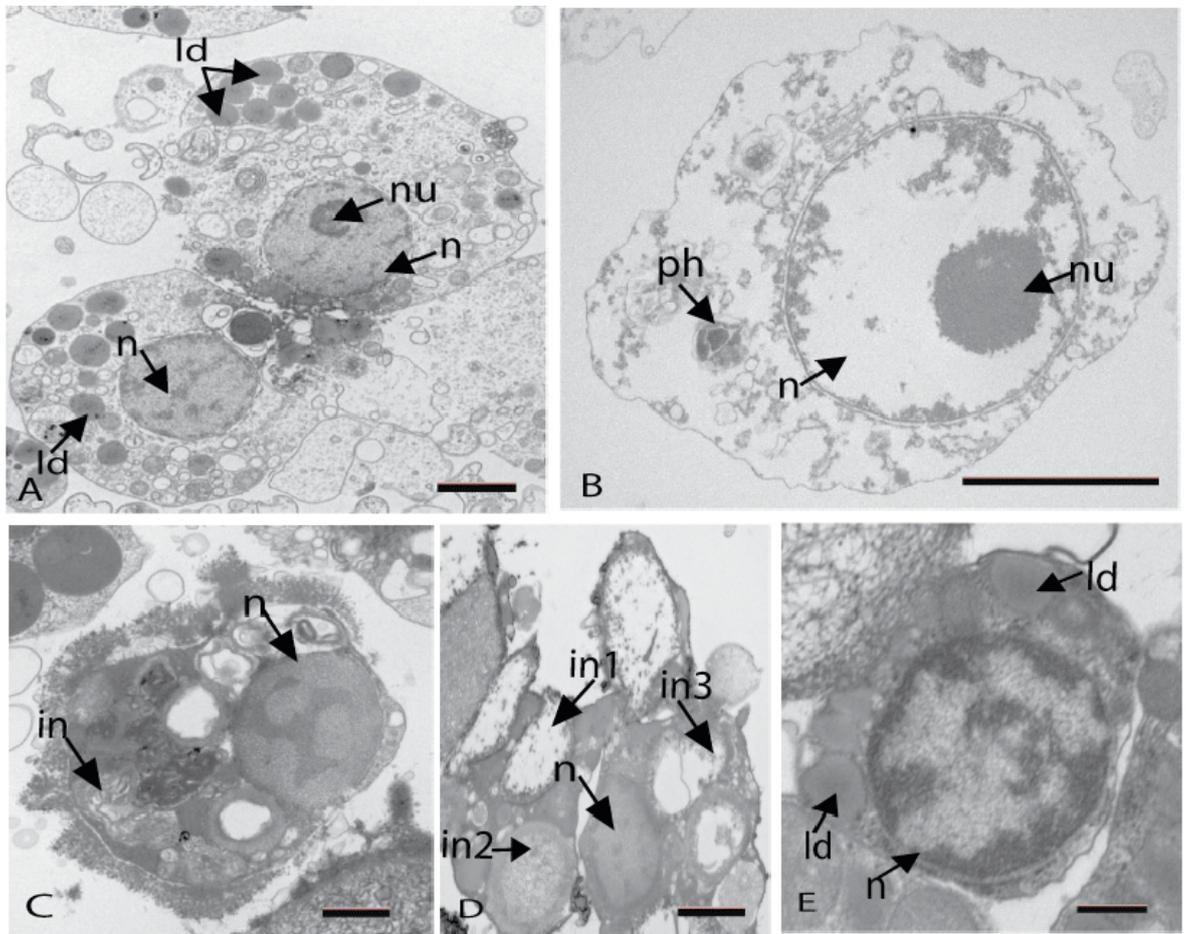
*Specimens;* Transmission electron microscopy (TEM) was employed to describe metamorphosis in *H. indistincta* (Morphotype 1) and the cellular dynamics from the free-swimming to sessile stage. Larvae were maintained in Petri dishes and monitored until they achieved the desired developmental stage (full details in Stephens et al., in press; Chapter 3). Two specimens from each of the following stages were sectioned for electron microscopy: Free-swimming stage 1 (one specimen fixed after approximately 24 hours post-release, and one immediately after release from parent (Chapter 3 for details of larval release)), free-swimming stage 2 (both fixed approximately 45 hours after release) and free-swimming stage 3 (both fixed approximately 49 hours after release), specimen settled for 1 hour, specimen settled for 24 hours, specimen settled for 48 hours, specimen settled for 96 hours, and specimen settled for 768 hours. A single additional specimen that had been settled for 192 hours was also sectioned.

*Fixation;* Specimens were placed in a primary fixation solution composed of 2% glutaraldehyde, 2% paraformaldehyde in 0.1M sodium cacodylate/HCL buffer for 24hours. Specimens were then postfixed in 1% osmium tetroxide in 0.1M sodium cacodylate/HCL buffer for about two hours. They were then dehydrated in a graded ethanol series and embedded in Agar low viscosity resin (R1078 resin kit (Agar Scientific)). Semi-thin and ultra-thin sections were obtained at 20-30micron increments, with a Reichert-Jung Z00M Stereo-Star Ultra-cut ultra-microtome. Ultra-thin sections were mounted on copper (200 $\mu$ m) mesh grids, and stained with Ultrastain 2 for 36 minutes via Leica EM (AC20). Hitachi H7000 Transmission Electron Microscope, operating at 75kV, was used to conduct the observations and imaging of the grids. In total, for all stages of development, approximately 9,150 images were obtained. All measurements of organelles and cells were taken at the longest diameter (averages and standard error were calculated in Excel).

**Results:**

*Free-swimming stage 1*; As previously reported, larvae immediately after release were oval in shape and completely and evenly ciliated (Stephens et al. (in press); Chapter 3). They also showed distinct anterior-posterior orientation, the anterior end (defined by the direction of movement), was wider than the posterior end. Five different cell types were present (i.e. ciliated epithelial cell, Type 1 and Type 2 Archeocyte-like cells, Type 1 and Type 2 cell with inclusions). The absence of spicules and spicule producing cells, as well as collagen secreting cells (e.g. collencytes), was very apparent. Ciliated epithelial cells comprised the epithelial layer and were fully described in Chapter 4.

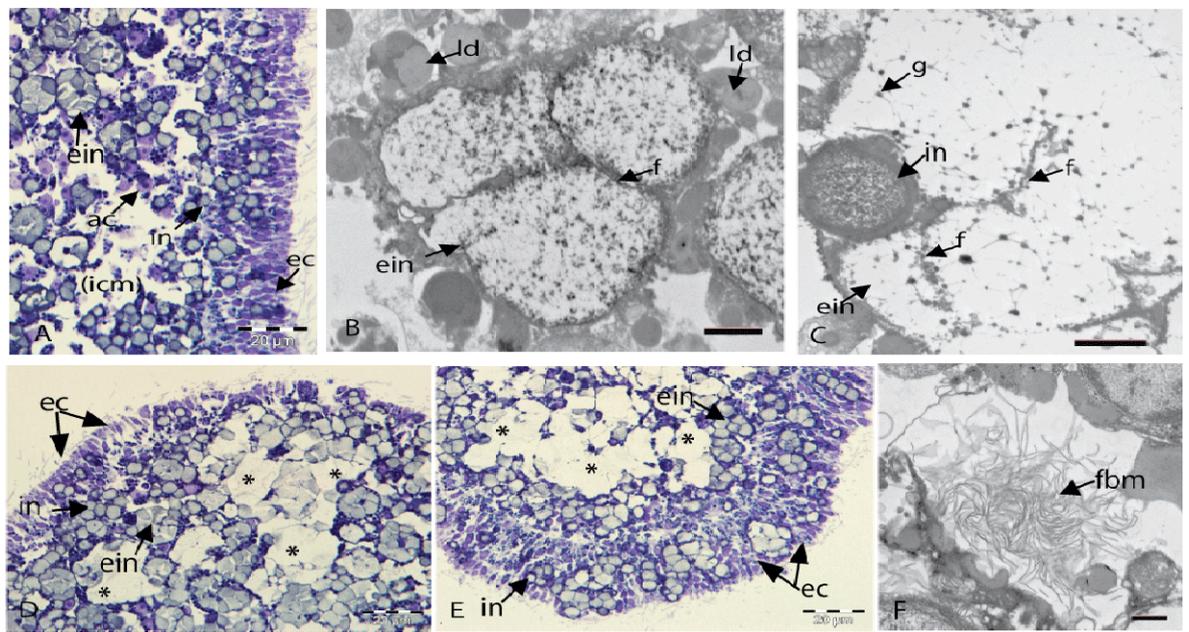
*Amoeboid archeocyte-like cells*; These cells displayed a prominent nucleus, lipid droplets, phagosomes, golgi apparatus, mitochondria and an endoplasmic reticulum (last four structures not visible in this image Figure 1A). These archeocyte-like cells varied in size from 6 $\mu$ m to 13 $\mu$ m in length (n=5, average of 9 $\mu$ m  $\pm$  3 $\mu$ m S.E.) and 3 $\mu$ m to 7 $\mu$ m width (average 5 $\mu$ m  $\pm$  2 $\mu$ m S.E.) and are here referred to as Type 1 archeocyte-like cells. A second amoeboid archeocyte-like cell observed (Type 2) varied in size from 4 $\mu$ m to 8 $\mu$ m in length (n=5, average of 6 $\mu$ m  $\pm$  4 $\mu$ m S.E.) and 3 $\mu$ m to 7 $\mu$ m width (average 4 $\mu$ m  $\pm$  2 $\mu$ m S.E.) and was square in shape. This cell type had a particularly large nucleus to cytoplasm ratio with comparatively small amount of cytoplasm that had few phagosomes and abundant endoplasmic reticulum (not visible in image used) (Figure 1B).



**Figure 1.** *H. indistincta* larvae free-swimming stage 1: Oval. **A** Typical Type 1 archeocyte-like cell. **B** Typical Type 2 archeocyte-like cell. **C** Type 1 cell with small inclusions. **D** Type 1 cells with large inclusions (3 different types: in1=fibrous with small granules; in2=fibrous with no or smaller granules; in3=thick fibres). **E** Typical Type 2 cell with inclusions. ld=lipid droplet; in=inclusion; n=nucleus; nu=nucleolus; ph=phagosomes. Scale bars: **A**=500nm, **B**=2µm, **C**-**E**=500nm.

*Cells with inclusions;* There were two general types of cells with inclusions observed (Figure 1C-D (Type 1); and E (Type 2)). Type 1 had a large prominent nucleus (non-nucleolated in figure), and produced inclusions (described in Chapter 4) that contained fibers and smaller granules which were prominent in all life stages of this species (Figure 1C). The inclusions were variable in number and appearance (e.g. have thick or thin fibrillar material within them) (Figure 1C-D; Figure 2). This cell type ranged in length from 4µm to 7µm (n=5, average 5µm ± 1µm S.E.) and 3µm to 5µm in width (average 4 µm ± 1µm S.E.). The cytoplasmic membrane was uneven and appeared to have particles adhered to the outside of it. The cytoplasm was irregular in shape due to the inclusions within the cytoplasm. The inclusions appeared to expand within in the cell and be released

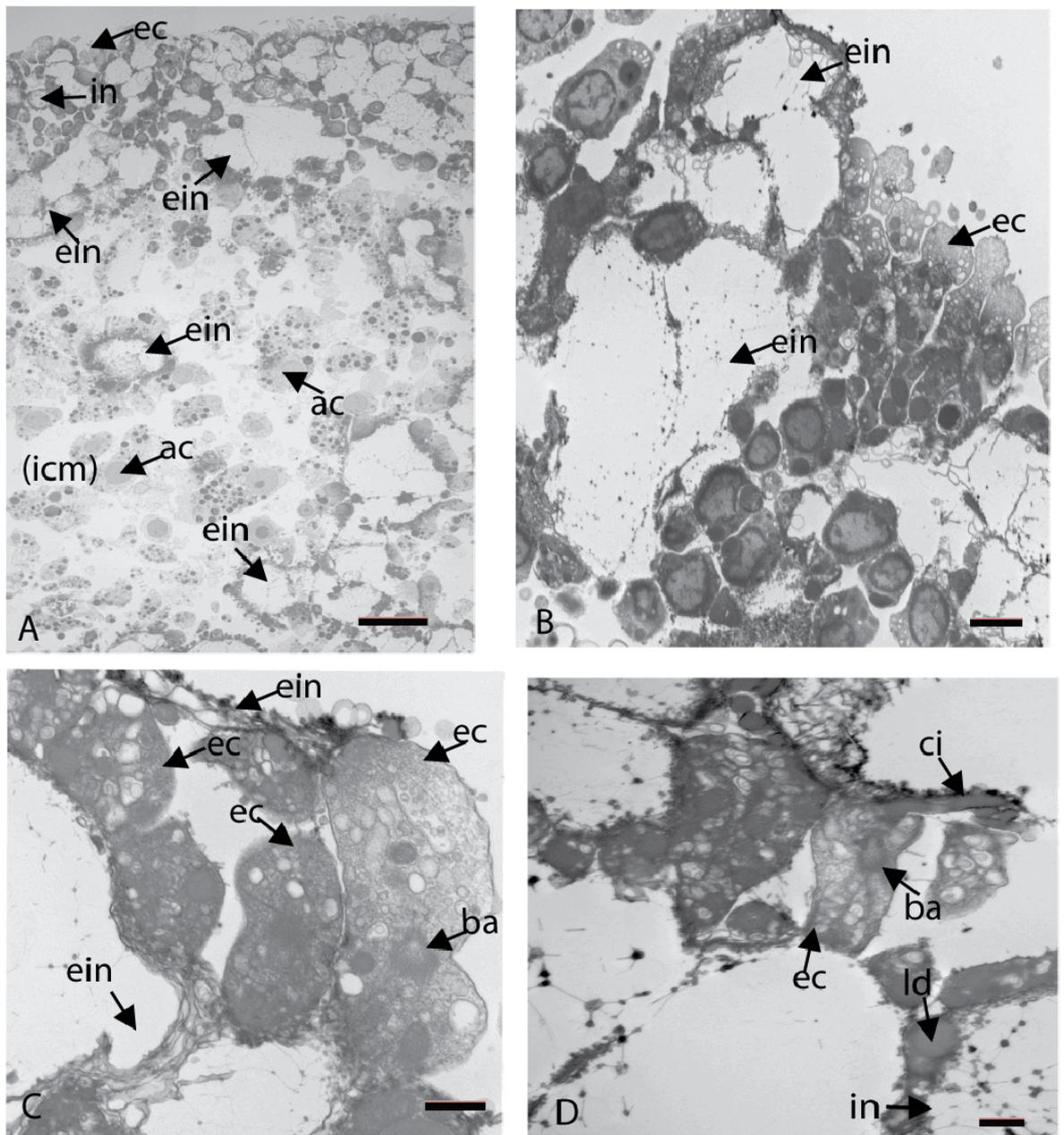
from the cell at different times. When expanded, the inclusions became less dense. The expanded inclusions appeared to become merged with other inclusions almost completely showing only traces of the outside layer that previously encapsulated the fibrillar-like material within the particle structures (Figure 2C). There was a concentration of these cells with inclusions at the anterior and posterior ends of the larvae (Figure 2 D-E). Type 2 cell with inclusions had a nucleus that was much larger in proportion to the amount of cytoplasm. It ranged in size from 2 $\mu$ m to 4 $\mu$ m in length (n=5, average 3 $\mu$ m  $\pm$  0.4 $\mu$ m S.E.) and 2 $\mu$ m to 3 $\mu$ m in width (average 2 $\mu$ m  $\pm$  0.4 $\mu$ m S.E.). The characteristic feature of this cell type was the lipid droplet-filled cytoplasm (Figure 1D). A non-cellular material, also observed within the inner cellular mass (ICM) of the larvae is what appeared to be an extracellular fibrillar material similar to inclusions released by spumeuse cells described in Donadey and Vacelet (1977) and Simpson (1984) (Figure 2F).



**Figure 2.** *H. indistincta* larvae free-swimming stage 1: Oval. **A** Semi-thin section (longitudinal) of an *H. indistincta* larva showing the flask-like ciliated epithelial cells at the epithelial zone, Type 1 cells with inclusions under the ciliated epithelial cells and amoeboid cells within the inner cellular mass (ICM) of the larvae. **B-C** Example of expanded inclusions, individual inclusion boundaries barely present. **D** Longitudinal semi-thin section showing the concentration of expanded inclusions at the anterior end of larvae. **E** Longitudinal semi-thin section showing the concentration of circular particle complexes at the posterior end of larvae. **F** Fibrillar material observed in inner cellular mass of the larvae. ac=amoeboid cell; ec=ciliated epithelial cells; ein=expanded inclusion; f=fibers; fbm= fibrillar material; g=granules; icm=inner cell mass; in=inclusion; ld=lipid droplet. Scale bars: A=20 $\mu$ m, B=500nm, C=2 $\mu$ m, D-E=20 $\mu$ m, F=500nm. \* denotes area formed by further expanded inclusions.

*Cellular organization in Stage 1;* Ciliated cells formed the epithelial layer. Type 1 cell with inclusions (i.e. organelles that appeared to contain smaller granules and fibers), were adjacent to each other and were positioned immediately below the epithelial cells (Figure 2A). There was a higher proportion of these cells with expanded inclusions at the anterior and posterior ends of the larvae; and constituted a greater proportion of the anterior end than the posterior end (Figures 2D-E). Small, dense inclusions approximately 2µm in size were also observed within the large areas of expanded inclusions. Apart from ciliated cells, all other cell types were primarily found in the ICM. Aside from the spaces created by the expanded inclusions (of Type 1 cell with inclusions), there was a lot of empty space between the cells within the ICM (Figure 2 A).

*Free-swimming stage 2: (approximately 45 hours post-release);* All elements present in Stage 1 (i.e. ciliated epithelial cells, amoeboid cells (Types 1 and 2), cells with inclusions (Types 1, and 2) were also found in Stage 2 with the addition of two secretory cell types (Table 1). Inclusions previously referred to were enlarged and more extensive than in Stage 1. Ciliated cells of the typical larval form were still observed at the external surface (Figure 3 A-B). Additionally, ciliated cells with an altered morphology were evident; including the shape of cell, absence of large tear-drop shaped nucleus and in some cases, no longer contained the cilium but still retained traces of the basal apparatus (Figure 3C-D).



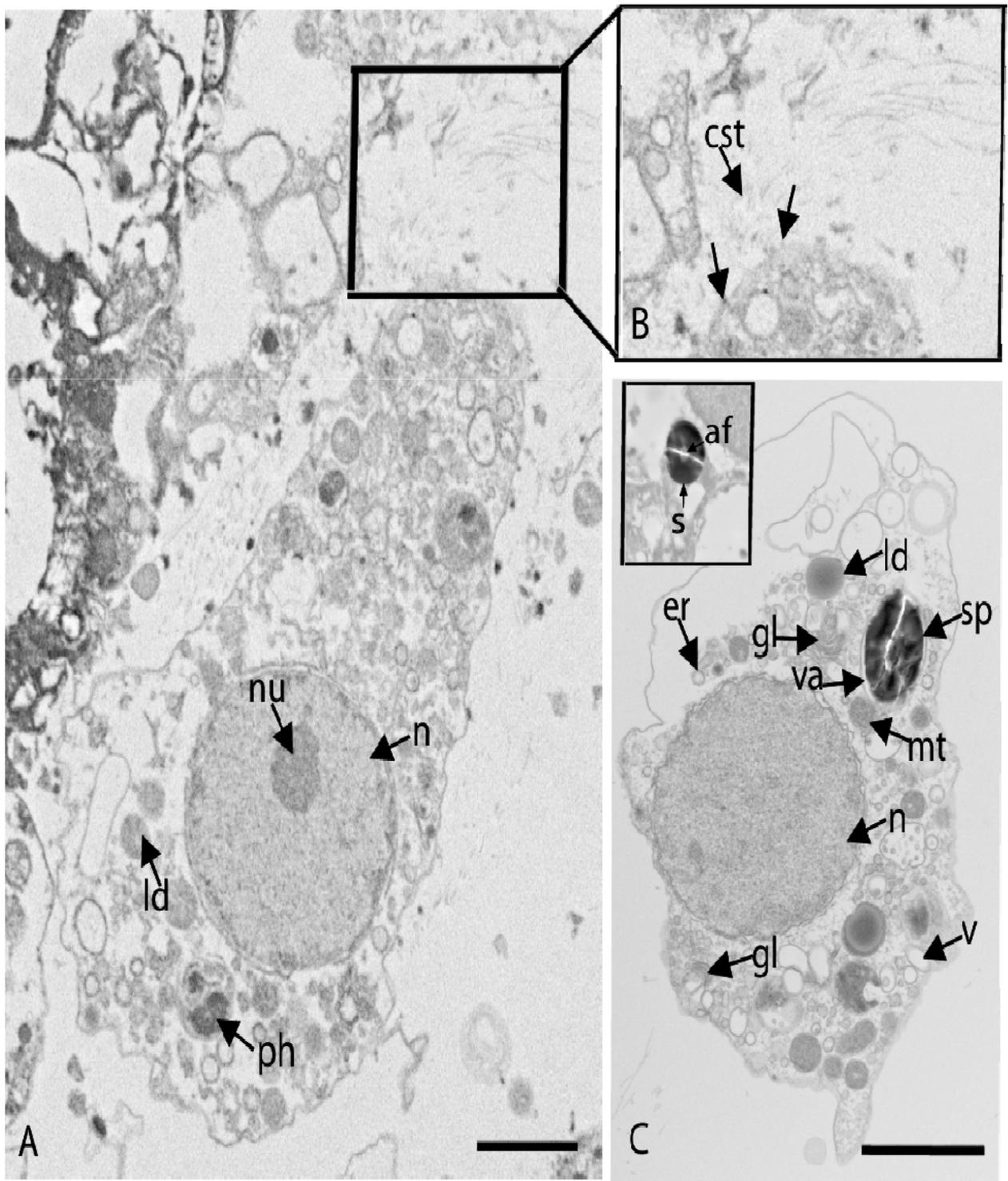
**Figure 3. *H. indistincta* larvae free-swimming stage 2: Compact angular.** **A** Cross-section showing from the epithelial layer to the inner cellular mass of the larva. Expanded inclusions (from Type 1 cell with inclusions) prominently positioned at the perimeter of the larvae at this stage. **B** Close-up of cross-section of the epithelial zone of the larvae showing expanded inclusions inhabiting the epithelial zone along with ciliated epithelial cells. **C** Example of expanded inclusions covering ciliated epithelial cells with the material from inside the inclusions. **D** Expanded inclusion material covering ciliated epithelial cells. ac= amoeboid cells; ba=basal apparatus; ci=cilium; ec=ciliated; epithelial cell; ein=expanded inclusions; icm=inner cell mass; in=inclusion. **A**=10 $\mu$ m, **B**=2 $\mu$ m **C-D**=500nm.

**Table 1.** Presence (+) or absence (-) of cell types and structures from pre- to post-settlement of *Haliclona indistincta*.  
 \*=altered morphology indicating transitional period for cell; ?=not observed.

Cell type/ Structure		Pre-settlement			Post-settlement					
		Stage			Hours					
		1	2	3	1	24	48	96	192	768
fibrillar material from “spumeuse cell”		+	+	+	+	+	+	+	+	+
Ciliated epithelial cells		+	+	+	-	-	-	-	-	-
Pinacocyte cells		-	-	-	+	+	+	+	+	+
Choanocyte cells		-	-	-	-	-	-	+	+	+
Archeocyte-like cells:	Type 1	+	+	+	+	+	+	+	+	+
	Type 2 (“polyblast”)	+	+	+	+	+	+	+	+	+
Cells with inclusions:	Type 1 (“fibrillar inclusion”)	+	+	+	+	+	+	+	+	+
	Type 2 (“lipid droplets”)	+	+	+	+	+	-	-	-	-
	Type 3 (“spumeuse cell”)	-	-	-	-	-	-	-	-	+
	Type 4 (“granular cell” 1: large granules)	-	-	-	-	-	-	-	-	+
	Type 5 (“granular cell” 2: small granules)	-	-	-	-	-	-	-	-	+
Secretory cells:	Type 1 (“lophocyte”)	-	+	+	+	+	+	+	+	+
	Type 2 (“sclerocytes”)	-	+	+	+	+	+	+	+	+
	Type 3 (“spongocytes”)	-	-	-	+	+	+	+	+	+
	Type 4 (“collencytes”)	-	-	-	-	-	-	-	-	+
Symbiotic Bacteria	Internal	-	-	-	-	-	-	-	+	+
	Superficial	-	-	-	-	-	-	+	+	+

*Secretory cells*; Type 1 secretory cell (Table 1) was a collagen producing cell (indicated by the trail of collagen strands) (Figure 4A-B). This cell type primarily inhabited the ICM, had archeocyte-like features with a large nucleus, a cytoplasm with phagosomes, and lipid droplets (Figure 4A). They were large amoeboid cells that ranged in length from 7µm to 14µm (average 9µm ± 3µm S.E.), and 5µm to 9µm (average 7µm ± 2µm S.E.) in width. Sclerocytes (Type 2 secretory cell: Table 1) were present at this stage (Figure 4C). Golgi

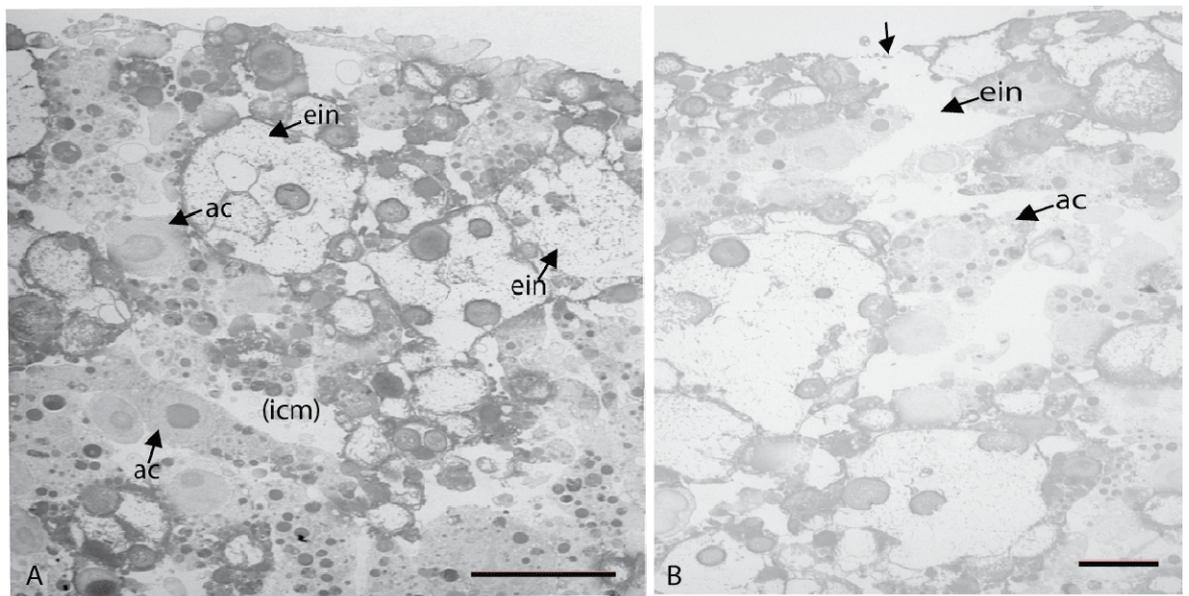
apparatus, mitochondria, endoplasmic reticulum were evident within the cytoplasm, as well as vesicles and lipid droplets. The spicules were observed in vacuoles, and more than one spicule was observed within these vacuoles, (but was a more common occurrence in the juvenile sponge than in the larvae). Commonly in demosponges, spicules have a hexagonally or triangle shaped axial filament (Simpson, 1984; Weaver and Morse, 2003; Maldonado and Riesgo, 2007) however, for *H. indistincta*, the axial filament was circular (Figure 4C). Spicules were sometimes observed within the nucleus of the cell and not within a vacuole within the sclerocyte's cytoplasm, an unusual occurrence also noted for the homoscleromorph sponge *Corticium candelabrum* (Maldonado and Riesgo, 2007). There did not appear to be a membrane around the spicules and it is unknown why they would occur in the nuclei of the cell. The length of the sclerocytes ranged from 7 $\mu$ m to 14 $\mu$ m (n=5, average 11 $\mu$ m  $\pm$  2 $\mu$ m S.E.), and 4 $\mu$ m to 7 $\mu$ m (average 5 $\mu$ m  $\pm$  1 $\mu$ m S.E.) in width.



**Figure 4. *H. indistincta* larvae free-swimming stage 2: Compact angular.** **A** Typical Type 1 secretory cell. **B** Close-up of collagen trail (arrows). **C** Typical Type 2 secretory cell (nucleolus not seen in this image). Box in the upper left-hand side of image shows silica covering a circular axial filament of a spicule. af=axial filament; cst=collagen strands; er=endoplasmic reticulum; gl=golgi apparatus; ld=lipid droplet; mt=mitochondria; n=nucleus; nu=nucleolus; ph=phagosomes; s=silica; sp=spicules; va=vacuoles; v=vesicles. Scale bars: A-C=2 $\mu$ m.

*Cellular organization in Stage 2;* Some epithelial cells were observed just inside the external layer with altered morphology (Figure 3C-D). The external surface of the larvae was covered with a membrane derived from the expanded inclusions of Type 1 cell with inclusions. This membrane surrounded and covered the external ciliated epithelial cells in some locations (Figure 3C-D). The large areas of expanded inclusions were also present within the ICM at this stage (Figure 3A). Only epithelial cells and expanded inclusions (of Type 1 cell with inclusions) were at the epithelial zone of the larvae, all other cell types were present in the ICM.

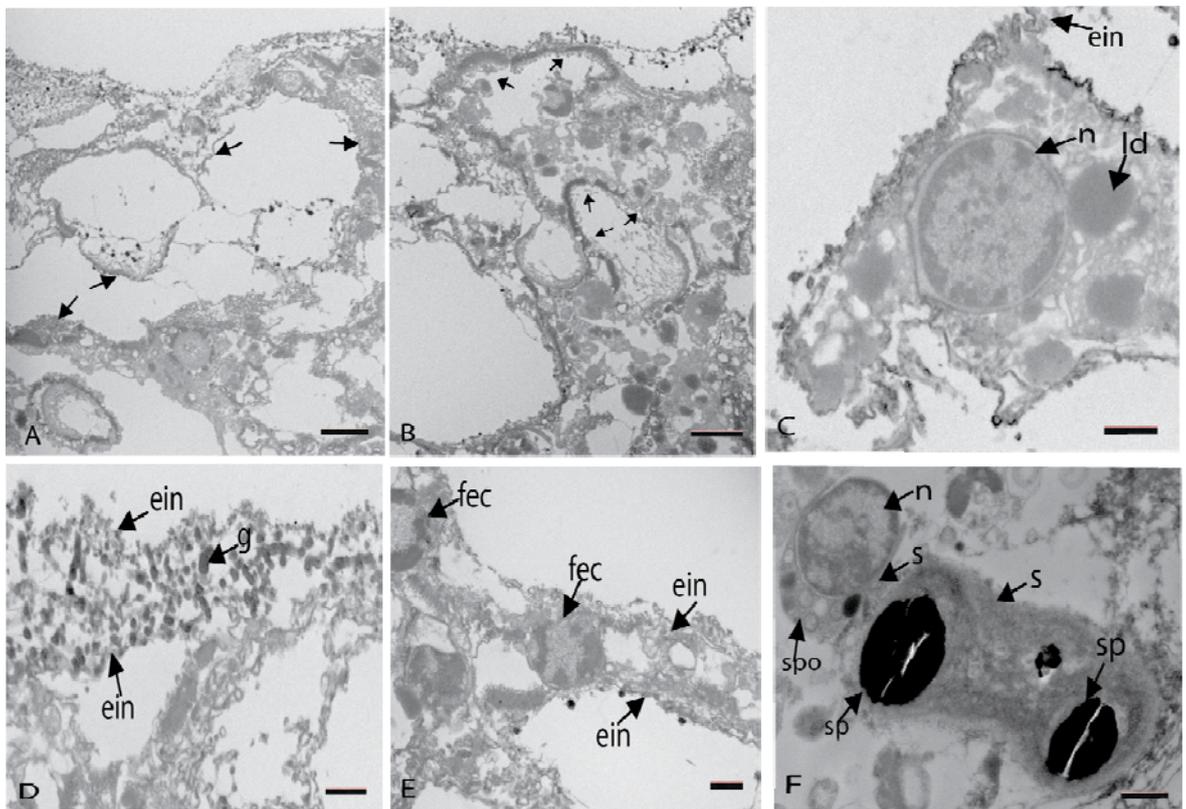
*Free-swimming stage 3 (approximately 49 hours post-release), and cellular organization;* All previously described cell types were present in the third free-swimming stage with no new cell types present (Table 1). Larval ciliated cells were still present at the external surface but not as much as compared to those in the previous stages. However, the shape of the ciliated epithelial cells was altered as compared to previous stages (Table 1). Inclusions of Type 1 cell with inclusions had expanded significantly forming large spaces (Figure 5A-B), and cytological structures (e.g. nuclei), were still associated with the inclusions. Amoeboid cells were plentiful and were heavily concentrated within the ICM, and were frequently in contact with the Type 1 cells with inclusions (as was observed in previous stages) (Figure 5A-B).



**Figure 5. *H. indistincta* larvae free-swimming stage 3: Circular.** **A** Section showing the epithelial layer to the inner cellular mass of the larvae. Expanded inclusions (of Type 1 cell with inclusions) prominently positioned at the perimeter of the larvae at this stage. Also showing cells more closely compacted together than previous stages and showing cells associated with Type 1 cells with inclusions. **B** Example of the epithelial zone of this stage, ciliated epithelial cells still present but expanded inclusions are heavily concentrated in this area right up to the external surface (arrow). Observed are cells commonly associated with Type 1 cells with inclusions. ac=amoeboid cell; ein=expanded inclusions; icm=inner cell mass. Scale bars: **A-B**=10 $\mu$ m.

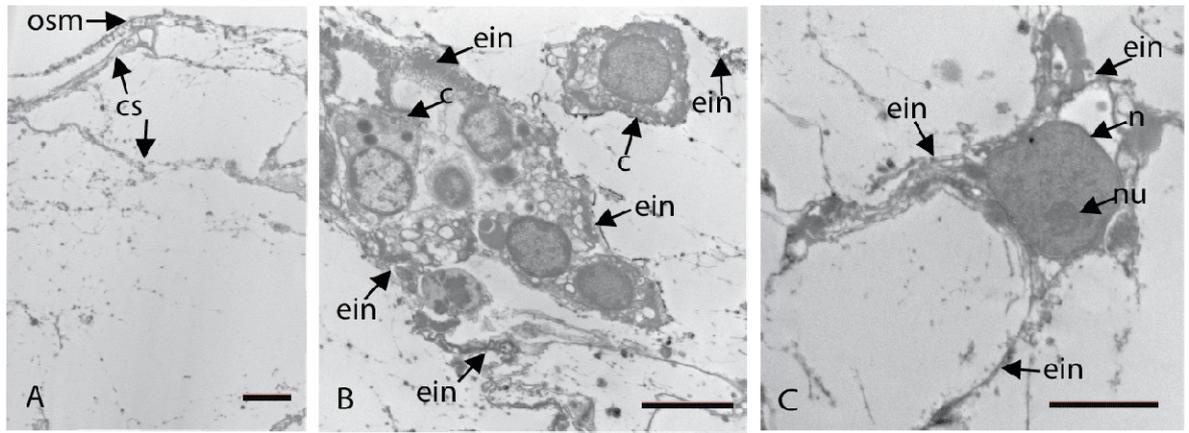
*1 hour settled;* The external surface of the juvenile was covered with a membrane derived from the expanded inclusions (of Type 1 cell with inclusions) (Figure 6A-E). Embedded within these layers of material were modified epithelial cells, perhaps in the process of differentiating into early pinacocytes (Figure 6E). In areas of the juvenile where expanded inclusions were no longer associated with cellular structures such as nuclei, the membranes of the inclusions seemed to line early canals (Figure 6B). The cell types observed in previous stages, showed altered morphologies (e.g. irregularly shaped cytoplasm) (except for the ciliated epithelial cells which were no longer present). These cells with altered morphology were located in the areas separated by canals (i.e. within the developing mesohyl) (e.g. Figure 6C), as were cell components (e.g. lipid droplets). Although sclerocytes were present from the second free-swimming stage, spongocytes, were not present until this stage (Type 3 secreting cell, Table 1) (Figure 6F) (*sensu* Simpson, 1984). There is nothing of the spongocytes' morphology that obviously distinguished them from archeocyte-like cells other than their position (i.e. immediately beside spicules). The length

range from 3 $\mu$ m to 10 $\mu$ m (n=5, average 6 $\mu$ m  $\pm$  3 $\mu$ m S.E.), and 2 $\mu$ m to 10 $\mu$ m (average 5 $\mu$ m  $\pm$  3 $\mu$ m S.E.) in width. The appearance of these putative early pinacocyte cells and the Type 3 secretory cell were the only new cell types observed in this stage than what were present in the second and third free-swimming stages.



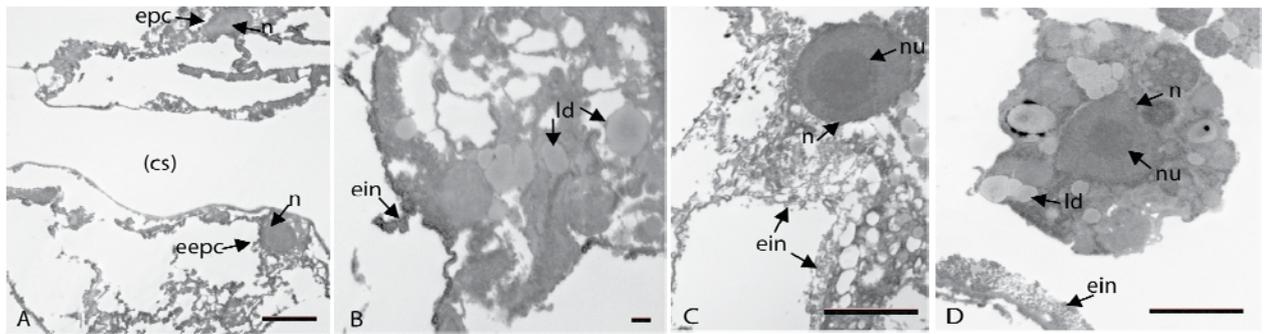
**Figure 6. *H. indistincta* 1 hour post-settlement: Juvenile.** **A** Longitudinal section showing early canal system in juvenile (arrows indicate direction of expansion). **B** Expanded inclusions (from Type 1 cell with inclusions) elaborating the early canal system (arrows indicate direction of expansion). **C** Amoeboid cell surrounded by expanded inclusion material **D** Images showing how the dermal membrane of the juvenile formed from the progressive expanding of inclusions. **E** Flattening epithelial cells contained within the layers of expanded inclusions. **F** Type 3 secretory cell secreting spongin around spicules. ein=expanded inclusions; fec=flattening epithelial cell; g=granule; ld=lipid droplet; n=nucleus; s=spongin; sp=spicules; spo=spongocytes. Scale bars: A-B=2 $\mu$ m C-F=500nm.

*1 day (24 hours) settled;* After 24 hours of settlement, the body was more flattened than in the previous time point (1 hour of settlement), the early canal system was more extensive and the early oscule mound could be clearly distinguished (Figure 7A). Many cells within the early mesohyl were irregular in shape and still apparently in a transitional stage (Figure 7B-C). No additional cell types were observed during this time point since the previous stage (i.e. 1 hour settled) (Table 1).



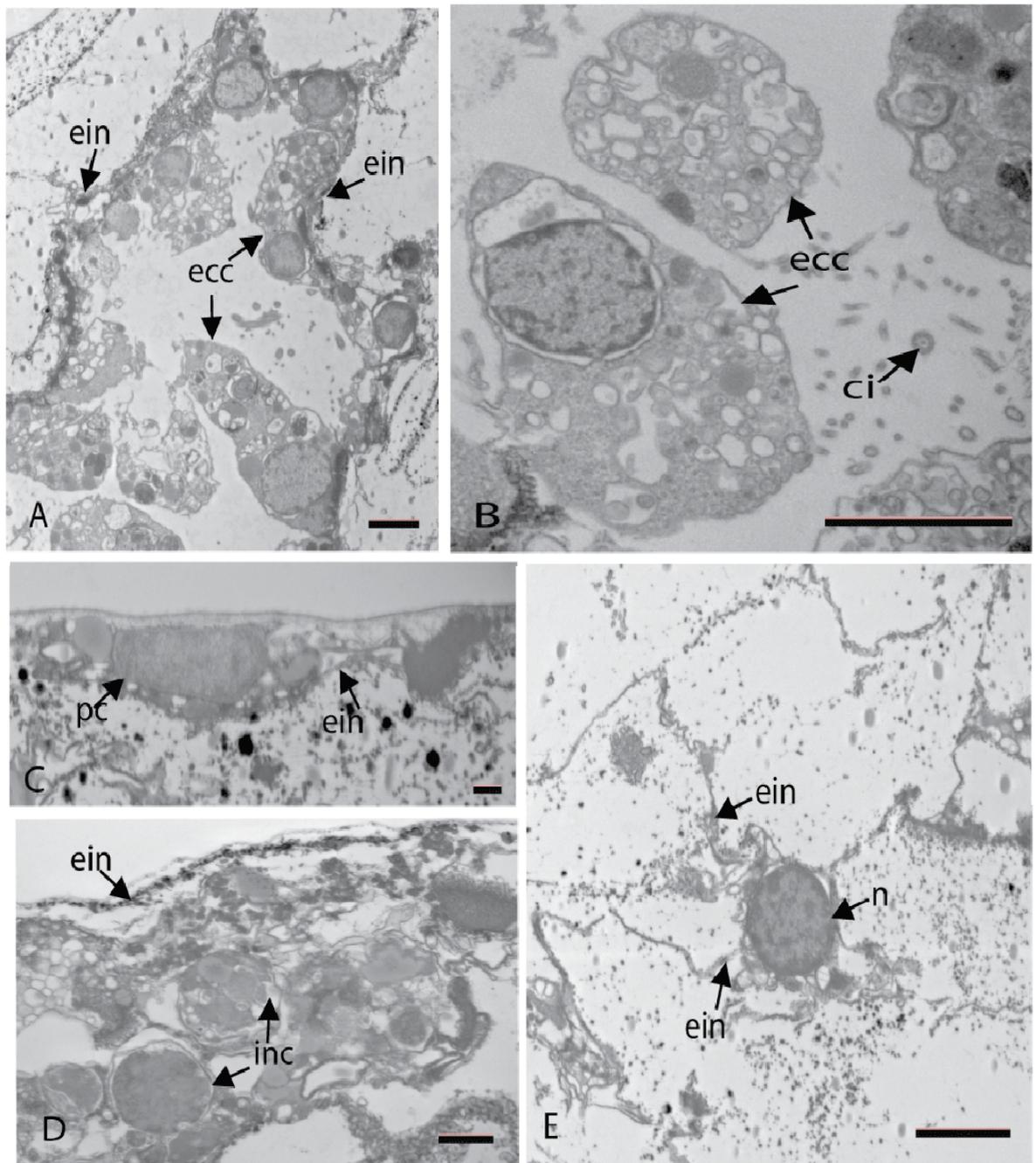
**Figure 7. *H. indistincta* 1 days post-settlement: Juvenile.** **A** Longitudinal section showing early canal system and early oscular mound. **B** Cells within mesohyl. **C** Amoeboid cell with little cytoplasm surrounded by expanded inclusions. c=cell; cs=canal system; ein=expanded inclusions (from Type 1 cell with inclusions); n=nucleus; nu=nucleolus; osm=oscular mound. Scale bars: A-C=2 $\mu$ m.

*2 days (48 hours) settled;* There was very little developmental difference between one, and two days of settlement. The overall body morphology was flat, however, oscular mounds were distinguishable, as well as the excurrent canal system (Figure 8A). The numerous pockets of space created by the expanded inclusions (of Type 1 cell with inclusions) from previous stages were still evident. Membranes of the inclusions encased nuclei and other organelles (e.g. lipid droplets) within the mesohyl (Figure 8B). There were numerous “disassembled” or “intermediate” cells with nuclei that had very little, or no, cytoplasm with few lipid droplets, and other inclusions, surrounding the nuclei (Figure 8C). However, there were also cells that looked like the archaeocyte-like cells of the pre-metamorphosis stages, but still did not appear to be “normal” (e.g. irregularly shaped cytoplasm) (Figure 8D) (Table 1). The absence of Type 2 cell with inclusions (which had large stores of lipid droplets) was the only noticeable change (i.e. appearance or disappearance) in cell types at this stage compared to the previous two stages (i.e. 1 and 24 hours settled) (Table 1).



**Figure 8. *H. indistincta* 2 days post-settlement: Juvenile.** **A** Longitudinal section showing developing canal system and early exo- and endo-pinacocyte cells. **B** Expanded inclusion material surrounding lipid droplets. **C** Cell with altered morphology surrounded by expanded inclusions. **D** Amoeboid cell. cs=canal system; eepc=early endo-pinacocyte cell; epc=early exo-pinacocyte cell; ein=expanded inclusions; ld=lipid droplet; n=nucleus; nu=nucleolus. Scale bars: **A**=2 $\mu$ m, **B**=500nm, **C-D**=2 $\mu$ m.

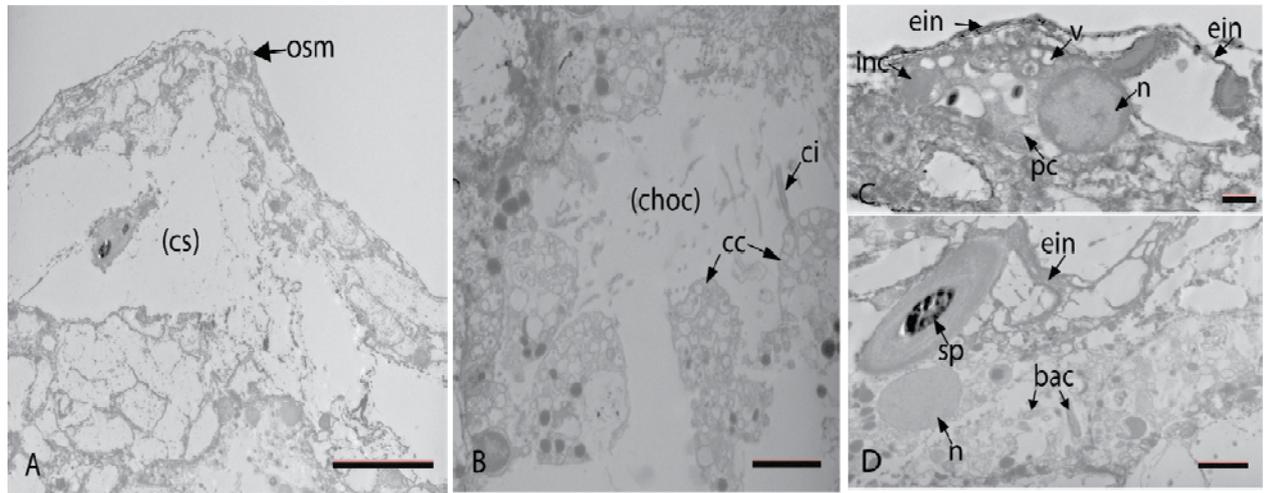
*4 days (96 hours) settled;* After four days of settlement, the body was noticeably more organized than the previous settled stages. Choanocyte chambers lined with early choanocyte cells were numerous (Figure 9A-B). Cells appeared to largely be past the transitional stage and have a more “normal” appearance (e.g. characteristic shape). However, the shape of choanocytes in many cases were not like that described in Chapter 4 (from 8 and 32 days settled specimens). The choanocytes present at this stage were less regular in shape, indicating that the trans-differentiation process was not yet complete. Early choanocyte cells attached to canal systems (Figure 9A), as well as early un-attached choanocytes were observed forming chambers within the mesohyl (Figure 9B). Cells such as pinacocyte cells (exo-, endo- and baso-), were the expected shape (i.e. elongate and flattened) (Figure 9C), however, there were still extracellular pockets of lipid droplets and other inclusions (Figure 9D) present in the mesohyl. As observed in other stages of development, large expanded inclusions were associated with nuclei and other cellular structures (e.g. lipid droplets), which was consistent with the structures being apart of one large cell (Figure 9E). Bacteria were present superficially on the underside of the juvenile and appeared to be surrounded by a thin dermal membrane which was perhaps an extension of the layers created by the expanded inclusions (of Type 1 cell with inclusions) that surrounded the juvenile. The only new cell type present at this stage were choanocytes (Table 1).



**Figure 9. *H. indistincta* 4 days post-settlement: Juvenile.** **A** Early choanocyte cells attached to expanded inclusions (from Type 1 cell with inclusions). **B** Early choanocyte cells not associated with expanded inclusions. **C** Pinacocyte cell. **D** Extracellular inclusions. **E** Type 1 cell with inclusions with expanded inclusions note large nucleus positioned in the middle of expanded inclusions. ci=cilium; ein=expanded inclusions; ecc=early choanocyte cell; inc=inclusion; n=nucleus; pc=pinacocyte cell. Scale bars: **A-B**=2 $\mu$ m, **C-D**=500nm, **E**=2 $\mu$ m.

*8 days (192 hours) settled;* After being settled for 192 hours (8 days), the body of the juvenile was thicker and had higher oscular mounds (Figure 10A) and choanocyte chambers lined with choanocyte cells that had numerous vacuoles present in the cytoplasm (Figure 10B). The general shape of the choanocytes is like that described for the choanocytes in Chapter 4. Cells such as pinacocytes had distinguishable cytoplasmic

inclusions (e.g. vacuoles) (Figure 10C). Internal bacteria were abundant (Figure 10D). No additional cell types were observed (Table 1).



**Figure 10.** *H. indistincta* 8 days post-settlement: Juvenile. **A** Longitudinal section showing well developed canal system and oscular mound. **B** Choanocyte chamber. **C** Pinacocyte cell. **D** Bacteria. bac=bacteria; cc=choanocyte cell; ci=cilium; choc=choanocyte chamber; cs=canal system; ein=expanded inclusions (from Type 1 cell with inclusions); inc=inclusion; osm=oscular mound; n=nucleus; sp=spicule; v=vesicles; pc=pinacocyte cell. Scale bars: **A**=10 $\mu$ m, **B-D**=2 $\mu$ m.

*32 days (768 hours) settled;* The body of the juvenile after 32 days (768 hours) of being settled was well organized with the presence of most cell types that were present in the previous time points (i.e. choanocytes, pinacocytes, Types 1-2 amoeboid archeocyte-like cells, Type 1 cell with inclusions and Types 1-3 secretory cells: Table 1).

Cells consistent with spumeuse cells (*sensu* Simpson, 1984) (Type 4 cell with inclusion: Table 1), were also present at this stage of development (Figure 11A). The cytoplasm was packed with inclusions that were membrane-bound and filled with a fibrillar material. Before a spumeuse cell releases its contents into the mesohyl, the inclusions fuse together. This fusion was observed (see Figure 11A), the membranes were ruptured and the contents merged. Figure 11B shows a closer view of a spumeuse cell that has inclusions that were not as advanced in development as in Figure 11A, as the membranes of the individual inclusions were clearly defined. The shape of the cell was circular to slightly elongate and

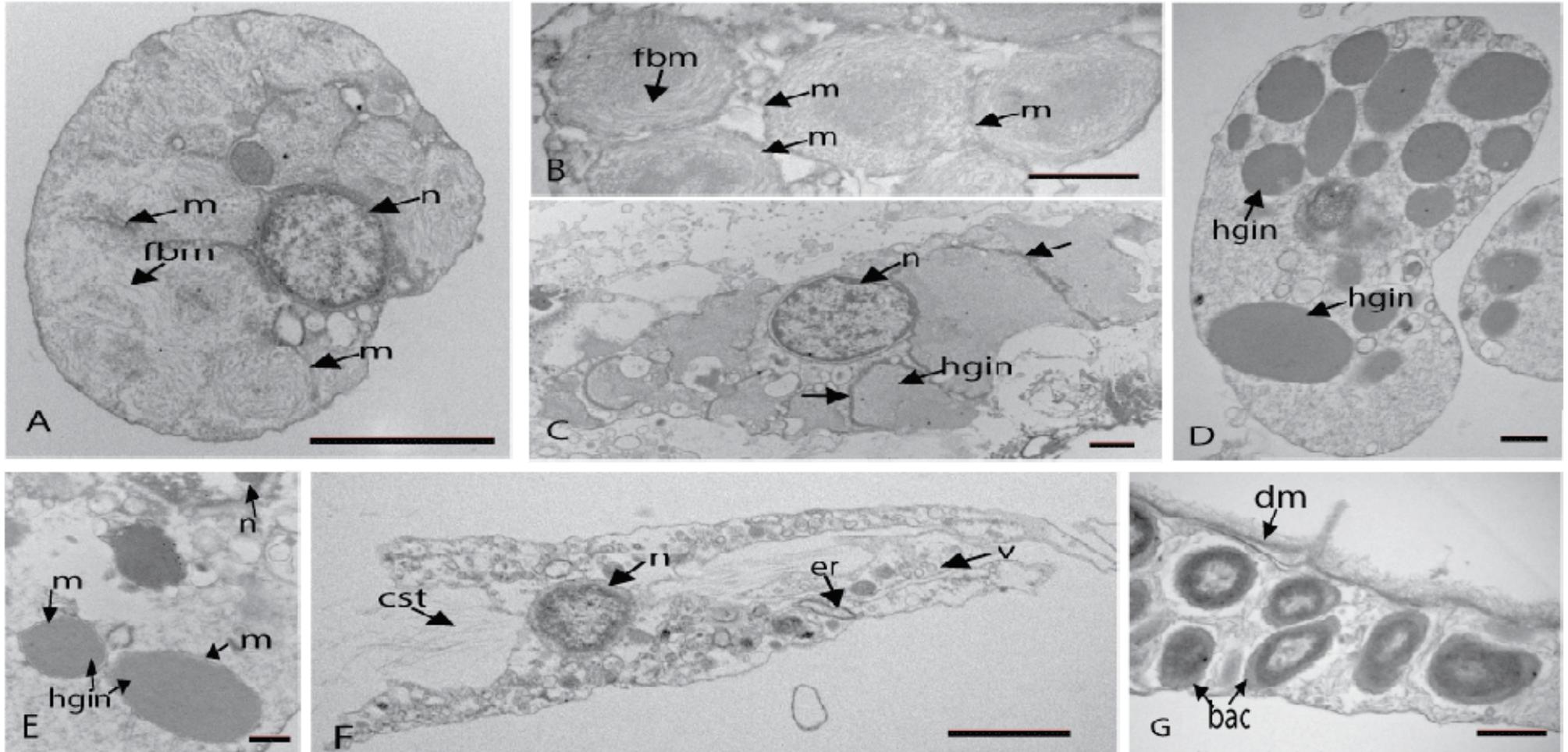
ranged in length from 5 $\mu$ m to 7 $\mu$ m (n=5, average 6 $\mu$ m  $\pm$  1 $\mu$ m S.E.), and 3 $\mu$ m to 6 $\mu$ m (average 4 $\mu$ m  $\pm$  2 $\mu$ m S.E.) in width. Much of the cytoplasm was obscured by the fibrillar inclusions and organelles were not apparent. This cell type was commonly seen within the mesohyl of the juvenile, as well as at the perimeter.

There were two additional types of granular cells. The first type, (Type 5 cell with inclusion: Table 1), was elongate with a small anucleolated nucleus surrounded by large homogeneous granular inclusions (Figure 11C). The granular inclusions were more dense at the perimeter of the inclusions, which made them appear to have a thick barrier surrounding the inclusions. The inclusions were typically packed tightly together. The cytoplasm was obscured by the inclusions and few to no phagosomes were present. From the longest axis it ranged from 5 $\mu$ m to 10 $\mu$ m (n=5, average 7 $\mu$ m  $\pm$  2 $\mu$ m S.E.) in length, and 3 $\mu$ m to 6 $\mu$ m (average 4 $\mu$ m  $\pm$  1 $\mu$ m S.E.) in width. The second new granular-like cell (cell with inclusion Type 6: Table 1) (Figure 11D) had much smaller granules by comparison, that were much more dense. Each inclusion was surrounded by a membrane (Figure 11E). Much more of the cytoplasm was visible and the overall shape of the cell was ovate and more consistent. The cell ranged in length from 4 $\mu$ m to 8 $\mu$ m (n=5, average 6 $\mu$ m  $\pm$  1 $\mu$ m S.E.), and 3 $\mu$ m to 6 $\mu$ m (average 5 $\mu$ m  $\pm$  1 $\mu$ m S.E.) in width. Both types of granular cells were typically found toward the middle of the juvenile body.

Type 4 secretory cell (Table 1) was elongated, with trails of fibers (Figure 11F). These cells showed features characteristic of collencytes (*sensu* Borojevic, 1966; Simpson, 1984). The shape of the cells were fusiform to amoeboid and ranged from 8 $\mu$ m to 15 $\mu$ m (n=5, average 13 $\mu$ m  $\pm$  3 $\mu$ m S.E.) in length and 2 $\mu$ m to 4 $\mu$ m (average 3 $\mu$ m  $\pm$  1 $\mu$ m S.E.) in width. Situated approximately in the middle of the cell, was a small, rounded nucleus. Within the cytoplasm, mitochondria, and numerous vesicles were apparent, with endoplasmic

reticulum being particularly prominent, there are few to no inclusions. The cells consistent with collencytes were primarily seen around the perimeter of the juvenile.

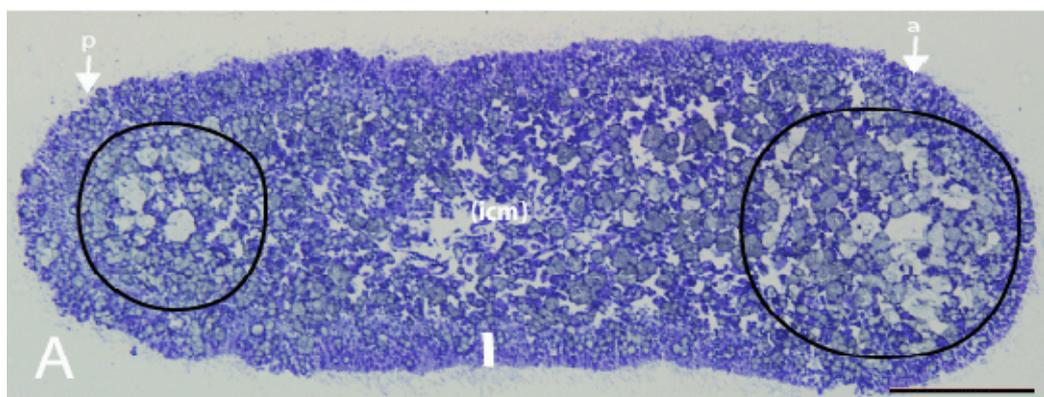
Internal bacteria were not plentiful, however, superficial bacteria were gathered in large clusters beneath the juvenile (Figure 11G). There was an obvious membrane around the bacteria, much more pronounced than in earlier stages, however, there was a distinct dermal layer between them and the juvenile body (Figure 11G).



**Figure 11. *H. indistincta* 32 days post-settlement: Juvenile.** **A** Type 3 cell with inclusions. **B** Close-up of inclusions from a Type 3 cell with inclusions showing fibrillar material as well as the membranes surrounding the inclusions. **C** Type 4 cell with inclusions showing large homogenous granular inclusions that are very dense around the edges of the inclusions (arrows). **D** Type 5 cell with inclusions. **E** Close up of inclusions of Type 5 cell with inclusions showing membrane surrounding each inclusion. **F** Type 4 secretory cell. **G** Superficial bacteria surrounded by a thick membrane. bac=bacteria; cst=collagen strands; dm=dermal membrane; fbm= fibrillar material; fbin=fibrillar inclusions; m=membrane; n=nucleus; hgin=homogenous granular inclusion. v=vesicles. Scale bars: **A**=2 $\mu$ m, **B-E**=500nm, **F**=2 $\mu$ m, **G**=500nm.

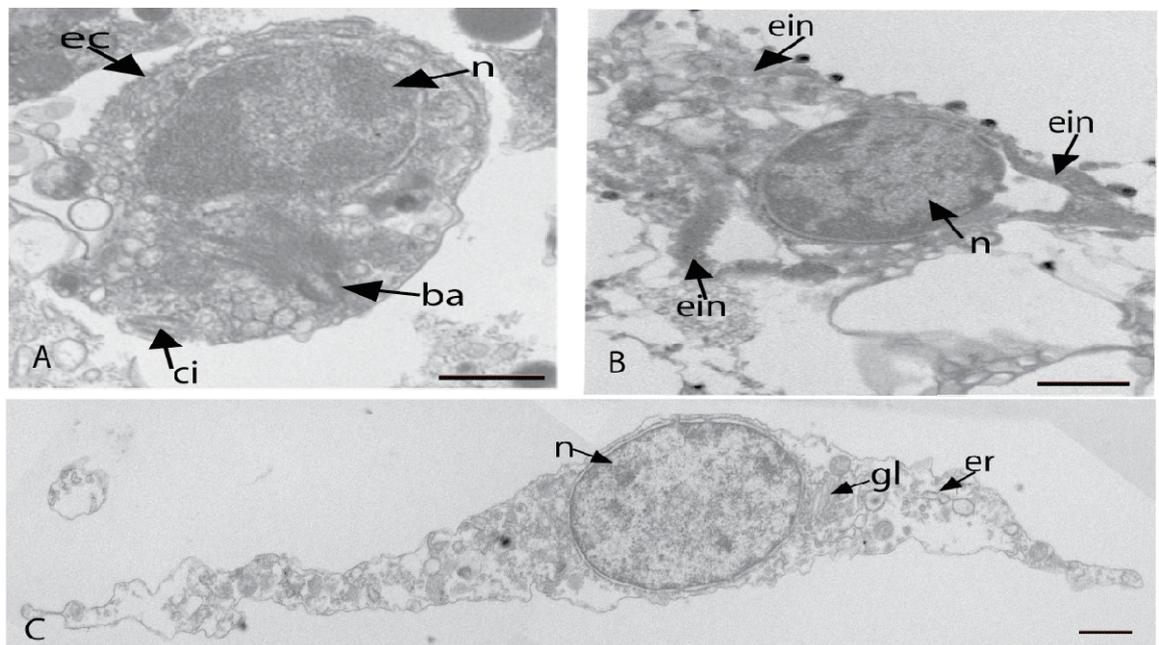
*Metamorphosis*; In summary, there were 14 different cell types observed throughout the pre- and post-settled stages of *H. indistincta* (Table 1). These included: epithelial cells, pinacocyte cells, choanocyte cells, 2 archeocyte-like cells, 5 cell types with inclusions, and 4 separate secretory cell types. In the first pre-settled stage (Stage 1) there were 5 different cell types, in Stage 2 and Stage 3 (pre-settlement) there were 7 different cell types. In both the 1 hour and 24 hour settled juveniles, there were 8 different cell types. There were 7 different cell types in the 48 hour settled juveniles (due to the loss of Type 2 cell with inclusions). In the 96 and 192 hours settled juveniles there were 8 different cell types (due to the appearance of choanocytes). After 768 hours of settlement there were 12 different cell types present (Table 1). There was a non-cellular material present in the ICM of the larvae and the mesohyl of the juvenile (i.e. the fibrillar material (Figure 1F)).

The shape and arrangement of cells in the first larval stage can be seen in Figure 12. The expanded inclusions (of Type 1 cell with inclusions) had delineated pathways in the larva by Stage 3 and also appeared to form the dermal membrane of the juvenile within one hour of settlement. The membrane, or layers, of expanded inclusion material became thicker as development progressed.



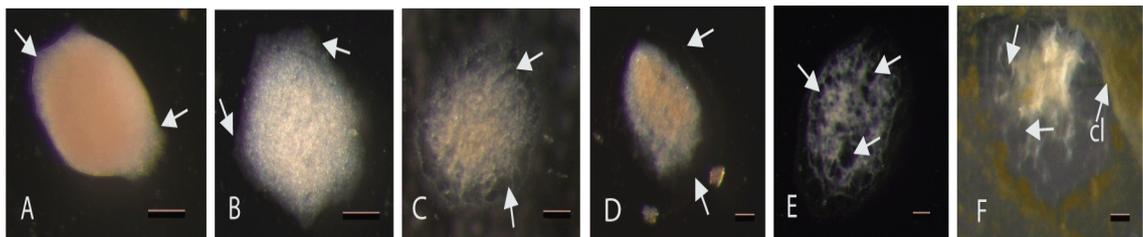
**Figure 12. Semi-thin sections of the three free-swimming larval stages of *H. indistincta*.** A Longitudinal section of Stage 1 free-swimming larvae. Circles mark general areas (anterior and posterior ends) of the larvae where there is a particular concentration of circular particle complexes (with separate and merged circular particles). (White bar indicates epithelial zone). a=anterior; icm=inner cellular mass; p=posterior. Scale bars: A=100 $\mu$ m.

The majority of cells present in the larval stages began to differentiate from the third free-swimming stage to the fourth day of settlement (96 hours) when there were fewer cells with altered-looking morphology. After 96 hours of settlement, the choanocyte cells were observed within the mesohyl of the juvenile. Prior to that, ciliated epithelial cells were observed within the mesohyl of the juvenile. Prior to that, ciliated epithelial cells were present internally throughout the pre-settlement stages (Figure 13A). Cells were flattening at the epithelium as well as next to early canal system (i.e. early exo-, endo-, and baso-pinacocyte cells), within the first hour of settlement. The pinacocyte cells changed throughout the post-settlement stages. The early form, as seen in the first hour of settlement and up to 2 days of settlement, was little more than a rounded nucleus with lipid droplets in the cytoplasm (lipid droplets not shown in figure) (Figure 13 B), when compared to the later developed pinacocyte cell of 32 days post-settlement as seen in (Figure 13C) which was flattened and elongated with an oval nucleus surrounded by Golgi apparatus either side and endoplasmic reticulum.

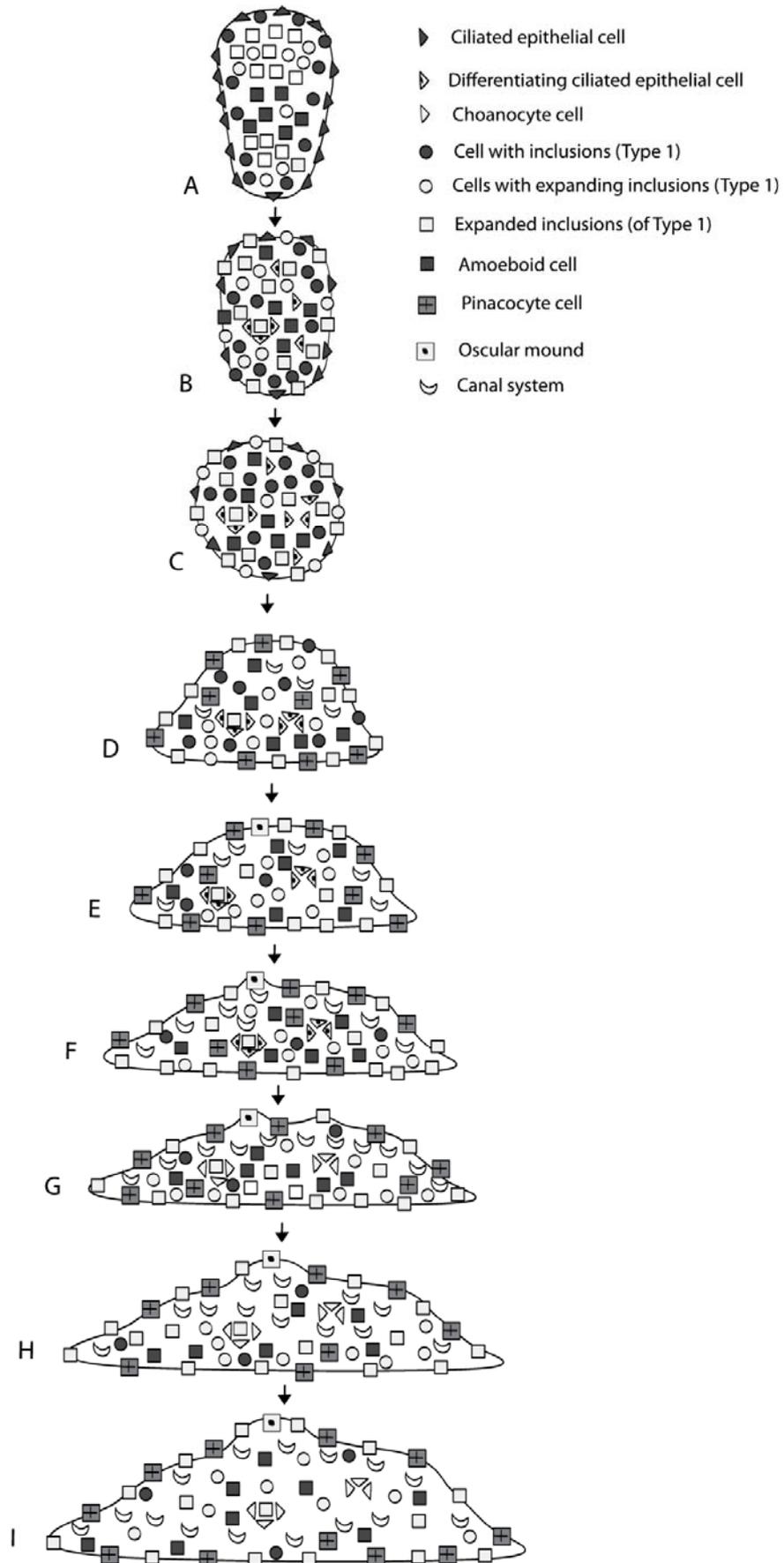


**Figure 13. *H. indistincta*: body rearrangements from the free-swimming to sessile.** A Ciliated epithelial cell within the inner cellular mass of the third stage free-swimming larvae. B Pinacocyte cell from juvenile settled for 1 hour. C Pinacocyte cell from juvenile settled for 768 hours (32 days). ba=basal apparatus; ci=cilium; ec=ciliated epithelial cells; ein=expanded inclusions (from Type 1 cell with inclusions); er=endoplasmic reticulum; gl=Golgi apparatus; n=nucleus. Scale bars: A-B=500nm, C=2 $\mu$ m.

Figures 14A-F show the external changes in the juvenile from within one hour to 768 hours post-settlement. The juvenile initially spread out and became flattened before it began to become more cushion shaped (around 4 days post-settlement). Between 8 and 32 days of settlement, a thin sheet of material covered the juvenile, this material is presumed to be a collagen as collagen secreting cells were abundant in this area (i.e. Type 3 cell with inclusion, Type 4 cell with inclusion, and Type 4 secreting cell). Figure 15 is a simplified schematic showing the general changes to the body shape and accompanying cellular re-arrangements observed during metamorphosis of *H. indistincta*.



**Figure 14. *H. indistincta*: light microscope images of the post-settlement time points.** **A** Just settled one hour. Arrows indicating flattening and spreading out of juvenile that commenced upon contact with substrate. **B** 24 hours (one day) post-settlement. Arrows indicate borders of specimen at this stage. **C** 48 hours (two days) post-settlement. Arrows indicate borders of specimen at this stage. Note the body shape of the juvenile is still quite flat at this time point. **D** 96 hours (four days) post-settlement. Arrows indicate borders of specimen at this stage. Centre of specimen becoming more raised. **E** 192 hours (eight days) post-settlement. Arrows indicate oscular openings. **F** 768 hours (thirty-two days) post-settlement. Arrows indicate oscular openings. Note the collagen layer that covered the juvenile (partially torn away and thus only covering a quarter of the juvenile). cl=collagen layer. Scale bars: **A-E** 100 $\mu$ m, **F**=200 $\mu$ m.



**Figure 15. A simplified schematic of the changes in body shape and accompanying cellular re-arrangements during the metamorphosis of *H. indistincta*. A Free-swimming stage 1. B Free-swimming stage 2. C Free-swimming stage 3. D Juvenile settled 1 hour. E Juvenile settled 24 hours (one day). F Juvenile settled 48 hours (two days). G Juvenile settled 96 hours (four days). H Juvenile settled 192 hours (eight days). I Juvenile settled 768 hours (32 days).**

**Discussion:**

One of the most interesting and novel aspects of this work are the Type 1 cell with inclusions and the apparent role of this cell type in the development of the canal system and mesohyl delamination, excretion of waste products, and cell fate during metamorphosis. It is possible that the canal-like passages observed at either end of the larvae, may have served at least two functions, 1) to directly deployed contents of the inclusions to the surface of the larvae which may allow the waste products to be expelled from the larvae, 2) to form an outside layer of the larvae and the juveniles (e.g. Figures 3, 5-6, 9). Cells were often closely associated with Type 1 cells with inclusions. This association suggesting cellular communication and/or transferral of waste (or other) products. Interestingly, expanded inclusions (of Type 1 cell with inclusions) were associated with amoeboid cells also in adult *H. indistincta* (Appendix 7A-B), as seen in the larval and juvenile stages, perhaps suggesting a similar function in adults.

When attached to circular particles, the morphology of cells appeared altered as if the cells were being “disassembled”. Furthermore, I have found evidence to suggest that in addition to the fates described in Stephens et al. (submitted) (Chapter 4) for the ciliated epithelial cells (i.e. becoming phagocytized by amoeboid cells as well as differentiating into the choanocyte cells), that pinacocyte cells may also be derived from them. This was indicated by a number of ciliated cells remaining at the surface of the larvae that appeared to be undergoing significant morphological changes expected of a cell undergoing trans-differentiation (e.g. internalizing their cilium).

Certain haplosclerids (i.e. *Petrosia ficiformis*) have very simple cytology, exhibiting an entirely ciliated solid larval stage with no cell differentiation (Maldonado and Riesgo, 2009). However, this is not a universal feature, with at least eleven cell types identified in

other haplosclerids (i.e. *A. queenslandica* (*sensu* Leys and Degnan, 2001,2002). The use of sponge larval morphology as a taxonomic tool is contentious (e.g. Ilan and Loya, 1990) because other than lecithotrophy and the lack of sensory cells, few larval characteristics are so far consistent across sponge larvae (Maldonado et al., 2003; Maldonado, 2004). However, some parallels can be drawn between studies on haplosclerid species, for example, Maldonado et al. (2003) found cells in *Haliclona caerulea* (formerly *Sigmatocia caeruleai*) (Haplosclerida) that were similar to the mucus cell described in *A. queenslandica* in Leys and Degnan (2001), however, the function of this cell is unknown (Maldonado, 2003).

Results from the present study show seven distinct differentiated larval cell types found in *H. indistincta*, two of which were not present until the second free-swimming stage (i.e. lophocytes and sclerocytes). Four cell types: ciliated epithelial cells, archaeocyte-like cells, and sclerocytes, correspond to described cell types (in Leys and Degnan, 2001; 2002). In Leys and Degnan, (2001; 2002) the cells (for *A. queenslandica*) included: ciliated epithelial cells, sclerocytes, pigment cells, vesicular cells, spherulous cells, mucus cells, and “at least four types of amoeboid cells that inhabit the interior of the larvae”. Two of the larval cell types observed in *H. indistincta* (i.e. Type 1 and Type 2 cells with inclusions) are not (to the author’s knowledge) analogous to previously described Haplosclerid larval cell types (e.g Boury-Esnault, 1976; Wollacott, 1993; Leys and Degnan, 2001; Maldonado et al., 2003).

It is possible that the Type 1 cell with inclusions, that appears to be so key to the development of this species, is a taxonomic character as (to the authors knowledge) no other study has described this cell type. However, it is difficult to assess the phylogenetic value of the larval cell types (e.g. Type 1 and 2 cell with inclusions), identified as unique

to *H. indistincta*. The newly identified cell types may reflect a lack of investigation into sponge larval cell differentiation rather than novel characters for *H. indistincta*.

Sponge larvae being lecithotrophic is interesting from an evolutionary point of view as lecithotrophy has been suggested to be derived from a planktotrophic state (Strathmann, 1978; Maldonado and Young, 1999; Pernet, 2003; Maldonado, 2006). Maldonado and Young's (1999) study of the lecithotrophy of *H. caerulea*, showed that the larvae that remained free-swimming longer (using up more nutrition stores (i.e. yolk granules/lipid droplets)) became inferior juveniles to those that had a shorter free-swimming stage (and used up less of the nutrient stores). In this study, it was shown that *H. indistincta*'s primary food stores (i.e. suggested to be Type 2 cell with inclusion (which contained primarily lipid droplets)) were most useful to the specimens, prior to, during and directly after settlement as the Type 2 cells with inclusions were completely gone after one day of settlement (Table 1). The presence of lecithotrophy in this species was further demonstrated by the archeocyte-like cells in the pre-settlement stages that had cytoplasm that were noticeably more full of lipid droplets while the archeocyte-like cells of the juveniles had more phagocytes. However, effects of a lengthened larval stage on the viability of the juveniles was not tested.

*Cell differentiations;* Choanocyte cells were first observed after four days of settlement (96 hours) (Table 1). Type 2 amoeboid cell fits the description for a cell type known as a polyblast, which is an archeocyte-like cell that is distinguished by having few or no phagosomes and large nuclear volume in comparison to cytoplasm volume (Connes, 1968; Thiney, 1972; Connes et al., 1972; Simpson, 1984; Ereskovsky, 2010). It has been suggested that polyblasts are an intermediary stage between archeocytes and choanocytes (Connes et al., 1972). It has also been suggested that polyblasts can be derived from

endopinacocytes as well as choanocytes, especially in the case of bodily trauma (Diaz, 1977), while others believe them to be a derivative of archaeocytes, and therefore within the same cell category of ‘archeocytes’ (Thiney, 1972). Polyblasts are believed to be highly important for morphogenesis for storing nutrient reserves (Simpson, 1984). Whether or not polyblasts derive from endopinacocytes or choanocytes in other sponges is unknown, however, considering that polyblasts were present in the larvae of *H. indistincta*, where no choanocytes or endopinacocytes were present, if they are derived from any cell type, the cell type was an archeocyte.

In general, cells that secrete collagen and have archeocyte-like features are often termed ‘lophocytes’ however, Feige (1969) suggests that there is no need to distinguish them from archaeocytes as ‘lophocytes’ are merely collagen producing archaeocytes. By tracking the fate of archeocytes, Efremova (1967), termed the lophocyte morphology as a transient stage that archeocytes may faze in and out of. For *H. indistincta* (Type 1 secretory cell: Table 1), I suggest that this is the case due to the morphology being so similar to archaeocytes.

Cells possessing features described for collencytes (as per Borojevic, 1966) were present after 768 hours (32 days) of settlement (Simpson, 1984) (Type 4 secretory cell: Table 1). The ‘fibre producing cell’ category of which this cell type belongs, is a confusing group because a number of the general cytological features are shared (Simpson, 1984). Even the name “collencyte” has been applied in two senses, the generalized “collencyte” is very poorly defined and thought to be immobile, the more specific definition of the term “collencytes” (as per Borojevic, 1966; Simpson, 1984) is considered in this study when describing the fibre-producing Type 4 secretory cell (Table 1). Among other features, the term “collencyte” with the more specific definition is thought to show collagen

fibrillogenesis, motility, and are presumptive pinacocytes (Simpson, 1984). It is possible that *H. indistincta* collencytes are, as Borojevic (1966) suggests, functional pinacocytes, as they are located at the perimeter of the juvenile, and look very similar to pinacocyte cells (except endoplasmic reticulum is far more abundant and golgi apparatus are not as numerous as in pinacocyte cells).

Spumeuse cells are packed with fibrillar material until they release it into the mesohyl. It is thought that the fibrillar material released by spumeuse cells is especially important during development of sponges for both interactions between cells, as well as mesohyl elaboration (Bretting et al., 1983; Simpson, 1984) which is possible considering it was plentiful during the early development of *H. indistincta*. When present, the fibrillar material in *H. indistincta* was positioned between cells, and always in contact with them. A fibrillar material which was produced and secreted by spumeuse cells, described by Simpson (1984) (Figure 2F), was seen from the first free-swimming stage, however, the cell that I suggest produces this fibrillar material (Figure 11A) did not appear until 768 hours (32 days) post-settlement. This indicates that this material was initially maternally derived.

The dynamic nature of sponge cells is emphasized most during the metamorphosis of the larva. The larval cell fate of *H. indistincta* does not follow the general trend observed in marine haplosclerids, as suggested in Ereskovsky's (2010) review of the literature on marine haplosclerid metamorphosis. Ciliated cells of *H. indistincta*, instead of differentiating into either choanocytes or sclerocytes as shown in Ereskovsky (2010), I suggest differentiate into both choanocytes and pinacocytes. I have shown that sclerocytes are first observed within the larvae during the second free-swimming stage (and possibly are originated from archaeocytes). My work is consistent with Ereskovsky

(2010) in that the archaeocytes differentiate into secretory cells as well as the cells with inclusions observed in the 32 days settled juvenile because they are the most similar in shape and size to the cells with inclusions than the other cells present before 32 days of settlement (Table 1).

Leys and Degnan (2001) have shown that sponges possess rudimentary body axes and tissue organization. While bilaterians show two body axes (i.e. anterior-posterior and dorsal-ventral), sponge larvae also clearly show axis polarity (i.e. anterior-posterior) (as shown by ciliation pattern, coloration, and shape differences, as well as one end always leading in forward movement) (e.g. *A. queenslandica* (Adamska et al., 2007; Ereskovsky et al., 2009)). *H. indistincta* larvae showed polarization, having distinct anterior end (as indicated by being larger, whiteish-pink in color (vs. dark pink), larger space filled with merged circular particles). I considered the possibility that because the anterior end became more noticeably diminished as the larva progressed to the second free-swimming stage, that there may be an anatomical difference between the ciliated epithelial cells of the anterior and posterior ends; however, no apparent difference between the anterior and posterior larval ciliated cells was observed.

As Stephens et al. (in press) (Chapter 3) described, the anterior end of the first stage of the free-swimming larvae, tilts upwards at an angle when in motion, and is completely erect when stationary; perhaps implying that the anterior end was more buoyant than the posterior end. This uneven buoyancy was possibly due (at least in part) to the larger spaces formed by the expanded inclusions (of Type 1 cell with inclusions) at the anterior end (Figures 2D-E, 12A). Maldonado (2006) suggested, that the internal cavity of freshwater demosponge parenchymella function either for osmoregulation or flotation, or a combination of the two. The typical ‘corkscrew’ swimming pattern in sponge larvae is

suggested to be influenced by spicules being concentrated at the posterior pole (e.g. Wollacott, 1993; Maldonado and Bergquist, 2002; Maldonado, 2006). In this case however, because there were no spicules present until the second free-swimming stage, this swimming pattern may be due to the larvae having a denser, posterior end.

Settlement of sponge larvae is dependent upon them ceasing swimming, and commencing substrate investigation (Maldonado et al., 1997; Maldonado, 2006; Mariani et al., 2006). To do this, physical changes within the larvae are required to make the larvae become less buoyant, and sink. Passive sinking mechanisms include: depletion of buoyant lipids (through phagocytosis), and increase in the synthesis of much less buoyant proteins, and spicule secretion (Maldonado et al., 1997; Maldonado, 2006; Mariani et al., 2006). Changes in geotactic responses with age has been commonly commented on (e.g. Wilson, 1935; Fell, 1974; Bergquist et al., 1979; Wapstra and van Soest, 1987; Maldonado et al., 1997). Maldonado et al. (1997) showed that with age, the larva of, *H. caerulea*, showed an increase in density, which was directly related to the secretion of spicules. Buoyancy modification strategies for larvae that do not have spicules are still somewhat ambiguous (Maldonado, 2006). I suggest that two strategies exist for *H. indistincta*. Firstly, settlement of *H. indistincta* larvae typically took place once the larvae went through three free-swimming stages wherein they progressively became spherical in shape and cells more tightly packed making the larvae denser. Subsequently, spicule secretion also commenced (at Stage 2) (Stephens et al., (in press); Chapter 3).

Bacteria are important to sponges providing nutrition by being ingested by sponge cells; furthermore, it has been suggested that bacteria undergo a level of disintegration which also possibly provides nutrients to the host sponge (Vacelet, 1975; Simpson, 1984). Bacteria are often gained through vertical transmission (e.g. by amoeboid nurse cells or

trophocytes that collect the bacteria and deliver it to the oocytes) (Maldonado, 2007), however, bacteria for *H. indistincta* were not maternally derived as bacteria were not present in *H. indistincta* larvae, but were only present post-settlement. Bacteria were found residing superficially, underneath the juvenile (four days settled), but were not abundant within the juvenile body until eight days of settlement suggesting the choanocytes and canal system were not fully developed until that point which would then allow bacteria to infiltrate the sponge body.

The main aim of this study was to describe the development of *H. indistincta* so that possible synapomorphies among the haplosclerids could be found; however, it was not possible to say with certainty what characters in *H. indistincta* have phylogenetic signal as there are too few studies on haplosclerids to compare at this level of detail. For example, even in Amano and Hori's (1996) study (one of the most detailed marine haplosclerid studies on metamorphosis (along with Leys and Degnan, 2002)), while providing great insights in the trans-differentiation process of ciliated epithelial cells into choanocyte cells of *H. permollis*, does not describe all cell types observed throughout the metamorphosis process. As shown in Chapter 2, *H. indistincta* (along with *H. viscosa*) are distantly related to *Haliclona* species (being more closely related to *Niphates* taxa). Therefore, to discover synapomorphies, additional species that are known to be closely related, such as *H. viscosa* and taxa from the genus *Niphates*, should be investigated. Additional aspects of this study to pursue is the further examination of the inclusions of the Type 1 cell with inclusions utilizing Raman spectroscopy, and histochemistry which may shed light on the chemistry of the inclusions and therefore may confirm some assumptions concerning their functions. Further investigations of adult *H. indistincta* utilizing TEM may show additional cell types commonly found in other demosponges that were not observed in the juveniles after 32 days of settlement (e.g. myocytes or

actioncytes, vacuolar, spherulous). When considering cell recognition and signalling that is known to occur in sponges, questions arose considering whether it is possible that *H. indistincta* may rely on cellular interactions to trigger cellular differentiation and preparations to commence a new developmental stage, the larvae of *H. indistincta* do respond to physical interactions with other larva (i.e. the merging behaviour described in Chapter 3, which appeared to occur when larvae were in proportionally high density situations). It is possible that fluorescent labelling may provide insights concerning this theory as cell migrations are easily traced using this methodology (as shown in Leys and Degnan, 2002).

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Chapter 6:

**General Discussion**

### **General Discussion:**

The primary aim of this project was to investigate the systematics, and reproductive and developmental biology of the marine sponge *Haliclona indistincta*. Phylogenetic studies concerning the order Haplosclerida have yielded many inconsistencies between morphology-based classification and molecular phylogenies (e.g. McCormack et al., 2002; Borchiellini et al., 2004; Erpenbeck et al., 2004; Nichols, 2005; Raleigh et al., 2007; Redmond et al., 2008; 2011). In this study, by focusing on a small group (i.e. “*H. rosea* group”) (Chapter 2) I have finally shown clear congruence between both types of data. Trees drawn from two independent gene loci (i.e. 28S rRNA gene and mtCO1), confirmed *H. indistincta*’s phylogenetic position within the marine haplosclerids (Chapter 2). Further, ribosomal data, showed *H. indistincta* (along with *H. viscosa* (a member of the “*rosea*” group)) more closely related to members of the family Niphatidae than the other chalinid species present in the tree (including type *Haliclona* species: *H. oculata*), which was supported by morphology (by reviewing patterns of skeletal structure in chalinids and niphatids). This indicates that *H. indistincta* would be more appropriately considered a member of the Niphatidae. Together, this data shows that molecular-based studies can be informed by morphological data (i.e. skeletal structure) and *vice versa*.

Morphological characters other than skeletal structure have shown to be useful in elucidating information concerning sponge phylogenetics. For example, the position of the Homoscleromorpha (i.e. within demosponges, or a class of their own) has been a subject of debate for a number of years. Molecular-based studies first suggested the homoscleromorphs are a separate group from the demosponges (e.g. Borchiellini et al., 2004). Further molecular data, and additional morphological, developmental, and reproductive characters, (i.e. occurrence of basement membranes, apical cell junctions,

unique rootlet system, skeletal differences, as well as embryonic developmental differences including multipolar egression) (Ereskovsky and Boury-Esnault, 2002; Gazave et al., 2012), has led to the recent suggestion by Gazave et al. (2012) to instate this group as the fourth class in the Phylum Porifera. A large part of this work was to discover synapomorphies among the haplosclerids; however, developmental processes, as well as aspects of parenchymella larval morphology require further mining for phylogenetic significance (Maldonado, 2004; Wörheide et al., 2012).

It is likely that many larval morphological characters are plastic; which I suggest is the case for ciliation patterns in larvae, as finding congruence between classification and ciliation patterns within the order Haplosclerida was difficult. Although the general shape of the choanocyte and ciliated epithelial cells of *H. Indistincta* may be similar to that of other sponge orders, the finer structure of the basal apparatus of either cell type showed great contrasts, even with other haplosclerids (e.g. *H. tubifera* (Woollacott, 1993; Woollacott and Pinto, 1995)) which supported the molecular data shown in Chapter 2 as *H. indistincta* grouped away from *H. tubifera* on the mtCO1 tree. This adds further support for the hypothesis that basal apparatus of ciliated cells can be phylogenetically informative, and the next step will be to compare species that are known to be closely related (i.e. *H. viscosa* and *H. indistincta*). However, as it stands, until more species are investigated, particular synapomorphies cannot be confirmed.

Aside from morphological plasticity, systematics research on sponges is also hampered by difficulties involved with applying molecular techniques to sponge DNA. Unfortunately, data yielded from *H. rosea* was unusable, all of the data returned were from contaminants, and I was unable to find fresh *H. rosea* specimens during any of my sampling trips, even to Lough Hyne where its presence had been previously documented

(i.e. Bell, 2007). I was unable to amplify non-contaminated mitochondrial (mtCO1) sequences from the cushion form of *H. indistincta* to include. It was thought that a more specific primer set would reduce the level of contamination observed, and therefore the author designed a new primer set. However, it was discovered that the “Folmer region” (the original region amplified by the primer set used) was the best region for amplification in the mtCO1 gene, as outside the “Folmer region” was too variable. Interestingly, Moniz et al. (accepted) has documented 66 species of algae in this cushion form over a year, and Murphy (unpublished thesis) documented conservative estimates of 21 species of fauna (e.g. copepods) (with an average of 6 different species in a single individual). Considering this, it is not surprising that it was difficult in obtaining non-contaminant sequences from the cushion form of *H. indistincta*; however, initially, I was unaware of the issue in using the “Folmer primers” (Folmer et al., 1994) (which are universal primers) as I had no idea of the vast number of species of algae and animals that are common inhabitants. However, Wolff et al. (unpublished data) are currently investigating the diversity observed within *H. indistincta* (cushion form) using 454 sequencing (or next generation sequencing).

There are additional areas where this study fell short of the objectives; this project was limited in the information obtained on embryogenesis of *H. indistincta* (Chapter 3). This was due to a material (that stained pink), which made it impossible to view fertilization, cleavage and early cellular differentiation (Chapter 3). When it was realized that describing the embryological development was not possible using histology, electron microscopy was attempted, however, the population that I had sampled from for the first two and a half years of my project (observing consistent populations) disappeared within six months, which coincided with a time of unusually high temperatures that was followed by frost. Through this study, this species was observed to have very specific

habitat preferences, and the harsh changes in weather observed at this time may have severely effected the population. Sections of maternal tissue already obtained were processed for TEM however, since the tissue had previously been fixed for histology, the tissue was too degraded to be of use. Therefore, this work should be carried out in the future when populations are readily available again, either in New Quay and Corranroo or elsewhere. Additionally, it would be of interest to carry out the larval studies on Type 2 morphotype (thin form) as well, when this future work is carried out.

In Chapter 3, larval behaviour of this species, including fusion of the larvae, was also discussed. It was suggested that in high-density situations, larvae would fuse together in response to physical interactions; similarly, it was also suggested that commencement of cellular differentiation, and metamorphosis was triggered by the larva's cells coming in contact with each other. This may be one explanation of why the larvae of *H. indistincta* (usually) progressed through three pre-settlement stages wherein their body (and cells) became more compacted together which caused the cells to come into contact with each other. Another possible explanation is that the body became denser which allowed the larvae to commence settlement; it is possible that the two systems for commencing metamorphosis work in tandem, however this remains to be resolved.

My work contributes to the growing body of work suggesting that gastrulation occurs via “inversion of layers” where the ciliated epithelial cells migrate inward and form an internal layer (i.e. choanoderm) (Brien, 1967; Fell, 1974; Simpson, 1984; Barnes and Harrison, 1991; Ruppert and Barnes, 1994; Gaino and Burlando, 1990) (Chapters 4 and 5). To resolve this debate, as previous authors have suggested, a redefinition of the expectations and definitions of the gastrulation process is required, as there are obvious similarities in sponge development to the rest of the animal kingdom, as well as drastic

differences. However, differences are not unexpected when considering how varied the developmental processes, and morphological characters can be, even among sponge groups. Additionally, evidence was shown that the ciliated cells of the *H. indistincta* larvae not only transdifferentiate into the choanocytes, but that they also transdifferentiate into pinacocytes of the juvenile (Chapter 5). The inclusions of Type 1 cell with inclusions (described in Chapter 5) observed using TEM were suggested to correlate to the pink-stained material observed using histology (Chapter 3); and it was further suggested to be the origin of the sticky mucus that is present covering the larvae as well as in the adult body. It was shown that the inclusions (of Type 1 cell with inclusions, Chapter 5) expanded and formed the canal system of the juvenile. It was further suggested in Chapter 5 that the expanded inclusions observed in the larval stages (that were concentrated at either end of the larvae) served further possible functions such as forming the early canal system that reached the surface of the larvae, perhaps as a means of depositing the mucus material, and/or waste products to the surface of the larvae, and also acting as a means of regulating the buoyancy of the larvae. It was further suggested that the Type 1 cell with inclusions may have a role in the arrangement of the cells (e.g. choanocyte chambers) during metamorphosis. The cell type that creates these inclusions, which are key to the developmental process of this species, needs to be further investigated in other species of haplosclerids (i.e. *H. viscosa*). Whether or not this cell type is present in *H. viscosa* would also be informative as to whether the inclusions are responsible for the sticky consistency of the adult (and outside of the larvae) because the tissue consistency of *H. viscosa* is not sticky.

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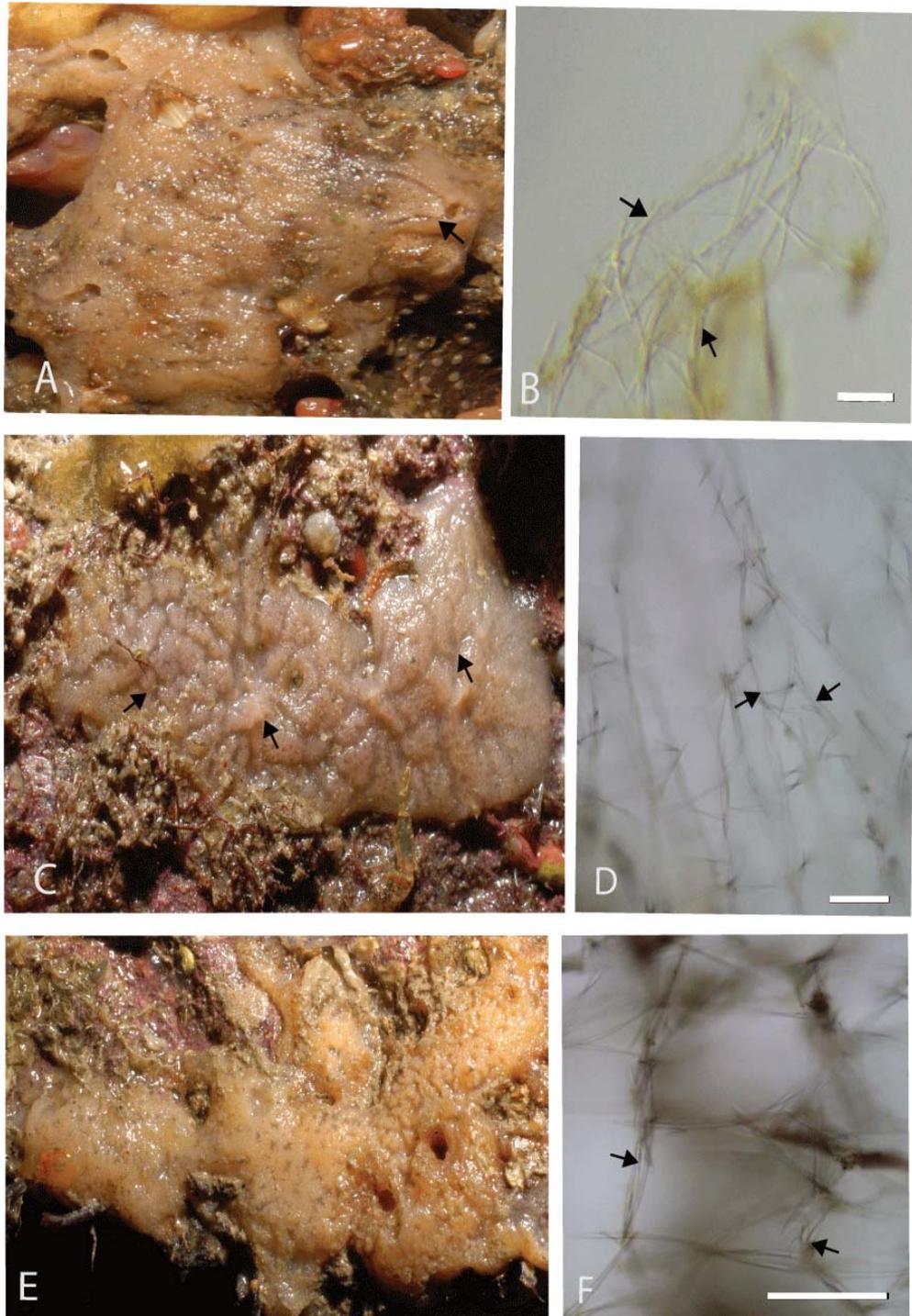
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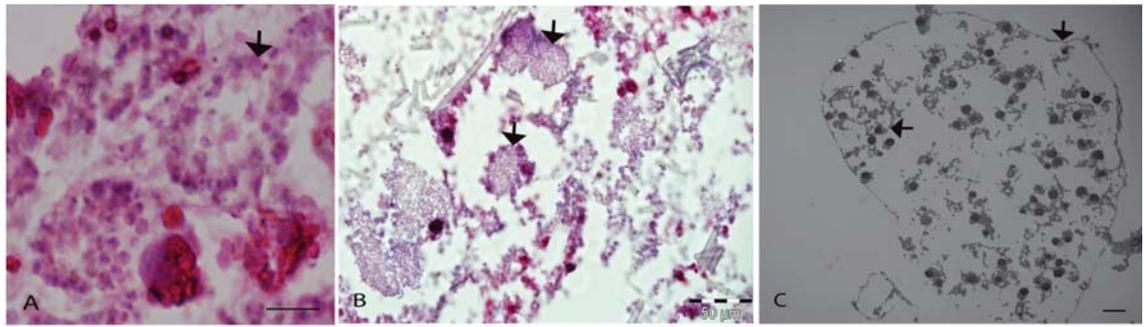
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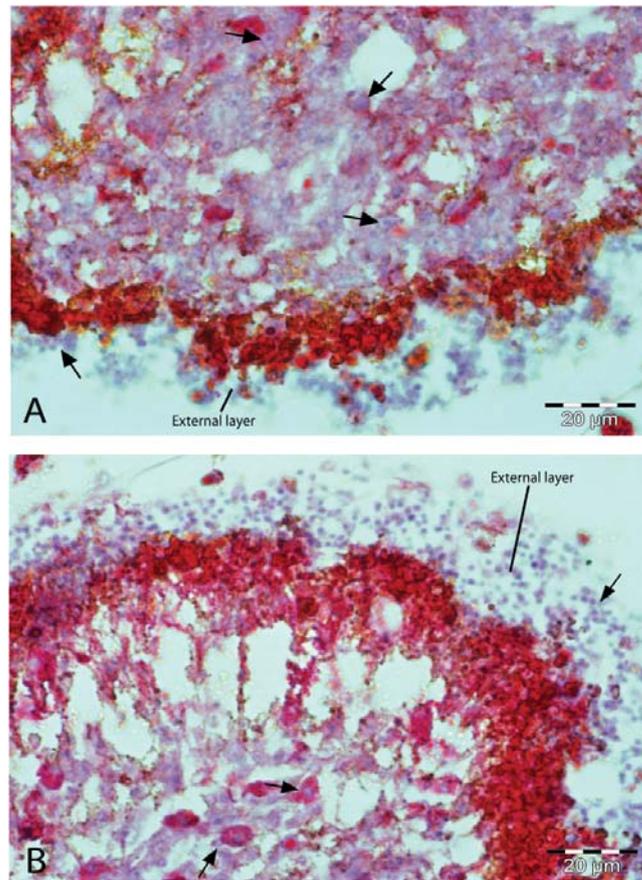
## **Appendices**



**Appendix 1.** (A) (Specimen B) *Haliclona indistincta* Morphotype 1 *in situ*. Arrows indicate membrane covering excurrent canal radiating from oscule. (B) Accompanying perpendicular skeletal section of *Haliclona indistincta* Morphotype 1 *in situ*. Arrow indicates multispicular primary lines. (C) (Specimen D) *Haliclona indistincta* Morphotype 2 *in situ*. Arrows indicate pockets or islands typical of Morphotype 2, that are a result of deep channels in the surface (D) Accompanying perpendicular skeletal section of *Haliclona indistincta* Morphotype 2 *in situ*. Arrows indicate multispicular primary lines. (E) (Specimen G) *Haliclona indistincta* Morphotype 2 *in situ*. Arrows indicate pockets or islands typical of Morphotype 2. (F) Accompanying perpendicular skeletal section of *Haliclona indistincta* Morphotype 2 *in situ*. Arrows indicate multispicular primary lines. **Scale bars**=100 $\mu$ m.



**Appendix 2.** Development of *H. indistincta* spermatozoa. (A) Choanocyte chambers filled with cells (arrow) Scale bar=10  $\mu\text{m}$  (B) Spermatocysts (arrows) (C) TEM image of sperm (arrows). **Scale bar**=500nm.



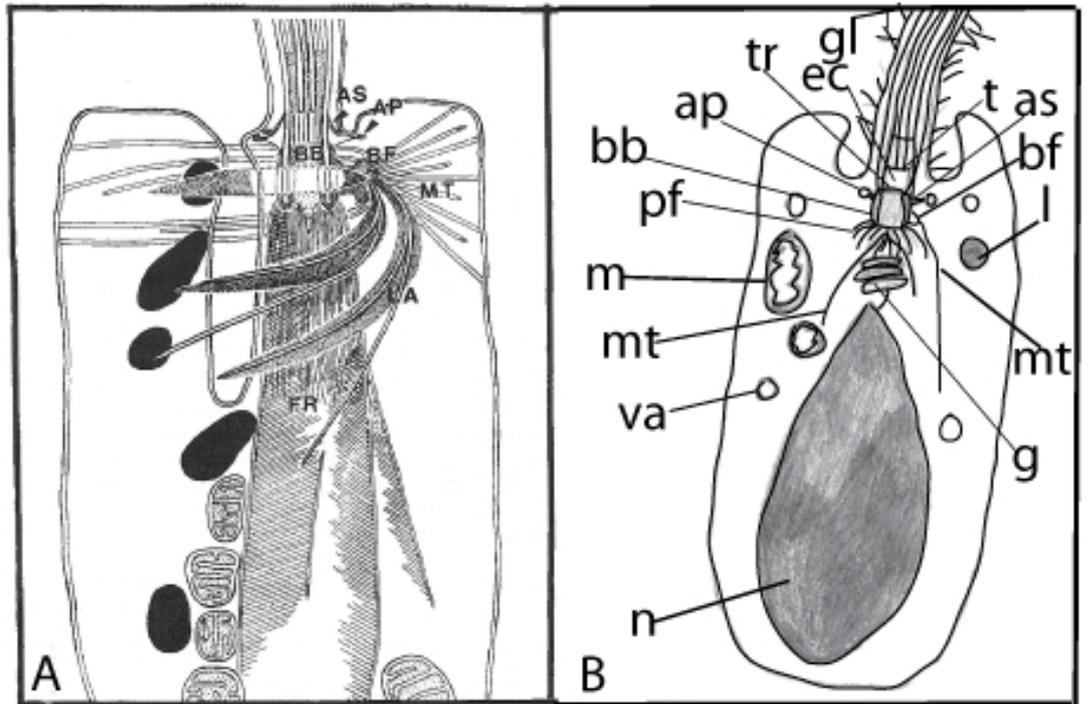
**Appendix 3.** *H. indistincta* larva sectioned at 4  $\mu\text{m}$ . (A) Posterolateral, (B) Anterior. Arrows indicate different cell types present. The external layer of cells is also indicated

Order	HAP (M)			HAP (FW)			HAL	POE		DEN	DIC	AST	CHO	HALI	HOM			
Species	<i>Haliclona indistincta</i>	<i>Haliclona tubifera</i>	<i>Haliclona permollis</i>	<i>Eunapius fragilis</i>	<i>Ephydatia muelleri</i>	<i>Spongilla lacustris</i>	<i>Halichondria melanadocia</i>	<i>Mycale cecilia</i>	<i>Mycale sylvina</i>	<i>Dendrilla cactus</i> (Aplysilla sp.)	<i>Ircinia oros</i>	<i>Thoosa mismalotti</i>	<i>Chondrilla australiensis</i>	<i>Halisarca dugardini</i>	<i>Oscarella lobularis</i>	<i>Oscarella imperialis</i>	<i>Oscarella tuberculata</i>	
Larval type	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA	HOP	CL (CO)	DI	CI	CI	CI	
Spicules present	N	Y	?	Y	Y	Y	N	Y	Y	N	N	Y	N	N	N	N	N	
Settlement: Anterior/Posterior	P	A	A	UA	UA	UA	UA	A	P	A	A	N/A	A	A	A	A	A	
Ciliation pattern	Uniform	Y	N	N	Y	Y	Y	N	N	N	N	N/A	N	N	N	Y	Y	
	Uniform w/bare (or few cilia at) posterior (pos.)	N	N	Y	N	N	N	N	Y	Y	N	N/A	N	Y	N	N	N	
	Uniform w/longer at pos.	N	N	N	N	N	N	Y	N	N	N	N/A	Y	N	N	N	N	
	Uniform w/ring of longer cilia around bare pos.	N	Y	N	N	N	N	N	N	N	Y	N/A	N	N	N	N	N	
	Uniform w/shorter at pos.	N	N	N	N	N	N	N	N	N	N	N/A	N	N	Y	N	N	
	A: little; L: Uniform; P: tufted	N	N	N	N	N	N	N	N	N	Y	N	N/A	N	N	N	N	N
Colour	PiW	C/PiW	RB	YI/G	YI/G	YI/G	YI	RO	?	Pi	GBB	B(?)	GB	MW	Pi	MW	Pi	
References	Stephens et al. (in press)	Woollacott, '93; Woollacott & Pinto, '95; Maldonado & Young, '96	Amamo & Hori, '96	Ivanova, '97a; '97b; Ereskovsky, '10	Ivanova, '97a; '97b; Ereskovsky, '10	Ivanova, '97a; '97b; Ereskovsky, '10	Woollacott & Pinto, '95; Ereskovsky, '10	Woollacott & Pinto, '95; Ereskovsky, '10	Wilson, 1935; Bergquist & Sinclair, 1968; Ereskovsky, 2010	Wilson, 1935; Bergquist & Sinclair, 1968; Ereskovsky, '10	Woollacott & Hadfield, '89; Woollacott & Pinto, '95; Ereskovsky, '10	Maldonado et al., 2003; Ereskovsky & Tokina 2004	Bautista-Guerrero et al., '10; Ereskovsky, '10	Usher & Ereskovsky, '05; Ereskovsky, '10	Gonobobleva, '07; Gonobobleva & Maldonado, '09; Ereskovsky, '10	Woollacott & Pinto, '95; Boursy-Esnault et al., '03; Ereskovsky, '10	Boursy-Esnault et al., '03; Ereskovsky, '10	Boursy-Esnault et al., '03; Ereskovsky et al., '07

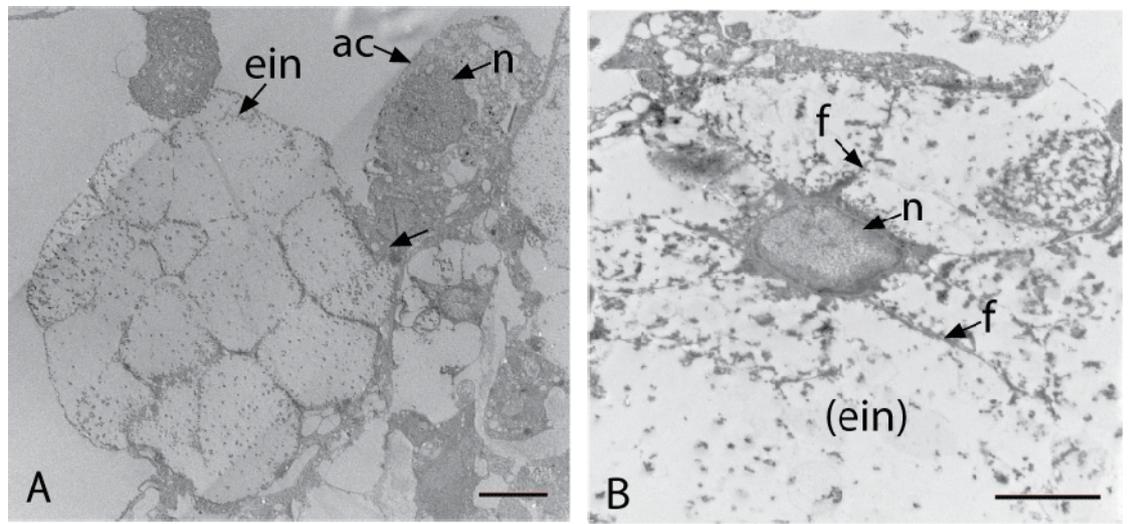
**Appendix 4.** Comparison of larval types found in demosponges: larval body. **HAP (M)**= Haplosclerida(marine); **HAP(FW)**=Haplosclerida(freshwater); **HAL**=Halichondrida; **POE**=Poecilosclerida; **DEN**=Dendroceratida; **DIC**=Dictyoceratida; **AST**=Astrophorida; **CHO**=Chondrosida; **HALI**=Halisarcida; **HOM**=Homosclerophorida; **PA**=Parenchymella; **HOP**=Hoplitomella; **CL**=Clavablastula; **CO**=Coeloblastula; **DI**=Dispherula; **CI**=Cinctoblastula; **N**=no; **Y**=yes; **N/A**=not applicable; **?**=missing or ambiguous information; **A**=anterior; **P**=posterior; **UA**=usually anterior; **PiW**=pink-white; **C/PiW**=cream to pink-white; **RB**=red-brown; **YI/G**=yellow to green; **YI**=yellow; **RO**=red-orange; **Pi**=pink; **GBB**=grey-black; **B**=brown; **GB**=grey-brown; **MW**=milky-white

Order	HAP (M)			HAP (FW)			HAL	POE		DEN	DIC	AST	CHO	HALI	HOM		
Species	<i>Haliciona indistincta</i>	<i>Haliciona tubifera</i>	<i>Haliciona permollis</i>	<i>Eunapius fragilis</i>	<i>Ephydatia muelleri</i>	<i>Spongilla laustris</i>	<i>Halichondria melandocia</i>	<i>Mycale cecilia</i>	<i>Mycale sylvia</i>	<i>Dendrilla cactus</i> (Abyssilla sp.)	<i>Ircinia oros</i>	<i>Thoosa miamolli</i>	<i>Chondrilla australensis</i>	<i>Halisarca dugardini</i>	<i>Oscarella lobularis</i>	<i>Oscarella imperialis</i>	<i>Oscarella tuberculata</i>
Larval type	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA	HOP	CL (CO)	DI	CI	CI	CI
Basal body Type	2	2	2	?	?	?	2	2	?	2	2(?)	N/A	2(?)	2	?	?	?
Basal foot	Y	Y	?	Y	?	?	Y	Y	?	Y	Y	N/A	Y	Y	Y	Y	Y
Basal foot shape	T	M	?	?	?	?	M	A	?	S	O	N/A	O	S	M	M	M
Accessory centriole	N	N	?	Y	Y	Y	Y	N	?	Y	Y	N/A	Y	Y	Y	Y	Y
Transverse cytoskeleton	N	Y	?	Y	?	?	Y	N	?	Y	Y	N/A	Y	Y	Y	Y	Y
Cross-striated rootlet(s)	N	N	?	?	?	?	N	N/A	?	N	N	N/A	N	N	N	N	N
Lateral arms	N	Y	?	?	?	?	N	N/A	?	Y	N	N/A	N	N	N	N	N
Basal foot microtubule(s)	Y	Y	?	?	?	?	Y	N/A	?	Y	N	N/A	N	Y	N	N	N
Longitudinal cytoskeleton	Y	Y	Y	Y	Y	Y	Y	Y	?	Y	Y	N/A	Y	Y	Y	Y	Y
Cross-striated rootlet(s)	N	N	N	N	N	N	N	N	?	N	N	N/A	N	N	Y	Y	Y
Fibrous rootlet(s)	Y	Y	Y	Y	Y	Y	Y	N	?	N	Y	N/A	Y	Y	N	N	N
Laminar sheets	N	N	N	N	N	N	N	Y	?	Y	N	N/A	N	N	N	N	N
Golgi associated with rootlet(s)	Y	N	N	Y (?)	?	?	Y	Y	?	Y	Y(?)	N/A	N	Y	Y	Y(?)	Y(?)
References	Stephens et al. (unpublished)	Woollacott & Pinto, '95; Maldonado & Young, '96	Amano & Hori, '96	Woollacott, '93; Woollacott & Pinto, '95; Woollacott & Young, '96	Ivanova, '97a; '97b; Ereskovsky, '10	Ivanova, '97a; '97b; Ereskovsky, '10	Woollacott & Pinto, '95; Ereskovsky, '10	Woollacott & Pinto, '95; Ereskovsky, '10	Wilson, '35	Woollacott & Hadfield, '89; Woollacott & Pinto, '95; Ereskovsky, '10	Ereskovsky & Tokina '04	Bautista-Guerrero et al., '10; Ereskovsky, '10	Usher & Ereskovsky, '05; Ereskovsky, '10	Gonoboleva, '07; Gonoboleva & Maldonado, '09; Ereskovsky, '10	Woollacott & Pinto, '95; Boury-Esnault et al., '03; Ereskovsky, '10	Boury-Esnault et al., '03; Ereskovsky, '10	Boury-Esnault et al., '03; Ereskovsky et al., '07

**Appendix 5.** Comparison of larval types found in demosponges: ciliated cells. **HAP (M)**= Haplosclerida(marine); **HAP(FW)**=Haplosclerida(freshwater); **HAL**=Halichondrida; **POE**=Poecilosclerida; **DEN**=Dendroceratida; **DIC**=Dictyoceratida; **AST**=Astrophorida; **CHO**=Chondrosida; **HALI**=Halisarcida; **HOM**=Homosclerophorida; **PA**=Parenchymella; **HOP**=Hoplitomella; **CL**=Clavablastula; **CO**=Coeloblastula; **DI**=Dispherula; **CI**=Cinctoblastula; **Y**=yes; **N**=no; **N/A**=not applicable; **?**=missing or ambiguous information; **basal body types 1 and 2**: *Sensu stricto* Woollacott and Pinto (1995); **T**=trapezoid; **M**=mushroom, stalked or campaign cork; **A**=articulated (*su stricto* Woollacott and Pinto, 1995); **S**=simple or circular; **O**=ovoid



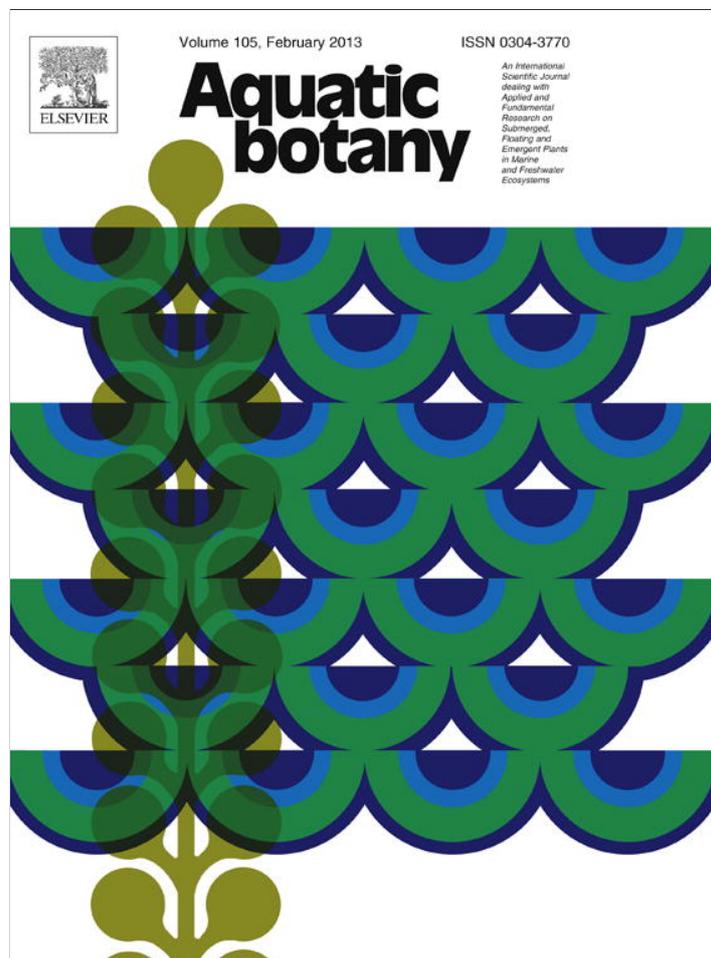
**Appendix 6.** Schematic drawings of ciliated epithelial cells of *H. tubifera* (A) (from Woollacott and Pinto, 1995), and *H. indistincta* (B) larvae.



**Appendix 7. *H. indistincta*.** A Expanded inclusions of Type 1 cell with inclusions with amoeboid cell in contact (arrows) (adult tissue). B Type 1 cell with inclusions. Nuclei in the middle of expanded inclusions (adult tissue). ac= amoeboid cell; ein=expanded inclusion; f=fibrillar material; n=nucleus. Scale bars: A-B=2 $\mu$ m.

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## Short communication

Composition and temporal variation of the algal assemblage associated with the haplosclerid sponge *Haliclona indistincta* (Bowerbank)Mónica B.J. Moniz<sup>a,\*</sup>, Fabio Rindi<sup>b</sup>, Kelly Stephens<sup>c</sup>, Elena Maggi<sup>d</sup>, Patrick Collins<sup>e</sup>, Grace P. McCormack<sup>c</sup><sup>a</sup> Irish Seaweed Research Group, Ryan Institute, Marine and Energy Research, National University of Ireland, Galway<sup>b</sup> Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy<sup>c</sup> Molecular Evolution and Systematics Laboratory, Ryan Institute, School of Natural Sciences, National University of Ireland, Galway<sup>d</sup> Dipartimento di Biologia, Università di Pisa, CoNISMa, Via Derna 1, 56126 Pisa, Italy<sup>e</sup> Benthic Ecology Unit, Ryan Institute, School of Natural Sciences, National University of Ireland, Galway

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## ABSTRACT

Although interactions between seaweeds and sponges have been studied in detail, general information concerning the whole epibiotic algal assemblage associated with a sponge species is virtually non-existent. We present here the first study in which the macroalgal community associated with a sponge, *Haliclona indistincta* (Bowerbank), was examined in detail. In the period October 2009–September 2010, the seaweed assemblage epibiotic on *H. indistincta* at a site of the Irish West coast was composed of 66 algal taxa (48 red algae, 7 green algae, 11 brown algae). The red algae *Gelidium spinosum* and *Rhodothamniella floridula* were the only epibionts associated with *H. indistincta* for the whole annual cycle. Most of the algal epibionts were filamentous species, which colonized the surface of the sponge and did not penetrate deeply into it. The algal assemblage was most abundant and species-diverse in the period late winter-spring; multivariate analyses revealed a significant variation of the community on the temporal scale of season and sampling date (weeks to months). The results indicate that the algal communities associated with sponges may be very diverse, showing that this type of assemblage deserves further detailed studies.

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## 1. Introduction

Macroalgae and sponges are ubiquitous in all marine coastal ecosystems where stable substrata exist. Given their widespread co-occurrence, these organisms may interact in many different ways, including complete symbiosis, mutualism, neutral tolerance, competition and parasitism. Neutral or negative interactions such as epibiosis and competition are the most common but have not been well studied (Wahl, 1989; Konar and Iken, 2005; Lopez-Victoria et al., 2006).

To date, the studies that have considered sponges and macroalgae in conjunction have focused on individual species, most frequently a species of alga and a species of sponge forming a strict association. A few investigations are available on the diversity of sponge assemblages associated with individual seaweed species (e.g., Tronchin et al., 2006; Avila et al., 2009). Conversely, detailed information about the algal assemblage associated with a certain sponge species is virtually non-existent. The information available

in this regard is scanty and based on low taxonomic resolution (e.g., Davis and White, 1994), quite possibly due to the fact that many species of sponges look superficially free of epibionts. It is well known that sponges produce many secondary metabolites with biological functions (Dobretsov et al., 2005; Taylor et al., 2007), including defence against epibionts and competitors; so it is probably assumed that sponges do not host a diverse assemblage of epibionts.

The first study examining in detail the diversity and temporal variation of the algal community associated with a sponge species is presented here. We examined the epibiotic assemblage of *Haliclona indistincta* (Bowerbank) at a site on the West Coast of Ireland. The genus *Haliclona* has been reported in frequent association with seaweeds (Boney and White, 1967; Tronchin et al., 2006; Avila et al., 2009) and this species was therefore considered a good candidate to host a rich and diverse vegetation of epibiotic algae.

## 2. Materials and methods

## 2.1. Study site

Sampling was performed at Corranroo, (53°9'6N, 9°0'59W), Galway Bay, Ireland, at low tide. At this site, *H. indistincta* is one of

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the dominant sponge species and forms a large population in the shallow subtidal zone. The site is a tidal inlet formed by a channel 50 m wide and is located approximately 7 km from the open part of Galway Bay. The rocky substratum consists of a mixture of granite and limestone. Due to its configuration, the site is subjected to wave-induced turbulence and strong tidal currents. Tidal amplitude ranges from about 5 m at spring tides to 2.5 m at neap tides. The historical range of water surface temperature in the study area is 5.5 °C in January/February to 16 °C in July (Guiry and Cunningham, 1984).

## 2.2. Sampling procedure

Sampling was carried out in the course of an annual cycle in an area of approximately 2000 m<sup>2</sup> in extent. For data analysis, the dates 8th October (date 1), 6th November (date 2) and 15th December 2009 (date 3) were considered autumn; the dates 29th January (date 4), 18th February (date 5) and 18th March 2010 (date 6), winter; 29th April (date 7), 25th May (date 8) and 24th June 2010 (date 9), spring; and 16th July (date 10), 16th August (date 11) and 9th September 2010 (date 12), summer. Thirty individuals of *H. indistincta* were collected on each sampling date. Samples were collected manually at low tide, removing sponge individuals in their entirety with a sharp knife. Each sponge individual was placed in a sealed plastic bag, which was kept in a cooler during transportation to the laboratory. In the laboratory, all visible algal epibionts (including those partly embedded in the tissue) were removed and identified immediately or frozen for subsequent identification. In order to make the data as comparable as possible, care was taken to collect for the study only sponges at least 10 cm in length and width.

Algal specimens were examined by light microscopy and identified based on morphological features. Voucher specimens for most of the species identified were deposited in the Phycological Herbarium of the National University of Ireland, Galway (GALW).

## 2.3. Data analysis

Due to the irregular shape of the sponge and the varied surface area of different sponge individuals, standardization of the abundance of the algal epibionts in terms of percentage cover, proved highly challenging. The small size of many epibionts also made it impractical to use biomass as a meaningful indicator of abundance. It was therefore decided to analyze the species richness as the total number of species in each sample. The structure and temporal variation of the community were analyzed in terms of presence/absence data. Because many species were only found in a relatively small number of samples, multivariate analyses were performed both on the complete species dataset and on a reduced dataset of 15 species (the most frequent and visually abundant). Species richness data were analyzed by ANOVA (Underwood, 1997), while multivariate data by means of permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001) based on Jaccard dissimilarities (Legendre and Legendre, 1998). In both cases a nested design was used, and there were two factors: Season (4 levels, fixed) and Date (3 levels, random, nested within Season), using 999 permutations. Patterns of multivariate data were visualized by means of nonmetric multidimensional scalings (nMDS). The centroids of the 30 replicate sponges on each date were used to display differences among dates and seasons. Centroids were calculated from the full set of principal coordinates obtained from the Jaccard dissimilarity matrix. Centroids and distances among them were obtained using the computer program PCO.exe (Anderson, 2003); nMDS plots were generated with PRIMER (Clarke and Gorley, 2006).

## 3. Results

Overall, 66 algal epibionts were recorded in the course of the study (Complementary Table 1). These consisted of 7 green algae (Chlorophyta), 11 brown algae (Phaeophyceae) and 48 red algae (Rhodophyta). About 9% of the taxa could not be identified reliably and it was therefore preferred not to separate them at species level. These were mainly algae belonging to morphologically simple genera (such as the green *Cladophora* and *Ulva*), whose identification is based on a limited set of morphological characters. At all times in the course of the study, most of the algal taxa found on *H. indistincta* ( $\geq 60\%$ ) belonged to the division Rhodophyta (Complementary Table 1). Except for the first sampling date of summer (date 10), the majority of the sponge individuals collected supported one or more algal epibionts. The highest numbers of sponge individuals with epibiontic algae were found in winter and spring whereas the lowest were found in summer (Table 1). The mean number of algal taxa per sponge individual was highest in spring, particularly early spring (Table 1). The highest number of taxa found on a single individual sponge (12 taxa) was recorded on dates 6 and 7, which correspond to late winter and early spring.

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.aquabot.2012.12.002>.

The only two species recorded on all sampling dates were the red seaweeds *Rhodothamniella floridula* and *Gelidium spinosum* (Complementary Table 1). Whereas specimens of *Cladophora* and leafy *Ulva* (Chlorophyta) were also recorded throughout the study period, these genera probably included several different species; it is likely that *C. hutchinsiae* and *U. lactuca* were the two most common. *Ceramium echionotum* was also very common, occurring as an epibiont of *H. indistincta* on all sampling dates except the first (Complementary Table 1). In general, many algal taxa occurred on a relatively low number of sponge individuals (data not shown). Many epibionts, especially filamentous species, were small-sized and/or in juvenile form. Usually, filamentous and leafy algae grew on the surface of the sponge and did not penetrate deeply into it. Conversely, some corticated species (in particular *G. spinosum*) were often embedded in the tissues of the sponge, sometimes to the extent that only a limited part of the alga could be observed externally. In the case of some leathery macrophytes (*Cystoseira* cf. *nodicaulis* and *Saccharina latissima*), articulated corallines (*Corallina* sp.) and corticated species (*Gastroclonium ovatum*, *Osmundea pinnatifida* and *Polysiphonia elongata*) the relationship established appeared to be coalescence rather than epibiosis. The sponge seemed to have gradually grown around the holdfast or stipe of the alga, surrounding it almost completely.

The ANOVA performed on species richness showed only a significant effect of the date of sampling ( $MS = 3.228$ ,  $F = 6.01$ ,  $p < 0.0001$ ; transformation:  $\ln(x+1)$ ), with no differences among seasons ( $MS = 4.908$ ,  $F = 1.52$ ,  $p > 0.2$ ; transformation:  $\ln(x+1)$ ). The results of the PERMANOVA performed on presence/absence data of the 15 most common species showed significant effects of both season ( $SS = 42,651$ ,  $MS = 14,217$ ,  $Pseudo-F = 1.6495$ ,  $p \leq 0.01$ ) and sampling date ( $SS = 68,952$ ,  $MS = 8619$ ,  $Pseudo-F = 2.2181$ ,  $p = 0.001$ ). The same results were obtained when the whole species dataset was analyzed (results not reported). The nMDS plot for the reduced species dataset reflected this pattern, showing that some dates were clearly separated from the others, even within the same season (Fig. 1). In terms of seasonality, a separation between summer and winter and between summer and autumn were the most apparent patterns (Fig. 1). *C. echionotum*, *Cladophora* spp., *Heterosiphonia japonica*, *Heterosiphonia plumosa* and *Corallina* sp. were more frequently present in winter than summer; *Cladophora* spp., *H. japonica*, *H. plumosa*, *R. floridula*, *Boergesenella fruticulosa*, *Ulva* spp. (blade forms) and *Ulva* spp. (*Enteromorpha* forms) were

**Table 1**  
Variation of some parameters of the structure of the epibiontic algal community of *Haliclona indistincta* in different seasons. Where appropriate, data are expressed as mean  $\pm$  standard error.

	Autumn	Winter	Spring	Summer
Number of Chlorophyta taxa	5	3	5	5
Number of Phaeophyceae taxa	4	5	9	6
Number of Rhodophyta taxa	36	33	29	20
Number of sponge individuals with $\geq 1$ algal taxon per sampling date	23.7 $\pm$ 4 (n=3)	24 $\pm$ 3 (n=3)	25 $\pm$ 3.4 (n=3)	17 $\pm$ 7.2 (n=3)
Number of algal taxa per sponge individual (not counting sponges devoid of algae)	3.5 $\pm$ 2.7 (n=71)	3.1 $\pm$ 2.5 (n=73)	3.8 $\pm$ 3.2 (n=75)	2.1 $\pm$ 2.7 (n=51)
Number of algal taxa per sponge individual (counting all sponges)	0.79 $\pm$ 0.13 (n=90)	0.80 $\pm$ 0.10 (n=90)	0.83 $\pm$ 0.11 (n=90)	0.57 $\pm$ 0.24 (n=90)

more frequently present in autumn than summer (Complementary Fig. 1).

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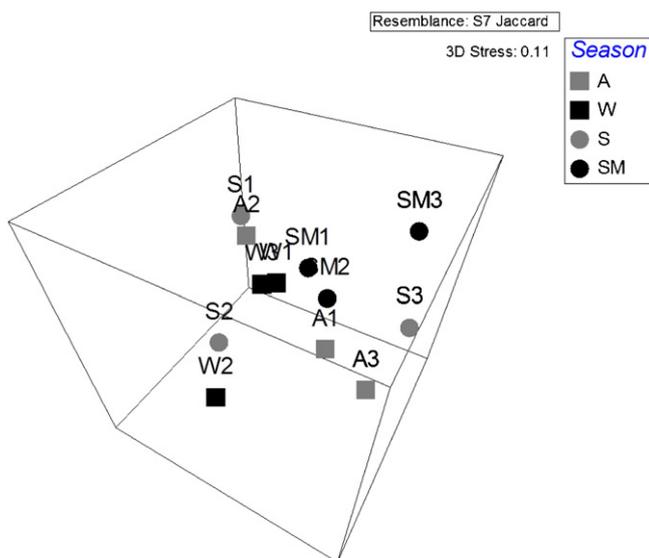
#### 4. Discussion

With 66 taxa recorded, the algal community associated with *H. indistincta* was unexpectedly diverse, even though comparisons with other sponge species are impossible due to the almost complete absence of published data. Apart from a few exceptions (such as *Anotrichium barbatum*), the algal species recorded are widespread on the shores of Ireland and Britain, and we observed many of them growing on rock or larger seaweeds close to *H. indistincta* at the study site. Only one, *Derbesia marina*, has been previously reported as epibiont of sponges (Brodie et al., 2007). For many species, especially filamentous, the presence on *H. indistincta* was occasional; these algae were found in small amounts, on a few sampling dates and on a small number of sponge individuals. In many cases they did not penetrate deeply into the body of *H. indistincta*, suggesting that they were serendipitous, opportunistic colonizers of the sponge surface. This appeared to be also the case for leafy species of *Ulva*, which reached some of the highest cover values observed in the assemblage in spring.

Interestingly, the two seaweeds most frequently found on *H. indistincta* were the red algae *R. floridula* and *G. spinosum*. *R. floridula* is a thin filamentous alga consisting of branched uniseriate axes

a few cm tall, widespread on rocky bottoms partially covered by sand in northern Europe. The gross morphology of *R. floridula* is identical to that of another widespread group of rhodophytes, the order Acrochaetiales. Species of Acrochaetiales (especially the genus *Acrochaetium*) are among the most common red algal endobionts, having been reported as endozoic in sponges (Boney and White, 1967; Rützler, 1990), hydroids (Sansón et al., 2002) and bryozoans (Dixon and Irvine, 1995) and endophytic in larger seaweeds (Dixon and Irvine, 1995). This indicates that this morphology is particularly suited for an endobiotic lifestyle, suggesting the possibility of beneficial effects for the alga and the host organism. *G. spinosum* is a corticated red alga with cylindrical or slightly flattened thallus variously branched, common in the subtidal zone of northern European shores. Thalli of *G. spinosum* were often enveloped by the tissues of *H. indistincta*; despite of this, the parts of the alga embedded within the sponge appeared perfectly vital and did not lose colour. A similar situation has been reported for other red algae that provide skeletal support to their sponge host (Rützler, 1990). *G. spinosum* seems therefore well integrated with *H. indistincta* and the relationships between these organisms are worthy of further study. Other red seaweeds with morphology similar to *G. spinosum* have superficial proliferations that represent favourable habitats for the settlement of sponge larvae (Phillips, 2002; Tronchin et al., 2006).

Temporal variation in the structure of the community was observed at season level and, most evidently, at sampling date level. When Davis and White (1994) looked to autumn's versus spring's epibiontic community of several sponge species in south-eastern Australia, these authors found that two sponge species were dominated by non-calcareous algae in the two seasons, whereas three other showed an increase in algal fouling in spring. The authors did not offer an explanation for this difference except that in general senescent basibionts tend to be more fouled. In the current study, the number of individuals fouled decreased in the summer (Table 1), but the variation between other times of the year was negligible. All sponges collected were fully-developed specimens, and therefore senescence and new generation of sponges are not an explanation for the lower number of sponges fouled in summer. Further studies are needed to understand why not all sponges were fouled and why this decrease. In general, the significant variability among seasons is probably due to differences in abundance of filamentous taxa (in particular *Cladophora* spp., *H. japonica* and *H. plumosa*). The fact that filamentous species were the most common may also explain why small scale temporal variation (weeks and months) was more significant than seasonal variation. Filamentous seaweeds usually have a quick growth, high inputs of propagules and quick life cycles with rapid transitions between different phases. The seasonal variance in the recruitment of foulers is probably explained by the seasonal variation in the phenology of the surrounding vegetation (Davis and White, 1994), since the life cycle of the epibiontic seaweeds is highly dependent on factors related to seasonality, such as day length, water temperature and amount of nutrients in the water column (Guiry and Cunningham, 1984).



**Fig. 1.** Two-factor nonmetric multidimensional scaling (nMDS) plot of centroids based on Jaccard dissimilarities calculated on presence/absence data for the 15 most common species. Factors considered are season and date; seasons are indicated as follows: autumn (A): grey squares; winter (W): black squares; spring (S): grey circles; summer (SM): black circles. Numbers in each label indicate the sampling date within the season.

Overall, the results indicate that the algal communities associated with sponges may be very diverse and include associations that may not be immediately observable with a superficial observation restricted to a single sampling time.

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# Reproductive cycle and larval characteristics of the sponge *Haliclona indistincta* (Porifera: Demospongiae)

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*Haliclona indistincta* has in some respects, a typical reproductive cycle for a marine haplosclerid sponge. We suggest that oocytes originate from archaeocytes and that spermatozoa originate from choanocytes. Oocytes were first seen in November and matured as eggs by May and June. Immature spermatid cysts were identified from February and mature cysts were present in May and June only. Of the individuals surveyed that had reproductive elements present (150/200), reproductive elements from a single sex were reported in over half of the specimens (59%) but there were also many hermaphrodites (41%). Embryos were first seen in June. Larvae were distributed throughout the mesohyl and were released from the end of June to the end of July. Three mobile larval stages and fusing of sibling larvae were observed. Post-settlement stages from early settlement to the development of oscula and excurrent canals are also shown. However, some elements of the larvae (uniform ciliation and no spicules at posterior pole) are not consistent with larvae from this genus.

**Keywords:** *Haliclona indistincta*, marine sponge, Porifera, reproduction, larval morphology, histology, microscopy

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## INTRODUCTION

Sponge reproduction has been a topic of study over several decades (e.g. Delage, 1892; Meewis, 1936; Leveaux, 1941; Lévi, 1956; Bergquist, 1979; Reischwig, 1983; Amano, 1986; Sarà, 1993; Mariani *et al.*, 2000, 2006; Ereskovsky, 2005; de Caralt *et al.*, 2007; Maldonado & Riesgo, 2009) but there are still reproductive processes that remain to be investigated, or are unconfirmed (Ereskovsky, 2010). Some sponge species are gonochoristic; however, most sponges are hermaphroditic, and this is also true of the marine haplosclerids (Bergquist, 1978; Ayling, 1980; Ilan & Loya, 1990a; Sarà, 1993; Maldonado & Riesgo, 2009). Viviparous and oviparous reproductive strategies are found in sponges (Bergquist, 1978). These traits were used to separate the marine Haplosclerida into two suborders: Haplosclerina (viviparous) and Petrosina (oviparous) (Lévi, 1956; Maldonado & Bergquist, 2002). Recently, molecular systematic approaches have indicated that both suborders are polyphyletic, suggesting that these traits have been acquired and lost over time in the marine haplosclerids (e.g. Borchiellini *et al.*, 2004; Raleigh *et al.*, 2007; Redmond *et al.*, 2007, 2011). The adult skeleton in haplosclerids forms the basis of the current classification, but is particularly simple leading to many problems in taxonomy and classification in the group. Molecular data suggests that some species are misplaced in the current classification (Raleigh *et al.*, 2007; Redmond *et al.*, 2011) and it is also likely that species have been misidentified and assigned to the wrong family or genus. A review of reproductive patterns

across the group is now highly recommended in parallel with classical morphological and molecular approaches given the new indications of phylogenetic relationships in this group.

Haplosclerid reproduction generally takes place during the warmer summer months. However, spawning often takes place during the winter months for oviparous haplosclerids such as *Petrosia ficiformis* (Maldonado & Riesgo, 2009) and some viviparous haplosclerids such as *Xestospongia testudinaria* and *Chalinula* sp. produce sperm year round (Fromont, 1988; Ilan & Loya, 1990a). Haplosclerids have parenchymella larvae (Ereskovsky, 1999), which in *Haliclona* as well as freshwater sponges at least, commonly possess a fringe of longer flagella that encircle a strongly pigmented posterior pole that is otherwise devoid of flagellated cells. Haplosclerid larvae have been described as possessing a dense bundle of oxeads located at the posterior end (Bergquist *et al.*, 1970; Simpson, 1984; Wapstra & Van Soest, 1987; Woollacott, 1993; Fromont, 1994; Ereskovsky, 1999). The presence of choanocyte chambers inside larvae is uncommon, but has been documented in two marine, haplosclerid species, *Haliclona limbata* and *Chalinula* sp. (Meewis, 1939; Ilan & Loya, 1990a). Once released, free-swimming larvae can remain mobile hours to weeks before they settle and develop into sessile juveniles (Maldonado *et al.*, 2008).

The origin of spermatogonia of freshwater haplosclerids is from flagellated choanocyte chambers (Efremova & Papkovskaya, 1980; Sukhodolskaya & Papkovskaya, 1985; Paulus & Weissenfels, 1986; Paulus, 1989; Weissenfels, 1989) but whether the suborder Spongillina belongs in the order Haplosclerida has yet to be conclusively determined (Redmond *et al.*, 2007; Sperling *et al.*, 2009). However, male gametes are also reported to develop from choanocytes in *Petrosia ficiformis*, a marine haplosclerid (Maldonado & Riesgo, 2009). Female gametes of haplosclerids are thought

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to develop from choanocytes, or archaeocytes (e.g. Tuzet, 1932; Meewis, 1936; Leveaux, 1941; Brien, 1967; Saller & Weissenfels, 1985; Saller, 1988; Weissenfels, 1989; Ereskovsky, 1999), and are associated with nurse cells; this is a greater specialization compared to other demosponge orders (Ereskovsky, 1999).

Reproductive biology and/or the form and behaviour of larvae have been described for quite a number of marine haplosclerid species, e.g. *H. oculata* and *H. xena* (Wapstra & Van Soest, 1987), *H. cinerea* (Meewis, 1941), *H. amboinensis* and *H. cymiformis* (Fromont, 1994), *Haliclona* spp. (Fromont, 1999; Whalan *et al.*, 2008), *H. loosanoffi* (Fell, 1976), *Callyspongia paralia*, *Niphates rowi*, *Petrosia elephantotus* and *Amphimedon chloros* (Ilan *et al.*, 2004; Ilan & Loya, 1990a, b), *H. permollis* (Elvin, 1976), *Xestospongia testudinaria*, *X. bergquistia*, and *X. exigua* (Fromont & Bergquist, 1994), *Petrosia ficiformis* (Maldonado & Young, 1996), *Amphimedon queenlandica* (Leys & Ereskovsky, 2006), and some of these studies provide excellent detail on the morphology throughout development, while others are limited in scope, or old and/or brief, providing considerably less insight, including the publications on *H. indistincta* (Bowerbank, 1866; Lévi, 1956). Furthermore, reports on the reproductive biology and form of the larva differ for some species (reviewed in Wapstra & Van Soest, 1987) suggesting the possibility of species misidentification in some cases. This is especially likely in species of the order Haplosclerida due to their notoriously simple skeleton and morphological plasticity.

We describe here the reproductive cycle and characteristics of the larva of *H. indistincta*, a species commonly found in the intertidal regions around Ireland and elsewhere (DeWeerd, 1986). The species name is derived from its variable form and the scarcity of defining characters (Bowerbank, 1866). Its growth form includes thin sheets or cushions, and the colour ranges from light tan to bright purple. Its most defining characteristic is its sticky mucous consistency (Lévi, 1956; DeWeerd, 1986). Lévi's (1956) description of reproduction in *H. indistincta* is focused upon the embryonic and larval stages and, though informative, is very brief. Our aim is to carry out a more detailed investigation of reproduction and development in this species and here we present data based upon histology and light microscopy.

## MATERIALS AND METHODS

### Material

To study the annual reproductive cycle of this species, *Haliclona indistincta* specimens (Figure 1A, identified via comparison of morphological characters with type material (DeWeerd, 1986) provided by the Zoological Museum University of Amsterdam) were collected from Corranroo, County Clare, Ireland (latitude 53° and longitude 9°). The sampling area was approximately 2000 m<sup>2</sup>. The tidal amplitude was a maximum of 5 m during spring tides and approximately 2.5 m at neap tides. In 2009–2010 the average surface water temperature (Malin Head station, 55°N and 7° W) ranged from 6.4°C in February to 15°C in August ([http://www.met.ie/marine/marine\\_climatology.asp](http://www.met.ie/marine/marine_climatology.asp)). The most prevalent rock types found in the area are granite and limestone, with limestone and sheets of algae as the preferred substrates of this species at this site. It can be an opportunistic

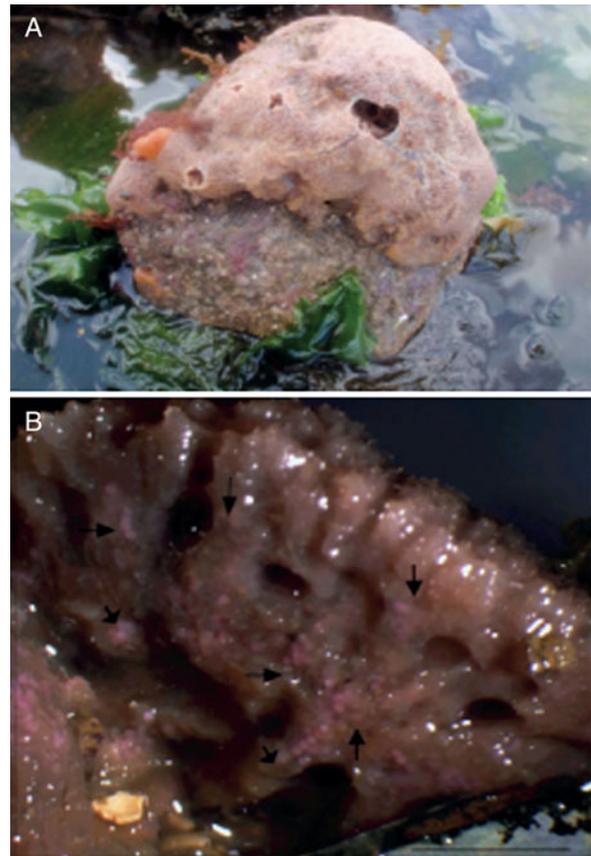


Fig. 1. *Haliclona indistincta*; (A) adult specimen *in situ*; (B) adult specimen with developing larvae (arrows) throughout the mesohyl. Scale bar: 5 mm.

settler as multiple specimens were also discovered growing upon the shells of bivalves.

Ten complete specimens ranging from 19 cm<sup>3</sup> to 30 cm<sup>3</sup> were collected monthly from September 2009 to September 2010 with an additional twenty specimens collected between April and July. Specimens were randomly sampled from just below the waterline during the low spring tides. The distance between sampled individuals was not measured but varied from approximately 30 cm (individuals on the same rock) to 300 m (individuals separated distantly on the same region of shoreline). From April onwards, multiple specimens were dissected to identify whether larvae were present and to observe the distribution of larvae throughout the parent sponge.

### Histological staining

Given that mature embryos/pre-released larvae were found distributed throughout the sponge bodies (Figure 1B), with a higher concentration seen in the mid-section, subsamples (5 mm<sup>3</sup>) were taken from the middle area of each specimen and fixed in Bouin's solution, and then transferred to 70% ethanol. The excised sections were washed with 70% ethanol until the Bouin's solution had been completely removed, and placed in histology cassettes in a histological tissue processor that moved the sections through a series of alcohol and Histoclear baths. The tissue was impregnated and blocked in paraffin wax. Successive serial sections (4 and 7 µm) were made with a microtome from each subsample, mounted on microscope slides, and stained with haematoxylin and eosin.

Slides were examined using light microscopy (Olympus BX51). Each specimen slide was examined for reproductive elements (oocytes, eggs, spermatid cysts, embryos and pre-released larvae). Ten representatives of each of the reproductive elements present (per slide) were measured where possible. All measurements were taken from the longest diameter of each structure using imaging software Cell<sup>^</sup>D (Olympus). The average size and number of the various reproductive elements of all the specimens per month was then obtained. Summary statistics and graphs were generated in Microsoft Excel.

## Larvae

Collection of larvae took place in the middle of the day during the low spring tides through the months May–August. Two methodologies were successfully employed to secure free-swimming larvae. Eight mature sponges were housed *in situ* by placing pyramid-shaped larval traps (made from nylon mesh with two collection tubes for larvae to swim into) over the rock containing the sponge. The larval collection tubes were checked at midday, five days a week during the last week of June and the first week of July, and once a week from the second week of July until the end of August. Collection tubes were replaced, and removed tubes were taken back to the laboratory where the contents were placed in 50 ml Petri dishes, and examined under a dissection microscope. We also induced release of larvae directly from parent sponges by cutting them open, allowing larvae to swim out into a container of water. Initially, larvae were held in varying densities in 25 ml Petri dishes in seawater. Subsequently, 69 larvae were individually maintained in 25 ml Petri dishes filled with filtered seawater. Specimens were allowed to settle and develop further. All stages were observed and photographed using a dissecting microscope (Olympus SZX16) and video recorded using a Panasonic 3CCD attached to a light microscope (Nikon SMZ-U).

## RESULTS

### Reproductive cycle

There was no evidence of reproductive elements in specimens collected in September, December and January (Table 1;

Figure 2A). All individuals collected between February and May contained reproductive material; this number dropped to 70% of individuals collected in July but was 100% in August (Figure 2A). The first sign of oogenesis was seen in October when one amoeboid oocyte (29  $\mu\text{m}$  in maximum diameter), without the presence of nurse cells, was observed in one specimen. Early oocytes were also seen in low numbers in November (one in each of two specimens, 28 and 32  $\mu\text{m}$ ). There were no early oocytes present in December and January. Of the 58 early amoeboid oocytes observed (and measured) in specimens from October through to August, the highest numbers were observed in March (14), April (18) and May (9). Early oocytes were still present in June–August, but were very few in number with three being present in June (sections from three specimens had one early oocyte each), one each in two July specimens, and four in August in three separate specimens.

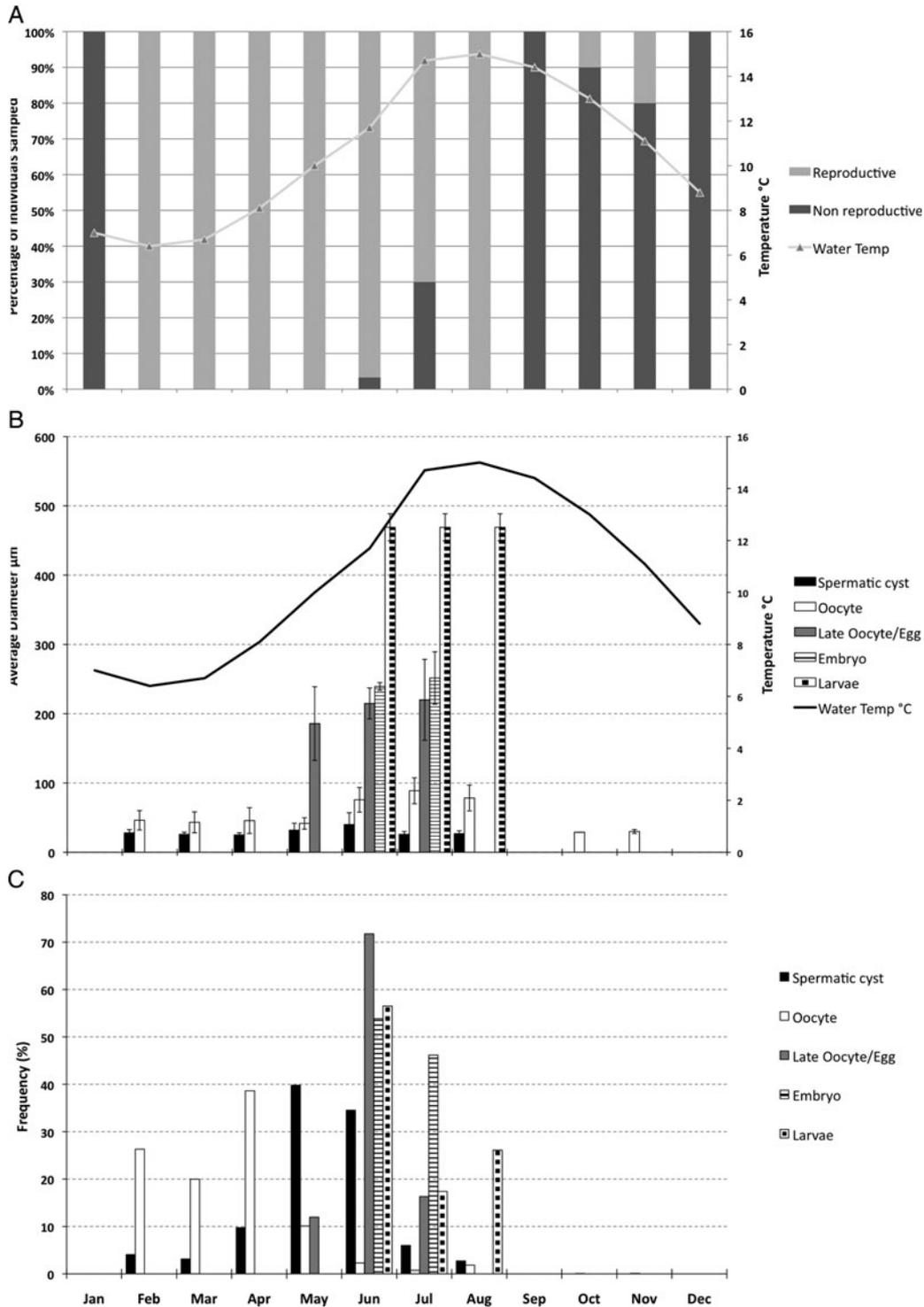
Reproductive elements increased in size from February through to August, in parallel to the rising water temperature (Figure 2B, C). The diameter of oocytes (Figure 3A) ranged from 29  $\mu\text{m}$  to 78  $\mu\text{m}$  ( $N = 1013$  mean 58  $\mu\text{m} \pm 27 \mu\text{m}$  standard error (SE)). Choanocyte chambers filled with cells and surrounded by a ring of choanocyte cells, suggestive of early spermatozoa (Supplementary Figure 1A), were seen from February through to the end of August. Fully developed spermatozoa were only seen during the months of May and June (Table 1) in one-sixth of the specimens surveyed.

Of a total of 200 mature *Haliclona indistincta* specimens examined throughout the year, 55 were hermaphrodites, 84 had female gametes only, while four had male reproductive tissue only (Table 1). A number of sponges contained developing oocytes and what we presume were early spermatozoa from February, and female-only sponges were also present at this time. By May, four sponges contained both fully developed spermatid cysts and late oocytes, while others still contained early spermatogonia along with early and late oocytes. Mature male-only sponges (i.e. with fully developed spermatid cysts, Supplementary Figure 1B) were seen first in June.

By the end of May, late oocytes (eggs) were seen in four out of the thirty specimens sampled and no embryos or larvae were seen. Eggs (Figure 3B), were circular and had an outer layer composed of yolk granules with an average size of 9  $\mu\text{m}$  ( $N = 10 \pm 1.7 \mu\text{m}$  (SE)) with nurse cells contained within the cytoplasm, as is typical of haplosclerids, with an

**Table 1.** Numbers of *Haliclona indistincta* specimens per month showing those that were found to be hermaphrodites (H), male (M), female (F) and those which had embryos and larvae of different stages. 0:10 = no specimen out of a total of ten specimens surveyed; 10:30 = ten specimens out of a total of thirty specimens surveyed.

Month	H	M	F	Late oocyte/eggs	Early embryos	Late embryos	Pre-released larvae
September	0:10	0:10	0:10	0:10	0:10	0:10	0:10
October	0:10	0:10	1:10	0:10	0:10	0:10	0:10
November	0:10	0:10	2:10	0:10	0:10	0:10	0:10
December	0:10	0:10	0:10	0:10	0:10	0:10	0:10
January	0:10	0:10	0:10	0:10	0:10	0:10	0:10
February	5:10	0:10	5:10	0:10	0:10	0:10	0:10
March	3:10	0:10	7:10	0:10	0:10	0:10	0:10
April	11:30	0:30	19:30	0:30	0:30	0:30	0:30
May	10:30	0:30	20:30	4:30	0:30	0:30	0:30
June	14:30	3:30	12:30	13:30	3:30	2:30	1:30
July	7:30	1:30	13:30	14:30	10:30	6:30	4:30
August	5:10	0:10	5:10	0:10	0:10	1:10	1:10



**Fig. 2.** (A) Graph showing (per month) the percentage of individuals sampled that were reproductive and non-reproductive. Also showing a plot of the water temperature over the same time period; (B) graph showing the average diameter of reproductive elements (standard deviation (error bars) included). Also showing a plot of the water temperature over the same time period; (C) graph showing the percentage frequency of each reproductive element of *Haliclona indistincta* over a twelve-month period, using 10 replicate individuals per month, e.g. 71% of all late oocytes/eggs were seen in June. The numbers of each reproductive element counted were: spermat. cysts N = 2166; oocytes N = 2133; late oocyte/egg N = 92; embryo N = 13; larvae (i.e. pre-released larvae) N = 23. For 'oocyte' both early and mid stages were included, and for 'spermat. cyst' both immature and mature stages were included.

average maximum diameter of 3 µm (N = 10 ± 1 µm (SE)), and a large amount of non-cellular material. The diameter of the eggs ranged from 186–220 µm (N = 261, mean 206 µm ± 45 µm (SE)). Embryos were similar in shape as they were also spherical but ranged in size from

239–252 µm (N = 64, mean 245 µm ± 22 (SE)). They did not possess the prominent layer of yolk granules on the exterior, and the internal cavity showed significantly less non-cellular material, being filled instead with dense clusters of cells. As the embryos matured inside the adult body, they

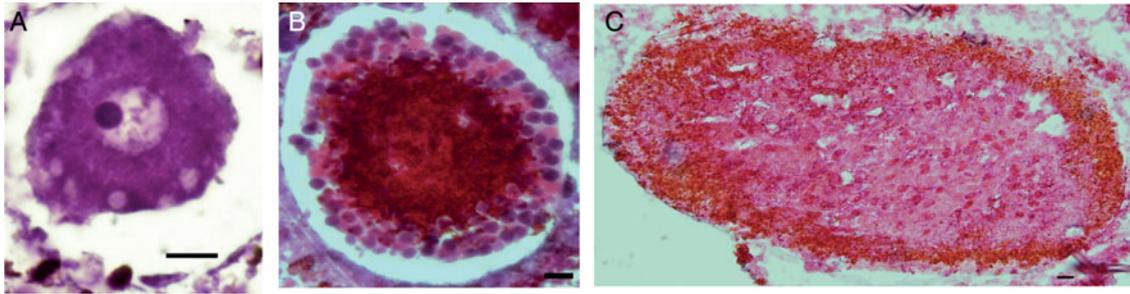


Fig. 3. (A) Early oocyte; (B) egg; (C) developed embryo, longitudinal-section. Scale bar: 10 µm.

attained a more elongated oval shape and, internally, the non-cellular material present formed a layer that separated an outer layer of epithelial cells and an internal area that contained a number of cell types (Figure 3C and Supplementary Figure 2). No spicules were present. The highest number of specimens containing mature embryos was in July, and by August, mature embryos were found in only one individual (Table 1). Mature embryos/pre-released larvae occurred in groups of varying numbers and were not held in defined brood chambers but were heavily surrounded by mucus (Figure 1B). The size of free-swimming larvae ranged from 414 µm to 477 µm ( $N = 41$ , mean  $469 \mu\text{m} \pm 20 \mu\text{m}$  (SE)).

## Larvae

Six larvae were retrieved from one larval trap on 29 June and over the six days between 1 July and 8 July a total of 67 larvae were collected from eight traps (i.e. eight sponges). The numbers of larvae collected in this way varied per day ranging from 0 larvae present in the traps to a maximum of 26 larvae present in a single trap. Only three sponges, out of the eight, released larvae over this time period. No larvae were present in the traps on 10 August. Most of the larvae used for observations were obtained through induced release in the laboratory. Three distinct free-swimming larval stages were observed. Figure 4A shows the initial free-swimming, mobile stage, which was oval and showed anterior–posterior orientation. The anterior end pointed upward when stationary, and at a slight angle when in motion (in the direction of movement). Larvae were found to swim in an anticlockwise, corkscrew pattern. The larvae were uniformly ciliated and the pink pigment associated with this species showed a higher concentration at the posterior pole. Figure 4B, shows a later stage where the shape of the larvae had changed to being compact and angular. The

pale anterior end diminished in size, but the anterior–posterior orientation was still apparent, especially when swimming. In a third stage (Figure 4C) the body was nearly perfectly circular, rotating on its axis, the pink colour of the free-swimming stage still apparent.

A fusing behaviour among the larvae was observed multiple times throughout this study; the highest number of events taking place in two separate (25 ml) Petri dishes holding 29 and 16 larvae, respectively. Of the dish containing 29 larvae, four separate fusing events were observed, with one fusion comprising three larvae (Figure 4D), and the remaining three events involving two larvae. In the dish containing 16 larvae, two separate fusion events each involving two larvae occurred. A third distinct fusing event was observed between two larvae when they were placed together in a drop of seawater (10 µl) for ten minutes. In all cases, larvae were observed to fuse and continue to develop to settlement stage.

Variability was seen in the amount of time it took for each larva to progress through the mobile stages. The shortest time from release to settlement was 25 hours (11/69 larvae), and longest time was 285 hours (3/69 larvae), with an average time of 161 hours (7 days). When about to settle, larvae ceased their horizontal forward corkscrew movement, though still spinning anti-clockwise, and eventually remained stationary, attached via the posterior pole and settled. Two hours after settlement, specimens began to flatten onto the substrate. After 24 hours, specimens were almost completely flattened. Subsequent days produced ostia formation (Figure 5A), the development of excurrent channels (Figure 5B) and oscular opening formation (Figure 5C). Sponge larvae, and newly settled sponges, were observed to be the dietary target of a water mite that could not be removed by filtering the water. No sponge survived beyond 39 days after release.

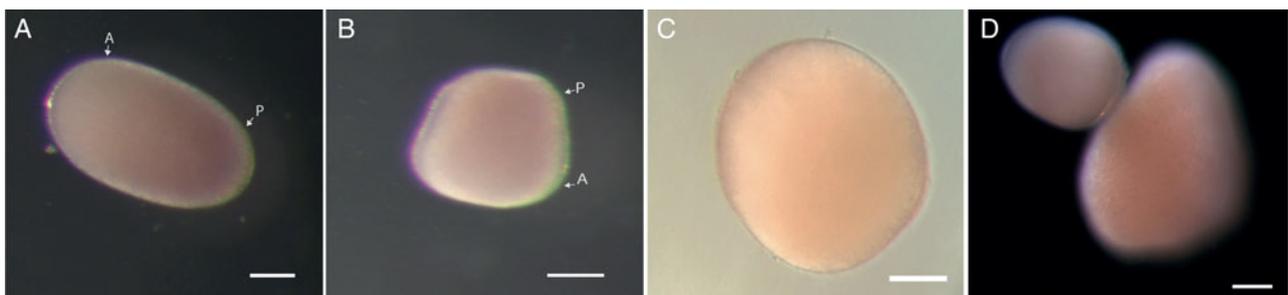
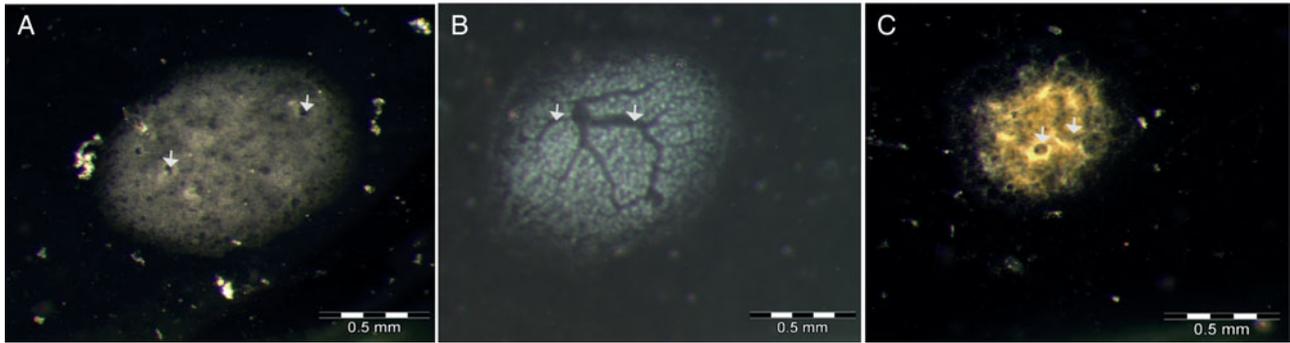


Fig. 4. *Haliclona indistincta* larvae: (A) free-swimming stage; (B) compact angular stage showing a diminishing anterior end; (C) semi-spherical ball stage; (D) free-swimming larvae fusing with two other already combined larvae. Scale bar: 100 µm.



**Fig. 5.** Post-settlement stages of *Haliclona indistincta*: (A) juvenile 37 hours post-settlement showing surface becoming punctated (arrows); (B) juvenile 39 hours post-settlement showing excurrent canals (arrows); (C) juvenile 115 hours post-settlement, formation of oscules and excurrent canals (arrows), colour change associated with addition of *Phaeodactylum tricornutum* (diatom used in feeding juvenile sponges).

## DISCUSSION

We have added to the current knowledge about the reproductive cycle and early development of *Haliclona indistincta* from that presented by Lévi (1956). We show the species to be primarily gonochoric but we cannot rule out that this species may be sequentially hermaphroditic as individual adult sponges were not sampled throughout the season. Female-only sponges appeared first and male-only sponges appeared later (in June), while hermaphrodites were present between February and August. Males were very few in number (4/200). It is possible that the focus on the middle section of the sponges resulted in missing male gamete production in the specimens surveyed. The asynchronous gamete production observed, a strategy commonly found in viviparous sponges (Simpson, 1980; Tarin & Cano, 2000), can result in extended larval release, as also seen in this species (mature larvae present from the end of June to early August).

The first appearance of identifiable oocytes showed amoeboid cells, which were very similar to archaeocytes though larger. Their form, and given that they were not found associated with choanocyte chambers, leads us to suggest an archaeocyte origin for oocytes, which is also consistent with other Haplosclerida (Leys & Ereskovsky, 2006). The identification of male reproductive elements was difficult, however, we suggest that spermatogenesis in this species is consistent with other haplosclerids. While the identity of mature sperm in the spermatid cysts were confirmed via transmission electron microscopy (TEM) (Supplementary Figure 1C), it was not possible to confirm the identity of developing spermatozoa in the same way. However, the presence of choanocyte-filled chambers (Supplementary Figure 1A) is consistent with the manner in which spermatid cysts develop in other sponges including the marine haplosclerid *Petrosia ficiformis* (Maldonado & Riesgo, 2009), and other haplosclerid species, as previously described (e.g. Efremova & Papkovskaya, 1980; Sukhodolskaya & Papkovskaya, 1985; Weissenfels, 1989; Ilan, *et al.*, 2004), as well as other orders (Halicondrida, (Barthel & Detmer, 1990) and Dictyoceratida (Kaye & Reising, 1991)).

We observed fewer embryos in the months June–July than oocytes or larvae. It is possible that oocytes were reabsorbed into the sponge body and did not contribute to the reproductive effort. It is also possible that through the sampling and sectioning approach adopted, some embryos were missed. In a similar manner to the embryos described by Lévi (1956), the embryos seen during the current study showed the

presence of non-cellular material associated with the oocytes, embryos and larvae from an early stage (February). This material made it difficult to document cleavage and early development using the approaches employed here and we are currently pursuing TEM for more clarity. Unidentified, dense, granular material has been found to be associated with developing oocytes of a number of sponge species including the marine haplosclerid *Haliclona cinerea* (Tuzet, 1947), and *Stelletta grubii* (Liaci & Sciscioli, 1967), *Suberites massa* (Diaz *et al.*, 1975), *Aplysina cavernicola* (Gallissian & Vacelet, 1976), *Hippospongia lachne*, *Spongia barbara*, *S. graminea* and *S. cheiris* (Kaye, 1991) and, though its function and composition is still unknown, it has been suggested to be important to oocyte development (Simpson, 1984; Kaye, 1991). We made initial attempts to identify the material (Masson's trichrome with Gomori's aldehyde fuchsin stain to identify collagen and PAS (periodic acid-Schiff) to identify carbohydrate) with no success; further work is ongoing on this material.

Increase in the numbers of reproductive individuals, as well as the diameter of reproductive elements (e.g. eggs and embryos) corresponded to an increase in water temperature (Figure 3A–D), a particularly important dynamic for initiating reproductive processes as shown by other authors (e.g. Fromont & Bergquist 1994; Witte *et al.*, 1994; Ereskovsky, 2000; Whalan *et al.*, 2007; Maldonado & Riesgo, 2008). The timing of larval release described here is shorter than described in Lévi (1956), as we did not find any mature larvae until the end of June while he suggested a period from May to July. It is possible that the geographical difference between the two study sites (Clare, Ireland versus Brittany, France) may have contributed to this difference. The size of the reproductive elements also differed between the studies; Lévi (1956) recorded an egg size of 300–350  $\mu\text{m}$ , which is larger than the eggs recorded in our study and the largest embryo we measured was 252  $\mu\text{m}$ . Lévi (1956) recorded a maximum length of larvae in Brittany to be 500  $\mu\text{m}$ , while the corresponding value for the Clare population is 477  $\mu\text{m}$ . The geographical difference could also contribute to this discrepancy in the size of reproductive elements. However, we have also observed two morphotypes currently ascribed to *H. indistincta*. Details of the morphology of the specimen used in Lévi's study are not provided (we investigated the cushion form), and it is possible that the larvae from the two studies are from different morphotypes. Further work on the two morphotypes of this species and

on specimens from different populations would shed light on this matter.

We have also observed the fusion of larvae in this species as has been described for other sponges (e.g. *Ophlitaspongia seriata* (Fry, 1971), *Crambe crambe* (Van der Vyver, 1970), *Halichondria panicea*, *Hymeniacidon perlevis*, *Pachymatisma johnstonia*, *Clathrina (Leucosolenia) coriacea*, *Iophon hyndmani*, and *Dercitus bucklandi* (Fell, 1974)), including haplosclerids (e.g. *Chalinula* sp. Ilan & Loya (1990b), *Petrosia ficiformis*, Maldonado & Riesgo (2009), *Haliclona* sp. (McGhee, 2006) but it is still relatively little studied.

All fusing larvae from *H. indistincta* seen in this study were from the same parent sponge. Only one-sixth of adult specimens collected in May and June possessed both spermatocysts and oocytes, therefore, while self-fertilization may be possible in these individuals (and yield more genetically similar offspring) it is unlikely to be the case for the entire population. Without genotyping adults and larvae from such fusion events it is difficult to predict whether they are chimeras of siblings that are more genetically similar than expected from sexual reproduction, or not. Ilan & Loya (1990b) and McGhee (2006) have shown that larva fuse indiscriminately with other genetically distinct larvae (and juveniles), but that adults will only fuse with fragments from genetically identical individuals, suggesting that the mechanism allowing distinction of self from non-self develops sometime after settlement (Ilan & Loya, 1990b). Fusion of sponge larvae and early juveniles may be an advantage as a higher rate of survivorship has been demonstrated for juvenile sponges that had previously formed fused groups (e.g. Fry, 1971; Connell, 1973; Highsmith, 1982; Ilan & Loya, 1990b). Also, a larger body size gained through fusion may allow an individual to be sexually mature sooner (Connell, 1973; Highsmith, 1982; Ilan & Loya, 1990b).

However, fusing appears to be more likely to occur if larvae are under some sort of stress, e.g. forcefully moved, overcrowded or restricted (Ilan & Loya, 1990b; McGhee, 2006) as was also seen during this study for *H. indistincta*. If close proximity of larvae induces the fusion of individuals, which our findings would suggest to be the case, then it could also be a competitive mechanism in environments where resources are limited (McGhee, 2006).

Ereskovsky (2010) described the early presence of a dense bunch of spicules at the posterior pole of haplosclerid larvae as a common feature of this group, and reported this in a number of *Haliclona* species, e.g. *H. aquaeductus*, *H. cinerea*, *H. ecbasis* and some fresh water sponges (Ereskovsky, 1999, 2010). However, spicules were not evident in the larvae of *H. indistincta* and were also not mentioned by Lévi (1956) in his work on this species. Ereskovsky (2010) also indicated that *Haliclona* larvae possess a ring of longer cilia at an otherwise bare posterior pole, however *H. indistincta* were uniformly ciliated (also described by Lévi, 1956). The external characteristics of *H. indistincta* larvae were also not reminiscent of the *Haliclona* sp., *Chalinula* sp. or *C. limbata* larvae illustrated by Mariani *et al.* (2005), lacking the longer posterior cilia and/or the pointed anterior pole. Because we observed this uniform ciliation both in larvae whose release from the parent sponge was induced, and from larvae collected in larval traps, the external morphology observed is not likely to be due to immaturity. Further, Maldonado & Bergquist (2002) also describe the larva of *H. petrosioides* as being uniformly ciliated.

Bergquist (1979) reported two larval types in the Haplosclerida. While she described *Callyspongia*, *Adocia* and *Haliclona* as having a ring of long cilia and a bare posterior pole, *Reniera*, which is now a synonym of *Haliclona* and *Chalinula* larvae both lacked this ring of longer cilia. While Wapstra & van Soest (1987) predicted that Bergquist's *Reniera* would belong to Halichondrida and *Chalinula* to the Poecilosclerida this was never subsequently determined to our knowledge. In any case, recent molecular phylogenies suggest that the family Chalinidae and the genus *Haliclona* are polyphyletic and that species of what were *Reniera* (e.g. *H. fulva*) are not closely related to the type species *H. oculata* (Redmond *et al.*, 2011).

Another unusual aspect of *H. indistincta* larvae is that they settled on the posterior pole rather than the anterior pole, which was also described by Wilson (1935) for the poecilosclerid *Mycale syrinx* but appears to be otherwise uncommon in sponges. Langenbruch & Jones (1990) suggested that adult *H. indistincta* had a different morphological structure from other *Haliclona* species after histological examination of the structure of the choanocyte chambers in particular. Given that recent molecular data suggest that *Haliclona* probably contains a large diversity of distantly related species (e.g. Raleigh *et al.*, 2007; Redmond *et al.*, 2007, 2011), our results may also support the suggestion that *H. indistincta* is not closely related to other typical *Haliclona* species (e.g. the type species *H. oculata*).

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## Supplementary materials and methods

The supplementary material referred to in this paper can be found online at [journals.cambridge.org/mbi](http://journals.cambridge.org/mbi).

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# Phylogenetic Relationships of the Marine Haplosclerida (Phylum Porifera) Employing Ribosomal (28S rRNA) and Mitochondrial (*cox1*, *nad1*) Gene Sequence Data

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## Abstract

The systematics of the poriferan Order Haplosclerida (Class Demospongiae) has been under scrutiny for a number of years without resolution. Molecular data suggests that the order needs revision at all taxonomic levels. Here, we provide a comprehensive view of the phylogenetic relationships of the marine Haplosclerida using many species from across the order, and three gene regions. Gene trees generated using 28S rRNA, *nad1* and *cox1* gene data, under maximum likelihood and Bayesian approaches, are highly congruent and suggest the presence of four clades. Clade A is comprised primarily of species of *Haliclona* and *Callyspongia*, and clade B is comprised of *H. simulans* and *H. vansoesti* (Family Chalinidae), *Amphimedon queenslandica* (Family Niphatidae) and *Tabulocalyx* (Family Phloeodictyidae), Clade C is comprised primarily of members of the Families Petrosiidae and Niphatidae, while Clade D is comprised of *Aka* species. The polyphyletic nature of the suborders, families and genera described in other studies is also found here.

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## Introduction

Haplosclerid sponges are extremely important in terms of numbers and diversity of species and habitats, as ecosystems, and as producers of bioactive compounds [1–4]. Taxonomically, they are also one of the most difficult and unstable groups of the Class Demospongiae *sensu stricto* [5] and a sound classification of the order is a long way from being established. This is because of low numbers of synapomorphies, plasticity of morphological characters, large number of species, and major discrepancies between morphological and molecular data. In the latest complete classification of the Porifera, *Systema Porifera: A guide to the classification of sponges*, the Order Haplosclerida Topsent, 1928 comprises three suborders; Haplosclerina Topsent, 1928, Petrosina Boury-Esnault and Van Beveren, 1982 and Spongillina Manconi and Pronzato, 2002; [6–8]. However, molecular data from ribosomal and mitochondrial genes and mitochondrial genomes have shown freshwater sponges (Suborder Spongillina) as closely allied to other demosponge orders including Poecilosclerida and Agelasida, [5,9–13], while nuclear protein coding data is consistent with a monophyletic Haplosclerida [14].

The taxonomic history of the marine species is complicated and many classification schemes have been proposed, [15–20]. In

*Systema Porifera* [21,22] each of the two marine haplosclerid suborders, i.e. Haplosclerina and Petrosina, were defined on the basis of skeletal architecture and reproductive strategy (e.g. members of the Haplosclerina are viviparous while those in the suborder Petrosina are oviparous), but they were also seen as being related due to similarities in spicule form and size and their shared chemistry [2,23]. The monophyly of the marine haplosclerids has been confirmed in a number of molecular-based studies but the monophyly of each of the two suborders, has been questioned suggesting that reproductive mode is not a good indicator of phylogenetic relationships for this group of sponges [5,9,10,24]. van Soest and Hooper [21,22] had already suggested that morphological synapomorphies supporting Haplosclerina and Petrosina as suborders were “vague and elusive, many of them being shared by sponges in other groups”, thus it may not be a surprise to find them to be polyphyletic.

Within the suborder Haplosclerina, the secondary ectosomal reticulation described by de Laubenfels [25] characterizes the family Callyspongiidae and should be enough to separate it from the Chalinidae and Niphatidae [18,26]. While Gray [27] and Lendenfeld [28] suggested that the Chalinidae contained highly unrelated sponges, De Weerd [29] took the opposite view and collapsed 27 chalinid genera to four and assigned six subgenera to

*Haliclona* [30]. Raleigh et al. [24] employing the Erpenbeck fragment of *cox1* [31] suggested that neither of the families Callyspongiidae and Chalinidae nor the genera *Callyspongia* and *Haliclona* were monophyletic. Difficulties at the species level for some *Haliclona* have also been indicated e.g. *H. oculata* and *H. cinerea*, [24,32].

The molecular evolution of the Haplosclerida has been described as ‘enigmatic’ because their ribosomal genes appear to evolve at a different rate and in a different manner to other demosponges [33,34] and also the mitochondrial genome of the target species of the sponge genome project (*Amphimedon queenslandica*, Family Niphathidae) has a number of features separating it from the mitochondrial genomes of other demosponges [35]. In this paper we further investigate the phylogenetic relationships in the marine members of this group using DNA sequences of the D1–D5 region of the 28S rRNA from a wide range of marine haplosclerid taxa and additional evidence from mitochondrial data (the Folmer fragment of *cox1* and *nad1*) while also exploring the evolution of these gene regions in haplosclerid taxa.

## Materials and Methods

### Specimens and DNA Extraction

Sponge specimens included in this study were acquired either from the Zoological Museum Amsterdam (ZMA), from the National Institute of Water and Atmospheric Research (NIWA), New Zealand, collected fresh by SCUBA by Dr. Marieke Koopmans (formerly of Wageningen University Research Centre, Netherlands), by Dr. Bernard Picton (National Museums Northern Ireland, UK), by the Biological Institute on Helgoland (BAH) in Germany, or collected in Ireland by Dr. Niamh Redmond and Dr. Grace McCormack. Details of all specimens are listed in Table S1. All specimens had been stored in 100% ethanol and/or 6 M-guanidinium chloride. In the majority of cases, DNA was extracted from the specimens by standard phenol-chloroform-isoamyl extraction followed by ethanol precipitation, otherwise the QIAGEN DNeasy™ Tissue kit was used. Extracted DNA from haplosclerid samples employed in Nichols [5] was also kindly provided by Scott Nichols. A number of marine haplosclerid sequences generated as part of the Porifera Tree of Life project were also kindly provided to help increase taxon sampling in the present study.

### PCR and DNA Sequencing

All primers used in PCR amplification are shown in Table S2. PCR amplification of the D1 to D5 region of the 28S rRNA gene was attempted in three overlapping fragments. Primers from Folmer et al. [36] were utilised for amplifying the 5' region of the *cox1*. PCR primers to amplify *nad1* were designed from the mitochondrial genomes of *Callyspongia plicifera*, *Xestospongia muta*, *Amphimedon compressa* and *Haliclona implexiformis* [37] using the online primer design program PriFi [38, <http://cqi-www.daimi.au.dk/cqi-chili/PriFi/main>] and a DNA calculator (<http://www.sigma-genosys.com/calc/DNAcalc.asp>). All gene fragments were amplified in 50 µl reactions, which comprised 5 µl 10X PCR Buffer (Promega), 10 mM dNTPs (Promega), 2 µM primers and 1 unit of *Taq* Polymerase (Promega and Biolabs). MgCl<sub>2</sub> concentration ranged from 1.5 mM to 3 mM. The temperature regime for the 28S rRNA and *cox1* genes was an initial denaturation of 94°C for 5 min followed by 30 cycles of 1 min at 94°C, 30 sec at annealing temperature (between 38°C to 50°C depending on the DNA template and primer combination) and 1 min to 1 min 30 sec at 72°C. A final extension step of 5 min at 72°C finished the regime. For the *nad1* gene the temperature regime was 10 mins at 94°C,

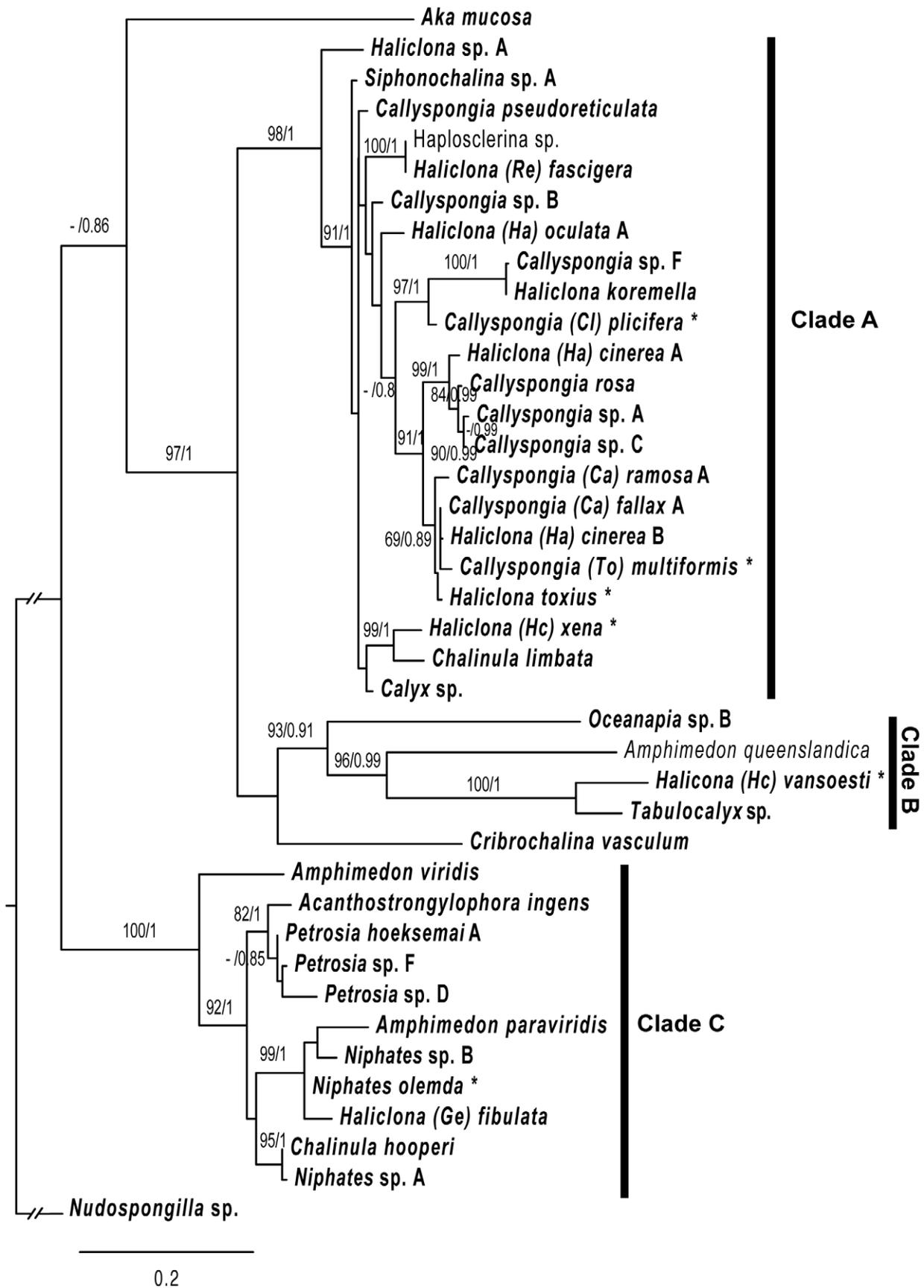
followed by 30 cycles of 30 sec at 94°C, 45 sec at an annealing temperature of 41°C and 90 sec at 72°C with a final extension step of 10 min at 72°C. All products were viewed on a 1% agarose gel stained with ethidium bromide or syber safe using a UV lightsource. PCR products were gel purified and automatically sequenced in both directions (by MWG-Biotech, Germany). It was not possible to amplify and sequence all gene regions from all specimens but from those that were sequenced, the resulting sequences were assembled into contigs using the SeqMan II software from the Lasergene package (DNASTAR Inc.) and the chromatograms were edited by eye. The fully edited consensus sequences were entered into a BLAST algorithm search [39] to check for possible contamination. All sequences have been deposited in GenBank (accession numbers JN178944-JN179046 (ribosomal sequences) and JN242192-JN242240 (mitochondrial sequences)). Additional sponge sequences for the various gene regions were downloaded from GenBank and used in phylogenetic analyses.

### Alignments

All multiple sequence alignments (including the additional sequences from previous studies that were submitted to GenBank) were assembled and edited in MacClade 4.0 [40]. The D2 region of the 28S rRNA gene was found to be hyper variable for some of the sequences and sequencing and analyses of this region was subsequently abandoned. Separate D1 (74 haplosclerid sequences) and D3–D5 (53 marine haplosclerid sequences) datasets were created. The full D1 alignment was 390 bp in length and 81 bp were removed due to ambiguous alignment (dataset S3). While the full D3 alignment was 735 bp, the alignment used for analysis was 518 bp (dataset S2). A concatenated dataset, (called D1–D5), was created by joining sequences of the D1 and D3–D5 regions from specimens that had both regions available (39 marine haplosclerids; dataset S1). In six cases the D1–D5 sequence originated from two separate individuals of the same species and these are marked with an asterisk on the tree produced. Freshwater sponges were initially chosen as outgroup for marine haplosclerids for all datasets as some data suggests that they are the closest sister group to the marine haplosclerids and, even if they are not the closest sistergroup, they are a monophyletic group within the G4 clade [5]. However, additional analyses were also carried out on the ribosomal and *cox1* datasets (datasets S4 and S5) using additional sequences from sponges who are part of the G4 clade [5] as outgroups. For the *nad1* alignment sequences from a range of other demosponges were included in the analyses using a *Plakinastrella* sequence (EU237487) as outgroup due to the low numbers of available sequences in GenBank for this region (dataset S6).

### Phylogenetic Analyses

Phylogenetic reconstruction was undertaken under a maximum likelihood framework implemented in RAxML 7.0.3 [41] using the GTR model of DNA substitution with model parameters optimised in RAxML, and with confidence levels estimated using bootstrap resampling (1000 replicates) and in PAUP\* 4.0b10 (Sinauer Assoc.) using model parameters estimated by jModelTest [42,43]. Inference under a Bayesian framework was undertaken using MrBayes 3.1.2 using the GTR substitution model with model parameters optimised by the program [44–46]. For each dataset, two runs of over 5 million generations were carried out with sampling every 100 generations. The appropriate burnin value was determined by examining the standard deviation of split frequencies to identify when convergence had occurred. A 50% majority rule consensus tree was constructed from all generations sampled after the burnin. Bayesian trees were also reconstructed using a covarion-like model, which allows substitution rates to vary across the tree.



**Figure 1. Maximum-likelihood phylogeny reconstructed using the D1–D5 region of the 28S rRNA gene.** The DNA substitution model parameters estimated by RAxML were: f(A) 0.24, f(C) 0.24, f(G) 0.33, f(T) 0.19; R(AC) 0.53, R(AG) 1.73, R(AAT) 1.0, R(CG) 0.52, R(CT) 5.71, R(GT) 1.0; alpha 0.52; pinvar 0.43. Sequences produced during this study are in bold. Sampling locations for each taxon are given in Table S1. Other sequences were downloaded from Genbank (*A. queenslandica*, EF654518, Haplosclerina sp., AY561860). Taxon labels showing an \* are those comprised of sequences from two specimens, in each case the D3 sequence was downloaded from Genbank (*C. multiformis*, AF441344 *C. plicifera*, AF441345; *H. toxius*, AF441342; *H. vansoesti*, AF441346; *N. olemda*, AF441353 and *H. xena*, AY319327). Numbers on the branches represent bootstrap proportions/posterior probabilities.  
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## Results

Although there were differences in the number and distribution of species between the datasets, overall, the tree topologies for the various gene loci were congruent with sequences falling in similar clades and positions. In addition there were very slight differences between ML and Bayesian analyses and between covarian and non covarian models in Bayesian analyses. The trees from RAxML are shown here along with bootstrap proportions (BP) and the posterior probabilities (PP) from the covarian Bayesian analyses (shown as BP/PP in the trees and remainder of the text).

In all trees there was a well supported clade (Clade A) containing multiple species of *Haliclona* and *Callyspongia*. Outside of this clade were a range of marine haplosclerid taxa from the genera *Petrosia*, *Oceanapia*, *Acanthostrongylophora*, *Amphimedon*, *Cribrochalina*, *Niphates* and *Xestospongia*. These sequences did not fall in a single clade and the relationships amongst themselves and with Clade A indicate a high diversity of marine haplosclerids. None of the gene trees supported the monophyly of the two marine suborders (Haplosclerina and Petrosina) or the five marine families examined, i.e. Callyspongiidae, Chalinidae, Niphatidae, Petrosiidae and Phloeodictyidae. A total of 12 genera, *Acanthostrongylophora*, *Amphimedon*, *Callyspongia*, *Chalinula*, *Cribrochalina*, *Haliclona*, *Neopetrosia*, *Niphates*, *Oceanapia*, *Petrosia*, *Siphonochalina* and *Xestospongia* were polyphyletic. The monophyletic status of the genera *Calyx*, *Cladocroce*, *Dasychalina*, *Dendroxea*, *Gelliodes*, *Hemigellius*, *Pachychalina* and *Tabulocalyx* could not be established as there was only one representative of these genera included in each of the various analyses. The genus *Aka* was found to be monophyletic but had few representatives.

### 28S rDNA phylogenies

The D1–D5 28S rDNA ML phylogeny is presented in Figure 1. This dataset had sequences from 39 marine haplosclerid species included from five families and 13 genera. Clade A was supported by 98/1 (BP/PP) and contained ten *Callyspongia* species, eight *Haliclona* species, *Chalinula limbata*, a *Calyx* sp., a *Siphonochalina* sp. and an individual identified as Haplosclerina sp. [9]. Smaller supported groupings of *Callyspongia* and *Haliclona* species were present within this clade. One such group had 69/0.89 support and contained *H. cinerea* B (POR14110), *H. toxius*, *C. fallax*, *C. multiformis* and *C. ramosa* A (MKB3142). Another clade (99/1) contained three *Callyspongia* species and *H. cinerea* A (POR17651). A third group with 97/1 support contained *C. plicifera*, *H. koremella* and *Callyspongia* sp. F ((MKB1668). Sequences from *Amphimedon queenslandica*, *Haliclona vansoesti*, *Tabulocalyx* sp. and *Oceanapia* sp. B (MKB586) formed another highly supported clade (Clade B, 93/0.91). Long branch lengths for this group suggest undersampling and/or high divergence. *Cribrochalina vasculum* was sister to Clade B but without support. A relationship between Clades A+B and *Cribrochalina vasculum* was also highly supported (97/1).

Within a third clade (Clade C supported by 100/1) three *Petrosia* species clustered with *Acanthostrongylophora ingens* (82/1). Two *Niphates* sequences were present in the same clade with *Haliclona fibulata* and *Amphimedon paraviridis* (99/1). *Chalinula hooperi* clustered

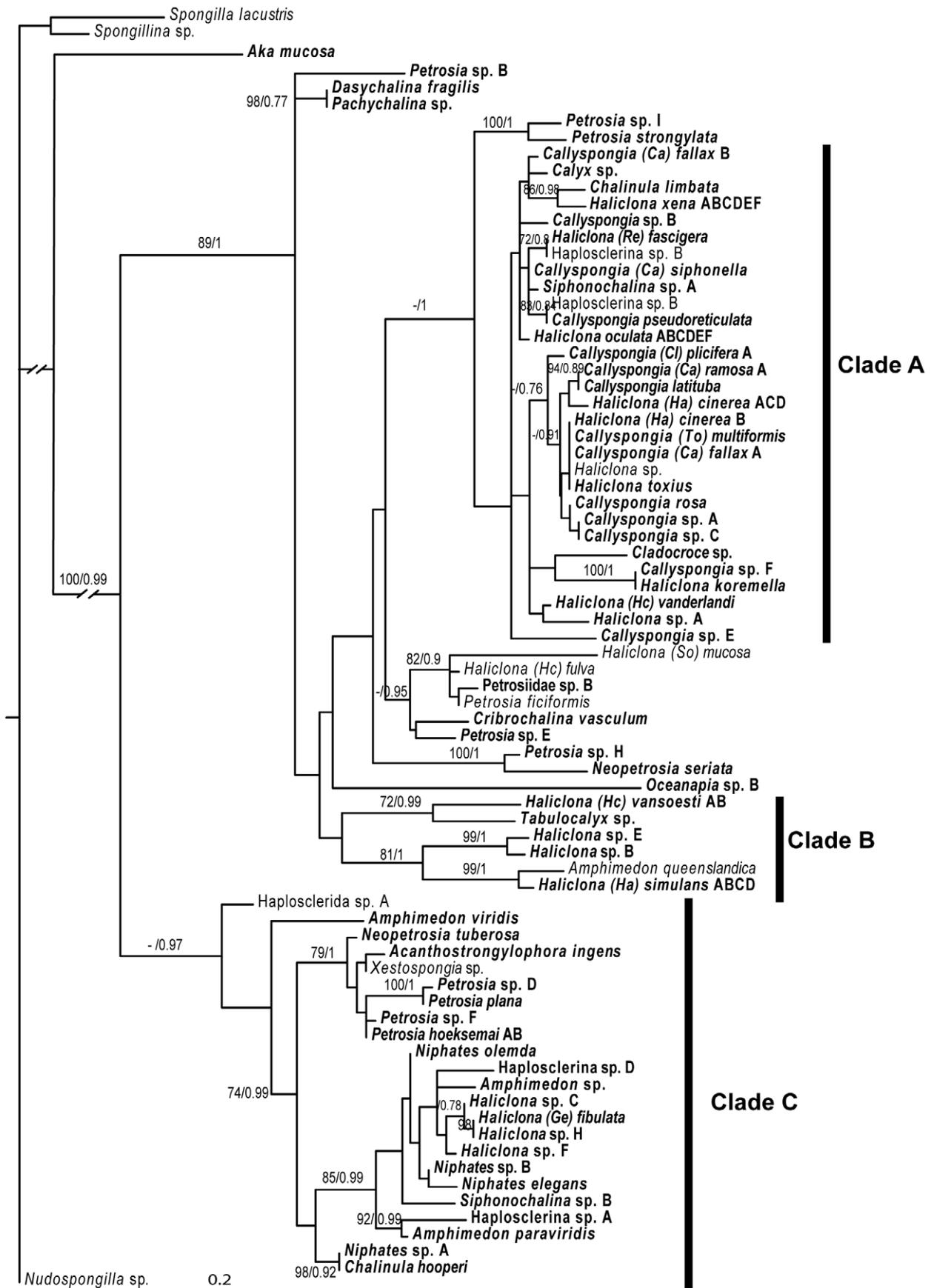
with a third *Niphates* (sp. A, POR14462) with 95/1 support rather than with *C. limbata*, which, as mentioned above, was in Clade A. The sequence from *Amphimedon viridis* grouped within this clade rather than with the *A. queenslandica* sequence in Clade B. *Aka mucosa* was in an isolated position.

Tree reconstructions of the 28S rDNA D3–D5 data (Figure S1) included sequences from an additional 14 marine haplosclerid specimens not included in the D1–D5 28S rRNA gene dataset. In Clade A there were additional sequences from *Neopetrosia subtriangularis*, *Haliclona manglaris* and another *C. fallax*. Clade B (83/0.95) contained the same four taxa as described previously with the addition of new sequences from *Xestospongia caminata* and *Haliclona vermeuleni*. The former was sister to the five other members of the clade and *H. vermeuleni* grouped with *A. queenslandica* with support of 100/1. In Clade C (100/1) the three additional *Amphimedon* sequences (*A. viridis*, AF441350 and *A. compressa*, AF441351 Genbank sequences and a newly generated *A. compressa* sequence (Table S1)) grouped with *A. paraviridis* and the newly added *N. erecta* sequence (94/1) and this clade was positioned distantly from the other *A. viridis* sequence. The additional *Acanthostrongylophora ashmorica* sequence grouped near the *A. ingens* sequence and *Haliclona walentinae* was also present in Clade C. Elsewhere, *A. mucosa* clustered with two congener sequences, *A. coralliphaga* and *Aka* sp., with support of 100/1 (Clade D).

The D1 dataset was the largest and most diverse marine haplosclerid dataset in this study as it contained sequences from 74 marine haplosclerid taxa from five families and 18 genera (Figure 2). However, it was also the shortest alignment being just over 300 bp long after variable bases were removed and should thus be used to give a broad indication of which clade sequences are allied to rather than a reliable picture of relationships, as there was no resolution at many of the internal branches. On the gene trees, the large clade containing subclades A+B+C. *vasculum* was highly supported with 89/1 (Figure 2). Relationships amongst sequences were broadly the same as the two previous datasets. *Cladocroce* is included for the first time in the analyses and was positioned amongst the Clade A sequences. Some of the new sequences grouped with *Cribrochalina vasculum*, such as *Haliclona mucosa*, *H. fulva*, *Petrosia ficiformis*, *Petrosia* sp. E (MKB1028) and *Petrosiidae* sp. B (MKB1785) however this grouping only had support in the Bayesian analyses (0.95). In Clade B *A. queenslandica* had 99/1 for a sister relationship with the newly added *H. simulans*, and a further two new *Haliclona* were sister to these two species (81/1). A different *Pachychalina* sp. was sequenced for this region (in comparison to the D3–D5 analyses) and was found in the larger Clade A+ Clade B rather than in Clade C. In Clade C there was high support for the grouping of *Petrosia* sp. D (MKB1020) and the additional *P. plana* sequence (100/1). A second *Siphonochalina* specimen included was positioned in Clade C rather than with the other *Siphonochalina* sp. A (POR14630), which was in Clade A.

### Mitochondrial gene phylogenies

The *cox1* tree reconstructed using a large dataset (containing cnidarian, homoscleromorph and demosponge sequences), shows



**Figure 2. Maximum-likelihood phylogeny reconstructed from the D1 region of the 28S rRNA gene.** The DNA substitution model parameters estimated by RAxML were: f(A) 0.26, f(C) 0.24, f(G) 0.33, f(T) 0.17; R(AC) 0.69, R(AG) 2.17, R(AAT) 1.39, R(CG) 0.61, R(CT) 5.12, R(GT) 1.0; alpha 0.61; pinvar 0.36. Sequences produced during this study are in bold. Sampling locations for each taxon are given in Table S1. Other sequences were downloaded from Genbank (Haplosclerida A, AY561856, Haplosclerina C AY561861, Haplosclerina B, AY561860, *Haliclona* sp, AY561862, *H. mucosa*, AJ225831, *H. fulva*, AJ225829, *P. ficiformis*, AJ225828, *A. queenslandica*, EF654518, *Xestospongia* sp., AY561853). Numbers on the branches represent bootstrap proportions/posterior probabilities.  
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the presence of very divergent patterns of sequence evolution in the *cox1* across different demosponges (Figure S2). A clade of anthozoan sequences was present within the demosponge clade. The divergent patterns of haplosclerid sequences first shown in Erpenbeck et al. [47] are also visible on this tree, however clades corresponding to those in the ribosomal gene trees are also recovered. There is a clade equivalent to Clade A supported by 96 BP and a number of smaller more divergent clades with high support associated with it. However, positioned distantly from this clade are four *Niphates* sequences (100 BP) and an additional clade containing *A. queenslandica*, *H. caerulea*, *C. vaginalis*, *C. armigera*, and *H. simulans* (corresponding to Clade B). An *A. compressa* sequence clusters very tightly within the clade of keratose sponges (99 BP). Thus very different sequence patterns are to be found amongst marine haplosclerid species in this gene region. Divergent patterns are also seen within the Poecilosclerida, Hadromerida and Halichondrida.

For the dataset that only included all marine haplosclerid *cox 1* sequences, using freshwater sponges as outgroups (Figure 3) we see that the *A. compressa* sequence falls in a basal position and has a long branch. The clade of *Niphates* sequences (100/1) are possibly attracted by the long branch of the clade containing the *A. queenslandica* sequence and these together have high support (82/1). They happen to fall well inside the marine haplosclerid set of sequences however, as a sister group to a clade containing *Oceanapia* and *Petrosia* sequences (amongst others), which is not unlike the pattern seen in ribosomal trees. There are a number of additional patterns in the *cox1* data however, that should be mentioned. Firstly, sequences from different species were found to be identical. These included those sequenced as part of the same study i.e. *N. erecta* B (EF519659) & *N. alba* (EF519654), and *H. tubifera* (EF519624) & *H. implexiformis* (EF519623) [47], *C. fallax* (GQ415412) and *C. vaginalis* A (GQ415417) [48] and those sequenced as part of different studies (i.e. *Eunapius* sp. (DQ167181) and *E. subterraneus* (FJ715439) (from Hess et al. (Direct submission in GenBank) and Harcet et al. [49] respectively) and all four sequences from *X. muta* (EF519699) [47], *N. proxima* (AM076980) [50], *X. bergquistia* A & B and *Petrosia* sp. G (this study). The second noteworthy pattern is of very different sequences being returned from the same species. For the case of *C. vaginalis* sequence (GQ415412) from the study of López-Legentil et al. [48] grouped in Clade A with three *C. fallax* sequences, being identical to one of them. A second *C. vaginalis* sequence (EF095182) [11] clustered with *X. bergquistia* while those from Erpenbeck et al. [47] and DeBiaise et al. [51] clustered in the same clade as *A. queenslandica* quite distantly from the rest of the marine haplosclerid sequences (Figure S2). All of the >200 sequences from the study of De Biasse et al. [51] clustered in this position (data not shown).

The *nad1* phylogeny (Figure S3) showed a monophyletic Clade A was retrieved with 100/1 support. Within this clade sequences generated from multiple specimens from *H. cinerea* were almost all identical as were two sequences from *H. oculata* and two from *H. xena*. The same pattern, seen in other gene trees, of smaller clades containing *Haliclona* and *Callyspongia* sequences were also evident on this phylogeny. *A. queenslandica* did not group with *A. compressa* and both had a very long branch. Instead *A. compressa* grouped

with *Petrosia plana* (99/1) in what might be Clade C but is very poorly sampled for this locus. The sequence generated from *Xestospongia bergquistia* was very similar to that from *X. muta* (100/0.99) and they grouped together on the tree with two *Haliclona* sp. sequences (88/0.86). The *Dasychalina fragilis* sequence was in an unsupported position.

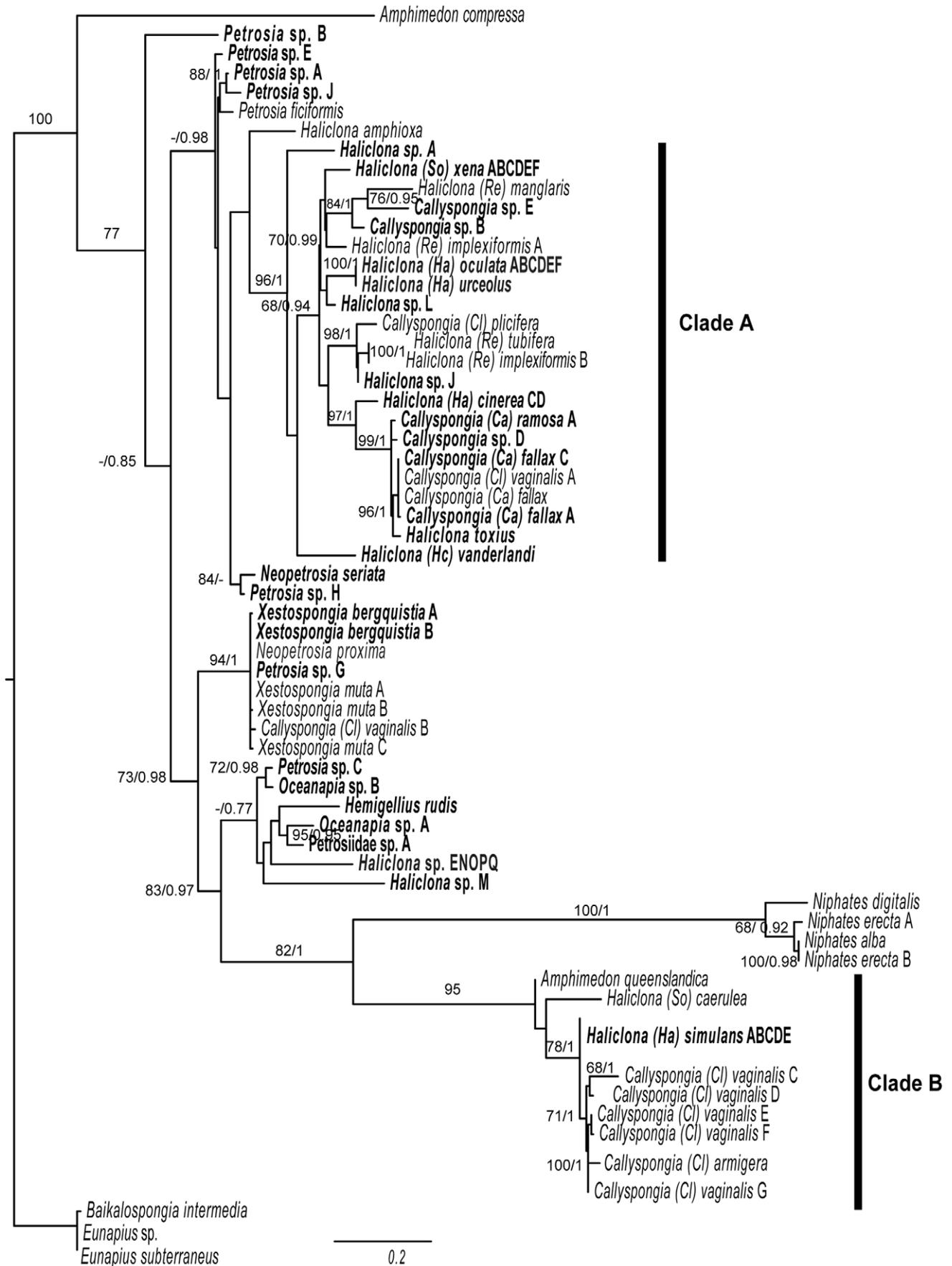
## Discussion

The gene trees shown here, generated from additional molecular data from three different genes, are highly congruent with phylogenies produced from 18S rDNA data and from data generated from the 3' (Erpenbeck) region of the *cox1* gene [10,24]. The data from all four genes (18S and 28S rRNA, *cox1* and *nad1*) suggests the presence of four clades.

The first, Clade A, is highly complex and dominated by species that have been identified as *Haliclona* in Family Chalinidae, and *Callyspongia* in Family Callyspongiidae. A few species of *Chalinula* and *Cladocroce* (Family Chalinidae) are also included in Clade A, along with *Siphonochalina* (Family Callyspongiidae) and a species identified as *Calyx* from the Family Phloeodictyidae. *Haliclona*, *Chalinula*, and *Siphonochalina* also appear in Clades B and C but to a much lesser extent than in Clade A. This clade would appear to be a combination of the two families Chalinidae and Callyspongiidae.

A range of smaller clades comprising species of *Petrosia*, *Acanthostrongylophora*, and *Xestospongia* (Family Petrosiidae), *Amphimedon*, *Cribrochalina*, *Niphates* (Family Niphatidae), and *Oceanapia* (Family Phloeodictyidae) were highly variable in their arrangement in relation to Clade A and to each other. Clade B was smaller, and consistently composed of the same two species of *Haliclona*, *H. simulans* and *H. vansoesti* (Family Chalinidae), *Amphimedon queenslandica* (Family Niphatidae), and a species of *Tabulocalyx* (Family Phloeodictyidae). Clade C differs considerably from Clade A in that it is dominated by species that have been identified as *Petrosia*, *Neopetrosia*, *Xestospongia*, and *Acanthostrongylophora* in the Family Petrosiidae, and *Niphates* and *Amphimedon* in the Family Niphatidae. This clade appears to be a combination of two families Niphatidae and Petrosiidae. A fourth clade was comprised of members of the genus *Aka*, a group of sponges that are presently classified with Family Phloeodictyidae, but which are very different from most Haplosclerida in that they excavate calcareous substrates and are externally visible only by their fistulose tubes.

The relationships between *Haliclona* and *Callyspongia* species and other Chalinidae and Callyspongiidae indicated in Clades A–C are perplexing because the genera are thought to be very clearly defined by morphological characteristics. These two families, with Phloeodictyidae, Niphatidae and Petrosiidae are recognisable along a gradient of decreasing spongin reinforcement (or increasing siliceousness), from Chalinidae to Petrosiidae. These trends are reflected in the overall structure of the molecular phylogenies, with Clade A dominated by Callyspongiidae and Chalinidae, and Clade C, dominated by Petrosiidae and Niphatidae. However, it is clear from these molecular data that the genera that form these families are not as easy to separate as was previously thought. This may be reflected also by the inherently great difficulties in the identification of 'transitional'



**Figure 3. Maximum-likelihood phylogeny reconstructed from the Folmer (5') region of the *cox1* gene from only Haplosclerida taxa.** The DNA substitution model parameters estimated by RAxML were; f(A) 0.26, f(C) 0.15, f(G) 0.22, f(T) 0.37; R(AC) 1.59, R(AG) 3.12, R(AT) 0.86, R(CG) 0.44, R(CT) 5.28, R(GT) 1.0; alpha 0.7; pinvar 0.44. Sequences produced during this study are in bold. Sampling locations for each taxon are given in Table S1. Other sequences were downloaded from Genbank (*A. compressa*, EF519558, *P. ficiformis*, EF519663, *H. amphioxo*, AJ843892, *H. manglaris*, EF519626, *H. implexiformis*, EF519625, *C. plicifera*, EU237477, *H. tubifera*, EF519624, *H. implexiformis* B, EF519623, *C. vaginalis* A, GQ415412, *C. fallax*, GQ415417, *N. proxima*, AM076980, *X. muta* A, EF519699, *X. muta* B, EU716650, *C. vaginalis* B, EF095182, *X. muta* C, EF095185, *N. digitalis*, EF519658, *N. erecta* A, EF519660, *N. erecta* B, EF519659, *N. alba*, EF519654, *A. queenslandica*, DQ915601, *H. caerulea*, EF519619, *C. armigera* EF519578, *C. vaginalis* C–G, EF519577, EF519579, EF519581, GQ304697, GQ304613). Numbers on the branches represent bootstrap proportions/posterior probabilities. doi:10.1371/journal.pone.0024344.g003

haplosclerid species. In the Indo-west Pacific, for example, there are numerous species that appear to transition between different genera including what we currently define as *Niphates*, *Amphimedon*, *Dasychalina* and *Pachychalina* and the numerous varied forms of supposed Callyspongiidae in this region (MK, Pers. Obs). However, it is encouraging to see molecular data consistently support the two New Zealand species currently recognised under the umbrella genus *Callyspongia* (*C. ramosa*, *C. latituba*) but which are clearly a new genus or subgenus. Similarly, two species of *Acanthostrongylophora* (*A. ingens*, *A. ashmorica*) previously thought to be an unusual form of *Xestospongia* or *Petrosia*, are also consistently grouped together, and with other Petrosiidae.

It is possible that re-evaluation of genus groups and their associated major biochemical components, including the metabolic pathways of various compounds, may provide additional support for the alternative relationships proposed by molecular data. The use of biochemical compounds for phylogenetic classification has been rejected by a number of authors due to disagreement between biochemical and morphological data [52,53], but Urban et al. [54] and Hu et al. [55] found that the disagreement between biochemical and morphological data is largely a problem of incorrect taxonomic identification of the sponge from which the compounds were identified. The problem of mis-identification is particularly acute amongst species in genera with a dearth of strong diagnostic morphological characters, and this is particularly so in the Haplosclerida with few megasclere and microsclere forms, and what appears to be a gradient of siliceous and fibrous development. The biochemical observations in marine haplosclerid taxa by Fromont et al. [56] and van Soest et al. [57] are largely congruent with molecular data.

The usefulness of developmental characters, also should not be ruled out. Bergquist et al. [58] distinguished two larval types in the Haplosclerida, one group represented by *Chalimula* and *Remiera* and the other by *Callyspongia*, *Adocia* and *Haliclona*. However, de Weerd [29] found it difficult to separate adults of *Adocia*, *Haliclona* and *Remiera* so placed them all into the genus *Haliclona* and suggested that differences in larval structure were not enough to separate them and were of minor importance. Re-investigating these larval characteristics may prove worthwhile in light of relationships indicated in this paper. We have shown for example that many *Callyspongia* and *Haliclona* sequences are associated with each other on all gene trees. Furthermore, on the D1 trees *Haliclona* (*Remiera*) *fulva* and *H. (Remiera) mucosa* sequences group away from this clade.

The Haplosclerida have long been recognised as a highly diverse group [2] and this is clearly demonstrated in this study. Suggestions of the polyphyletic nature of the various taxa within haplosclerids have been appearing in various publications since 2002 [5,9–11,24,59] with 18S, partial 28S rRNA genes and mitochondrial data. However, a number of authors have also suggested that the differing nature of the molecular phylogenies (when compared to morphology) may be due to a higher rate of substitution in the ribosomal and mitochondrial genes in marine haplosclerids when compared to other demopsponges and that as a result they are not suitable for phylogeny reconstruction in this

group [11,33,47]. Nonetheless, previous studies [9,11,33,59] had included fewer haplosclerid species, and, given the high diversity in the group we are not surprised that branch lengths were longer in some taxa. With the addition of a higher number of sequences and a longer region of 28S rDNA we find that the relationships remain congruent and branch lengths within the haplosclerids are comparable to those in other groups of sponges. Furthermore, Redmond and McCormack [34] showed that the variable indels found in the 18S rRNA gene are very important synapomorphies. Therefore although it may be difficult to use this region to compare haplosclerids with other sponges, due to the indels present in Clade A, 28S rDNA data shows strong phylogenetic signal for studying relationships within the marine Haplosclerida and highlights a very high diversity within this group, which is supported by other data.

Mitochondrial data is not straightforward for haplosclerid phylogenetics. Using the 5' end of the *cox1* Erpenbeck et al. [47] found the marine Haplosclerida to be polyphyletic in their study of Caribbean demopsponges. They pointed out the high evolutionary rate found in the mt genome of *A. queenslandica* [60] and concluded that the *cox1* would not be suitable to resolve Haplosclerida relationships sufficiently. The addition of further sequences through this study certainly suggests that this is true. We would suggest that the positions of the major clades within the order cannot be reliably determined using this locus but support is strong within those clades. In *cox1* and *nad1* gene trees branches leading to the *A. queenslandica* sequence were long but branch lengths leading to most other marine haplosclerid sequences were not. This pattern was also shown in Wang and Lavrov [37] who showed that *A. queenslandica* has an unusual mitochondrial genome, lacking genes, containing deletions in several genes, and displaying an accelerated rate of sequence evolution. Analysis from three additional marine haplosclerid species found no similar features indicating that the *A. queenslandica* mitochondrial genome has undergone unusual evolution and is a poor representative of the G3 clade [37]. Thus, as found in other metazoans (e.g. the nematode *Caenorhabditis elegans*) it is reasonable to suggest that the evolutionary rate in some species within a taxon may be higher than others and therefore inclusion of these taxa may lead to erroneous views of phylogeny [61]. The *cox1* data produced to date for marine haplosclerids has indicated a number of other species have unusual evolutionary patterns in their mitochondrial genomes (e.g. *C. vaginalis*, *N. erecta*, *H. simulans*). It is likely that additional demop sponge sequences should also be viewed with caution, including some of the poecilosclerid sequences from Erpenbeck et al. [47] that have exceedingly long branch lengths (e.g. *Mycale* and *Monanchora*, *Chondrosia*, *Chelonaphysila*). Despite the *cox1* gene evolving more slowly in sponges compared to other metazoans it is clear that it is not suitable for reconstructing relationships across an entire order and between orders. There are also clear examples of where the *cox1* data both assists and hinders barcoding by showing different species to be identical and the same species to have very different sequence patterns. It has been shown here and in other studies [24,34] that specimens identified

as *C. vaginalis*, *H. cinerea* and *H. oculata* may comprise of more than one OTU, and there are indications that species that should be closely related are in fact not. The evolution of mitochondrial genomes continues to be an exciting area of research and will offer further illumination in time.

Although all of the four genes employed to date are not all independent (two ribosomal genes and two mitochondrial genes), and there are problems in using these genes for certain taxa within the Haplosclerida, the patterns in all four genes are reasonably congruent lending support to suggestions that the suborders and some of the families and genera should be revised. There is also some support for the molecular phylogenetic patterns, not only from the general trends in morphological data, but also in secondary metabolite and biochemical signals, and possibly in reproductive traits. It is now time to employ alternative approaches to find synapomorphies between taxa suggested as being closely related by molecular data. Further acquisition of ribosomal and mitochondrial data is necessary of species that would potentially fall in the poorly represented clades within the Haplosclerida. Furthermore, data from nuclear protein coding genes would provide additional support for deep branches in the tree, confirm results from ribosomal and mitochondrial genes and help to find the sister group of the Order Haplosclerida.

## Supporting Information

**Figure S1 Maximum-likelihood phylogeny reconstructed from the D3-D5 region of the 28S rRNA gene.** The DNA substitution model parameters by RAxML were; f(A) 0.23, f(C) 0.24, f(G) 0.32, f(T) 0.21; R(AC) 0.57, R(AG) 2.03, R(AAT) 1.0, R(CG) 0.57, R(CT) 4.19, R(GT) 1.0; alpha 0.19. Sequences produced during this study are in bold. Sampling locations for each taxon are given in Table S1. Other sequences were downloaded from Genbank (*A. coralliphaga*, AF441345, *A. queenslandica*, EF654518, Haplosclerina B, AY561860, *C. plicifera*, AF441345, *H. toxius*, AF441342, *H. vansoesti*, AF441346, *N. olemda*, AF441353, *H. xena*, AY319327, *N. subtriangularis*, AF441341, *C. fallax*, AF441344, *X. caminata*, AF441348, *Pachychalina* sp. B, AF441352, *A. viridis*, AF441350, *A. compressa*, AF441351, *A. ashmorica*, AF441354, *H. vansoesti*, AF441346). Numbers on the branches represent bootstrap proportions/posterior probabilities. (TIF)

**Figure S2 Maximum-likelihood phylogeny reconstructed from the Folmer (5') region of the *cox1* gene.** The DNA substitution model parameters estimated by RAxML were; f(A) 0.26, f(C) 0.15, f(G) 0.22, f(T) 0.37; R(AC) 1.37, R(AG) 4.1, R(AT) 1.43, R(CG) 1.18, R(CT) 6.87, R(GT) 1.0; alpha 0.71; pinvar 0.41. Sequences produced during this study are in bold. Sampling locations for each taxon are given in Table S1. Other sequences were downloaded from Genbank. Numbers on the branches represent bootstrap proportions. (TIF)

**Figure S3 Maximum-likelihood phylogeny reconstructed from the *nad1* gene.** The DNA substitution model

parameters by RAxML were; f(A) 0.3, f(C) 0.1, f(G) 0.21, f(T) 0.39; R(AC) 4.18, R(AG) 6.72, R(AT) 1.0, R(CG) 4.18, R(CT) 11.9, R(GT) 1.0; alpha 0.26; pinvar 0.08. Sequences produced during this study are in bold. Sampling locations for each taxon are given in Table S1. Other sequences were downloaded from Genbank (*P. cf. ankodes*, EU237487, *X. muta*, EU237490, *A. queenslandica*, DQ915601, *C. plicifera*, EU237477, *A. compressa*, EU237474, *C. kuekenthalii*, EU237479, *G. neptuni*, AY320032, *E. muelleri*, EU237481, *T. actinia*, AY320033, *A. schmidtii*, EU237475, *A. corrugata*, AY791693, *H. dujardini*, EU237483, *Chondrilla* sp., EU237478, *T. ophiraphidites*, EU237482). Numbers on the branches represent bootstrap proportions/posterior probabilities. (TIF)

**Table S1 List of all the marine haplosclerid specimens sequenced in this study.** Sampling location, voucher number where available, and what gene regions were sequenced for each is included. (DOC)

**Table S2 Primer sequence information for each primer used in amplifying each gene region.** (DOC)

**Dataset S1** The concatenated dataset created by joining sequences of the D1 and D3–D5 regions from specimens that had both regions available. (NEX)

**Dataset S2** The final D3 alignment used for analysis. (NEX)

**Dataset S3** The final D1 alignment used for analysis. (NEX)

**Dataset S4** The *cox1* dataset including sequences from other demosponge orders and cnidarians. (NEX)

**Dataset S5** The *cox1* dataset including sequences from only Haplosclerida. (NEX)

**Dataset S6** The *nad1* alignment used for analysis. (NEX)

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## Author Contributions

Conceived and designed the experiments: NER GPM RWMvS. Performed the experiments: NER JR GPM BB SV KMS. Analyzed the data: NER GPM SAAT. Wrote the paper: NER GPM MK.

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