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Kelly M Stephens, Alexander Ereskovsky, Pierce P Lalor, Grace McCormack. Ultrastructure of the Ciliated Cells of the Free-Swimming Larva, and Sessile Stages, of the Marine Sponge *Haliclona indistincta* (Demospongiae: Haplosclerida). *Journal of morphology.*, 2013, 10.1002/jmor.20177 . hal-01456652

**HAL Id: hal-01456652**

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Submitted on 13 Apr 2018

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# Ultrastructure of the Ciliated Cells of the Free-Swimming Larva, and Sessile Stages, of the Marine Sponge *Haliclona indistincta* (Demospongiae: Haplosclerida)

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**ABSTRACT** We provide a detailed, comparative study of the ciliated cells of the marine haplosclerid sponge *Haliclona indistincta*, in order to make data available for future phylogenetic comparisons at the ultrastructural level. Our study focuses on the description and analysis of the larval epithelial cells, and choanocytes of the metamorphosed juvenile sponge. The ultrastructure of the two cell types is sufficiently different to prevent our ability to conclusively determine the origin of the choanocytes from the larval ciliated cells. However, ciliated, epithelial cells were observed in a migratory position within the inner cell mass of the larval stages. Some cilia were observed within the cell's cytoplasm, which is indicative of the ciliated epithelial cell undergoing transdifferentiation into a choanocyte; while traces of other ciliated epithelial cells were contained within phagosomes, suggesting they are phagocytosed. We compared our data with other species described in the literature. However, any phylogenetic inference must wait until further detailed comparisons can be made with species whose phylogenetic position has been determined by other means, such as phylogenomics, in order to more closely link genomic, and morphological information. *J. Morphol.* 000:000–000, 2013. © 2013 Wiley Periodicals, Inc.

**KEY WORDS:** *Haliclona indistincta*; sponge; larvae; ultrastructure; ciliated cells

## INTRODUCTION

The general organization of a typical demosponge comprises three layers: external and internal epithelial layers [sensu lato (Schmidt, 1866; Schneider, 1902; Starck and Siewing, 1980)], made up of exopinacocytes, basopinacocytes, and endopinacocytes, and an extracellular matrix, or mesohyl, which is composed of proteins, polysaccharides, and fibrous collagen (Bergquist, 1978; Ereskovsky, 2010). The term “epithelium” defined as closely associated, laterally contacted, polarized cells, (Schmidt,

1866; Schneider, 1902; Starck and Siewing, 1980; Nickel et al., 2011), was originally applied to sponges in a broad, functional sense (Nickel et al., 2011). It is currently a matter of some controversy whether or not sponges can be said to possess a “true” epithelium. This is primarily because, contrary to the more recent definition, most Porifera taxa lack the belt-like junctions between cells, as well as a basal lamina (Rieger, 1986; Ax et al., 1989; Ax, 1995; Tyler, 2003; Ereskovsky and Dondua, 2006; Leys et al., 2009; Adams et al., 2010; Nickel et al., 2011). In recent years however, data suggests the term “epithelium” can be applied to sponges, as their polarized epithelial cells show functional similarities to other animal epithelia (e.g., ion and solute transportation regulation), and should therefore, not be discounted (Leys et al., 2009; Adams et al., 2010; Nickel et al., 2011). It is generally accepted that sponges are the most basal of the metazoans (Nielsen, 2001; Philippe et al., 2009). 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 (Ruiz-Trillo et al., 2008; Fahey and Degnan, 2010; Ereskovsky, 2010).

Grant sponsor: National University of Ireland; Grant sponsor: Thomas Crawford Hayes Trust Fund Scheme (NUI Galway).

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Received 24 January 2013; Revised 28 May 2013; Accepted 31 May 2013.

Published online 00 Month 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jmor.20177

Flagella 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; we 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 in *Homoscleromorpha* and some *Demospongiae*, apopodocytes, spermatozooids and choanocytes (Boury-Esnault et al., 1999; De Vos et al., 1991; Ereskovsky, 2010). Choanocytes are ciliated, collared cells that utilize their cilium to facilitate ambient water in, and throughout, the body in a complex canal system. These cells are suggested to be a key cell type for understanding the evolutionary transition from a unicellular to a multicellular state (Karpov and Efremova, 1994; Gonobobleva and Maldonado, 2009). 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 adult 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 6 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, larvae of some sponge groups undergo “layer inversion” (Delage, 1892) during which the epithelial ciliated cells migrate inward and transdifferentiate into choanocytes (for review see: Maldonado, 2004; Leys and Ereskovsky, 2006; Ereskovsky, 2007; Ereskovsky et al., 2007, 2009, 2010). Central to studies investigating the transdifferentiation 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 choanocytes 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 sponge species (e.g., Fjerdingstad, 1961; Feige, 1969; Garrone, 1969; Efremova et al., 1988; 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 (e.g., Seravin, 1986; Efremova et al., 1988; Gonobobleva and Maldonado, 2009). The parenchymella larva of the marine haplosclerid sponge *Haliclona indistincta* (Bowerbank, 1866) is oval in shape (approximately 477  $\mu\text{m}$  in length and approximately 200  $\mu\text{m}$  in width) when first released from the parent sponge; and has a uniform ciliation pattern (Lévi, 1956; Stephens et al., 2012). The larva has distinct anterior and posterior ends that diminish as they progress through two additional mobile stages, becoming more compact and angular (Stage 2) and subsequently becoming spherical (Stage 3) just prior to settlement (Stephens et al., 2012). However, what occurs internally during this change in the larval morphology has not been investigated until now. Here, we describe and compare the ciliated epithelial cells during metamorphosis in this species. This study also aims to add new information to the limited data that exists of this nature, explore the potential for phylogenetic information in such characters (i.e., basal apparatus of ciliated cells), and build the basis for future ultrastructurally based, comparative, phylogenetic studies.

## MATERIALS AND METHODS

### Material

Larvae of *Haliclona indistincta* (Bowerbank, 1866) were collected from Corranroo (Clare, Ireland) ( $53^{\circ} 8'28.50''\text{N}$  and  $9^{\circ} 0'33.70''\text{W}$ ). Six free-swimming larvae and four postsettled juveniles were sectioned for electron microscopy (TEM).

### TEM

To describe and compare the ultrastructure of the ciliated epithelial cells of the free-swimming larvae and the choanocytes of the juvenile sponge, transmission TEM was employed. For ultrastructural observations, larvae were fixed in a primary fixation solution composed of 2.5% glutaraldehyde, 1% paraformaldehyde in 0.2 mol  $\text{l}^{-1}$  sodium cacodylate/HCL buffer (pH = 7.2) (1,400 mOsm) for 24 h. Specimens were then post-fixed in 1% osmium tetroxide in 0.2 mol  $\text{l}^{-1}$  sodium cacodylate/HCL buffer (pH = 7.2) (with a 1:1 volume ratio; 420 mOsm) for approximately 2 h. Fixed specimens were dehydrated in a graded ethanol series and embedded in agar low viscosity resin (R1078 resin kit, Agar Scientific, Essex, United Kingdom). 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 (Wetzlar, Germany). The sections were mounted on copper (200  $\mu\text{m}$ ) mesh grids, and stained with Ultrastain 2 for 36 min via Leica EM (AC20), Wetzlar, Germany. Hitachi H7000 TEM (Tokyo, Japan), operating at 75 kV, was used to conduct the observations of the grids. All

measurements of organelles and cells were taken at the longest diameter.

## RESULTS

### Ciliated Cells of the Larval Epithelial Layer

**General cytology.** The surface of the larva of *Haliclona indistincta* was densely covered by cilia (Fig. 1A). The ciliated epithelial cells of larvae were flask-like in shape, ranging from 5.4 to 8  $\mu\text{m}$  at the longest diameter and 1–2  $\mu\text{m}$  in width ( $n = 5$  average  $6.3 \pm 1.0$  Standard Error (SE) and  $1.5 \pm 0.4$   $\mu\text{m}$  (SE), respectively). There were no visible specialized junctions between these epithelial cells; however, they were usually in close contact with neighboring cells (Fig. 1B,C). Distinct apical-basal polarization was evident by localization of the cilium and its associated basal apparatus at the apical end (Fig. 1C). Nuclei are situated at different levels making the epithelium appear pseudostratified, although this may also be due fixation effects and requires further confirmation (Fig. 1B). There are no nucleoli. Within the nuclei are peripheral islands of heterochromatin (Fig. 1C). From the longest axis, the nuclei varied from 1.3 to 3  $\mu\text{m}$  [ $n = 5$  average  $2.4 \pm 0.7$  (SE)].

The Golgi bodies were generally located near the base of the basal apparatus but were often also seen in other locations in the cytoplasm. 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 to 475 nm [ $n = 5$  average  $371 \pm 60$  nm (SE)] or ovoid and ranged from 585 to 760 nm in length [ $n = 5$  average  $711 \pm 72$  nm (SE)] and 350–490 nm in width [ $412 \pm 51$  nm (SE)] in shape (Fig. 1C).

**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 324 nm [ $n = 5 \pm 18$  nm (SE); Fig. 2A]. The ciliary axoneme was composed of the typical 9+2 microtubule organization surrounded by the cytoplasm and covered in cell membrane with glycocalyx. Although mostly monociliated, multiple axonemes were seen inside the apical part of the cytoplasm in some cells (Fig. 2B,C). The central microtubules of an axoneme terminated just above the apical surface of the cell's cytoplasm (approximately 50 nm) and in the area of the terminating

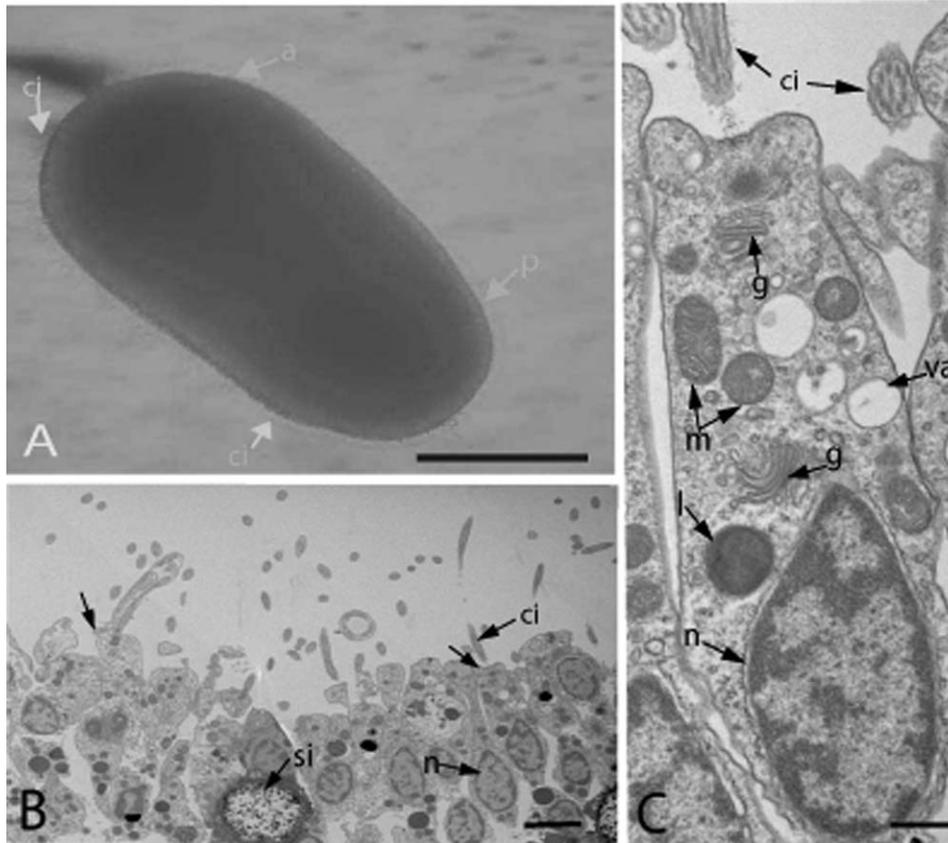


Fig. 1. *Haliclona indistincta* (A) Free-swimming larva showing a fully ciliated exterior (B) Longitudinal section of epithelial layer of cells. Arrows indicate the depth differences among the cells. (C) A ciliated epithelial cell of the larva (longitudinal section). a, anterior pole of larva; ci, cilia; g, Golgi apparatus; l, lipid inclusion; m, mitochondria; n, nucleus; p, posterior pole of larva; si, spherical inclusion; va, vacuole. Scale bars: A = 100  $\mu\text{m}$ , B = 2  $\mu\text{m}$ , C = 500 nm.

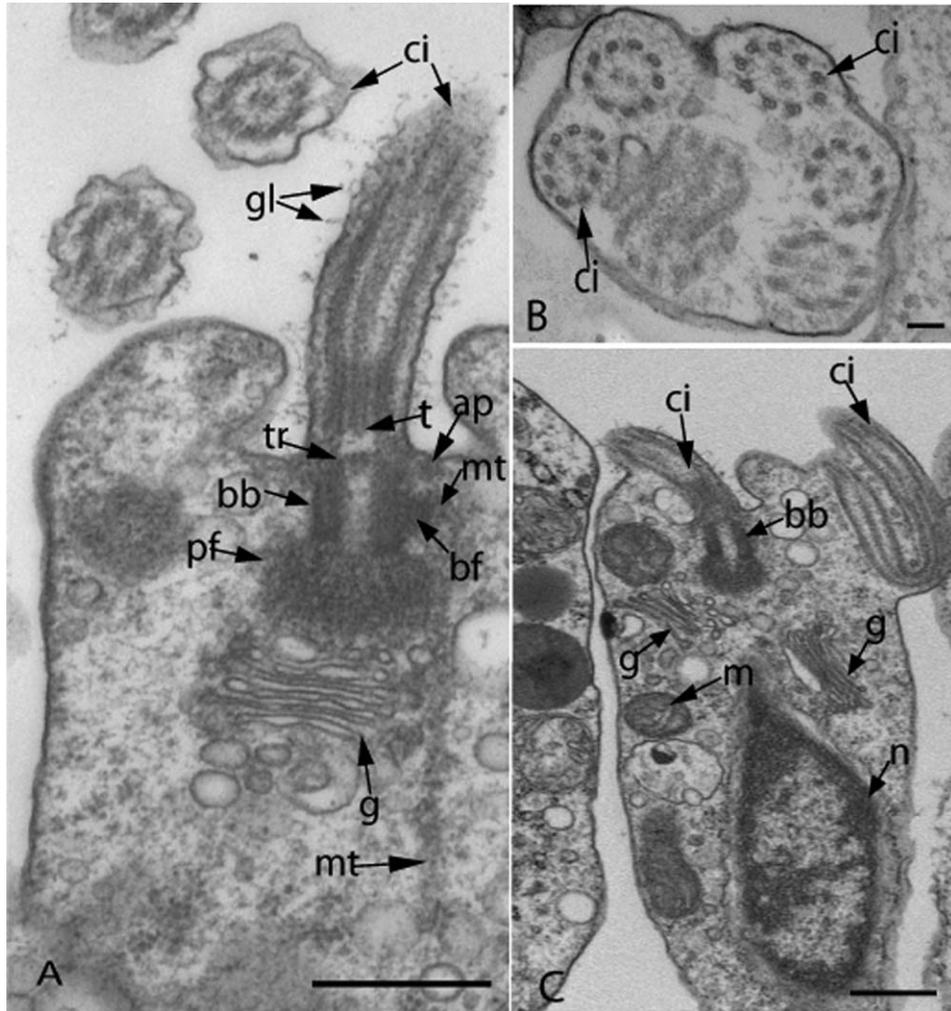


Fig. 2. *Haliclona indistincta* (A) Longitudinal section of apical part of an epithelial cell of a larva with the ciliary basal apparatus. (B) Cross-section of cilia showing the typical 9+2 configuration of the microtubules of the cilia. Also showing more than one cilium axoneme present in one cytoplasm. (C) Longitudinal sections of epithelial cells showing more than one cilium 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, glycoalkyx; m, mitochondria; mt, microtubules; n, nucleus. A = 500 nm, B = 100 nm; C = 500 nm.

microtubules, there was an electron-dense area, of approximately 150 nm in length, ascending up the shaft of the cilium. Directly below the terminated central microtubules, there was an electron-clear space approximately 41–81 nm in length and 97–114 nm in diameter between the external microtubule doublets that remain (Fig. 2A).

The basal body was located directly below the electron-clear zone and was in contact with the outer cilia microtubule doublets (Fig. 2A). Overall, the basal body was approximately 286 nm at the longest axis, and approximately 244 nm in width. A basal foot (approximately 94 nm long) emerged from the side of the basal body and was essentially trapezoid in shape, being approximately 110 nm in diameter at the base and approximately 104 nm at its apical end. An accessory centriole was not identified. Alar sheets radiated approximately 80 nm 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 75 nm in width on average [ $n = 5 \pm 13.5$  (SE); Fig. 3A,B]. The longitudinal cytoskeleton of the cilium and basal apparatus consisted of a microtubule that descended from the tip of the basal foot and ran parallel to the basal body deep into the cytoplasm of the cell toward the nucleus, as well as a skirt of parallel fibers, which were coupled with microtubule strands that were attached to the base of the basal body (Fig. 3C).

No differences were observed in the ultrastructure of the ciliated epithelial cells that occupied 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 (Fig. 4A). Remnants of ciliated cells were

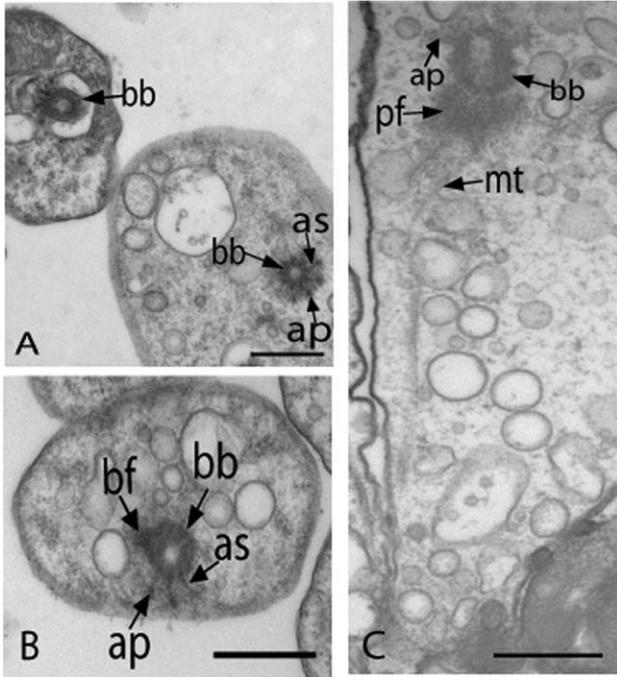


Fig. 3. *Haliclona indistincta* (A,B) Cross-section of the apical part of an epithelial cell of a showing the transitional region between the proximal end of the cilia shaft and the basal body. (C) Longitudinal section of anterior pole of larval epithelial cell showing the basal apparatus and microtubules 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-B = 500 nm, C = 100 nm.

also present within the ICM of the larvae, e.g., remnants of cilia basal apparatus were contained within phagosomes of amoeboid cells, and remnants of cilia were observed in cells (not within phagosomes), which were reminiscent of modified epithelial cells (e.g., Fig. 4B–D). Within the ICM, numerous larval ciliated cells were attached to cells containing spherical inclusions, which were very prevalent within the larvae (Fig. 5). The origin and chemical composition of these inclusions are so far unknown. They were variable in size 1–4  $\mu\text{m}$  ( $n = 5$  average  $2.9 \pm 1.2 \mu\text{m}$  (SE)), were often joined together to form complexes and appeared to contain fibrous material (Fig. 5).

### Ciliated Cells of the Juvenile *Haliclona indistincta*

**General cytology.** Choanocytes of the juvenile *Haliclona indistincta* were the only cell type with cilia. These cells were irregular in shape, however, two general forms existed; 1) pseudocylindrical with a length of 4–6  $\mu\text{m}$  [ $n = 5$  average  $4.5 \pm 0.85 \mu\text{m}$  (SE)] and width of 1.6–3  $\mu\text{m}$  ( $n = 5$  average  $2.4 \pm 0.7 \mu\text{m}$  (SE)) and 2) oval to rounded, 2.8–3.1  $\mu\text{m}$  in length [ $n = 5$  average  $2.9 \pm 0.1 \mu\text{m}$  (SE)] and 3–4.5  $\mu\text{m}$  in width [ $n = 5$  average  $3.5 \pm 0.6 \mu\text{m}$  (SE)]; (Fig. 6A,B). The shape of the choanocyte was not associated with their position

in the sponge body as both forms were observed lining choanocyte chambers. A spherical nucleus that ranged from 1.1 to 2  $\mu\text{m}$  in diameter [ $n = 5$  average  $1.5 \pm 0.2 \mu\text{m}$  (SE)] was located at the basal part of the cell (Fig. 6A,B). Mitochondria that ranged from 335 to 440 nm [ $n = 5$  average  $380 \text{ nm} \pm 37 \text{ nm}$  (SE)] were also seen within the cytoplasm (Fig. 6B) and were more consistent in shape (i.e., rounded) than those observed in the ciliated epithelial cells (which could be ovoid). As in the larval ciliated cells, numerous lipid droplets and electron clear vacuoles were seen throughout the cytoplasm, but were much more plentiful (Fig. 6A–D). 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 (Fig. 6C). The apical parts of the cells were orientated toward the lumen of the choanocyte chamber (Fig. 6D).

**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 cell's cytoplasm than in the larval ciliated cells (150 nm compared to 50 nm). As in the larval cells there was an electron dense area that extended from the terminating end of the microtubules to approximately 120 nm up the cilia shaft (Fig. 7A). Below the terminated central microtubules, there was a larger electron-clear space that ranged approximately from 102 to 173 nm in length and 61–123 nm in width between the external microtubule doublets that remained (Fig. 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 265 nm in length and approximately 240 nm in width. An accessory centriole in the ciliar basal apparatus of the choanocytes was absent. Alar sheets, radiated from the basal body (approximately 100 nm in length) and connected to electron-dense spheres, anchoring points, 61 nm 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 (Fig. 7B,C,E). Below the alar sheets, extended a basal foot approximately 95 nm from the side of the basal body. The basal foot was roughly pyramidal in shape, being approximately 60 nm in diameter at the apical end and approximately 105 nm at the base (Fig. 7A,D). Directly below the alar sheets, microtubules radiated in a spiral-like fashion, parallel to the surface (Fig. 7E), which may be due to the angle at which the cells were cut. These microtubules, and a

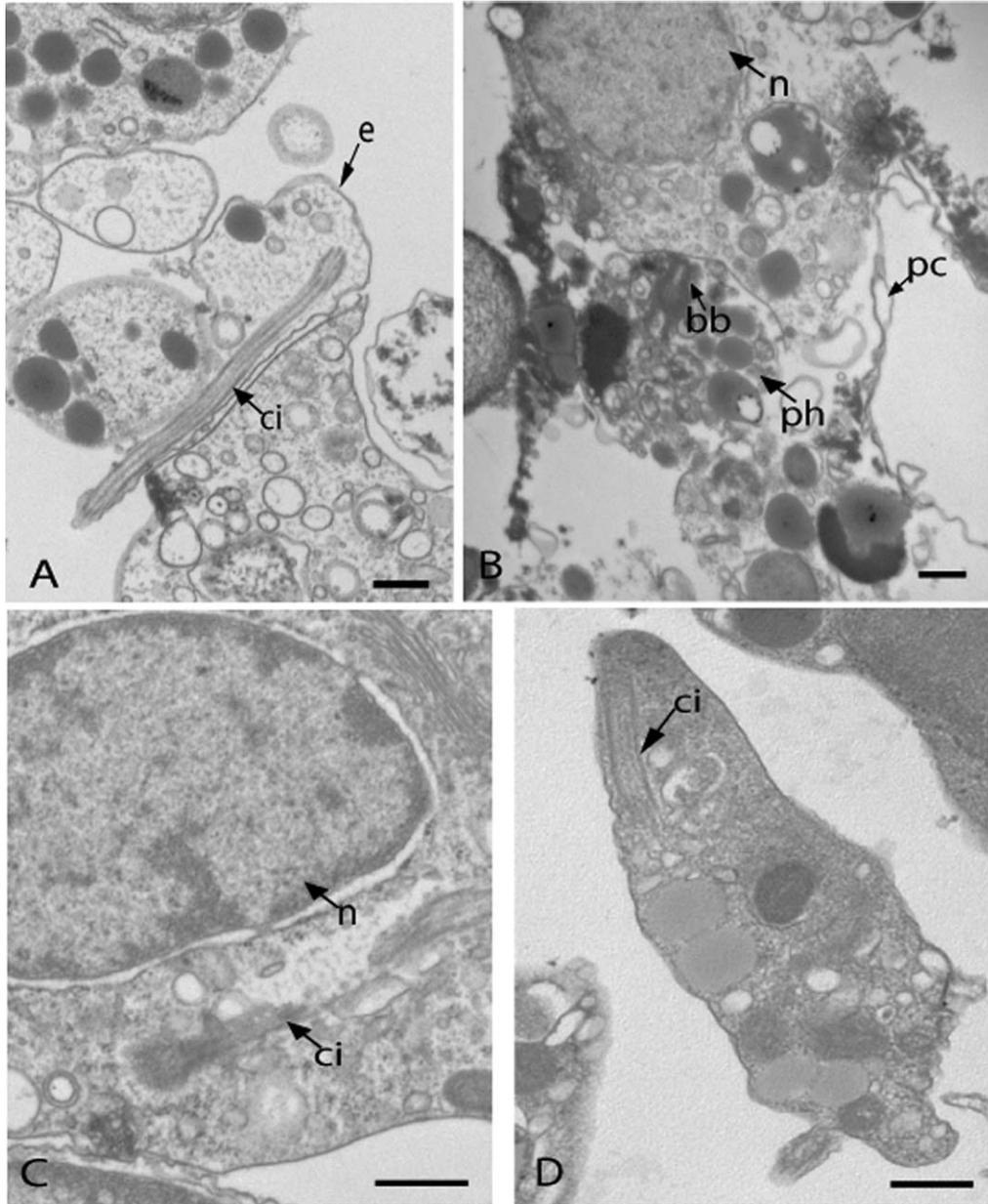


Fig. 4. *Haliclona indistincta* (A) Epithelial cell present within the ICM during the first free-swimming larval stage. (B) Longitudinal section of a cell within the ICM of a free-swimming stage larva with the basal apparatus (bb) of a ciliated epithelial cell within a phagosome (ph). (C) Longitudinal section showing epithelial cell of the first free-swimming larval stage of an *H. indistincta* larva with its cilium and basal apparatus inside the cytoplasm. (D) Longitudinal section of epithelial cell migrated inward during the third of the free-swimming stages of this species (note the cilia enveloped by the cytoplasm). ci, cilia; bb, basal body; e, epithelial cell; pc, phagocytosing cell; ph, phagosome; n, nucleus; Scale bars: A–D = 500 nm.

forked microtubule that extended from the tip of the basal foot, comprised the transverse cytoskeleton (Fig. 7D,E). There were three microtubules attached above the base of the basal body instead of only one as in the larval epithelial cells (attached to the basal foot). Two extended from the basal foot, one parallel to the basal body and one parallel to the surface of the apical region of the choanocyte (as mentioned above) (Fig. 7D). 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 (Fig. 7D). Attached at the tip of the basal body was a bundle of microtubules that extended to a Golgi apparatus, which along with the two longitudinally running microtubules mentioned previously, comprised the longitudinal cytoskeleton (Fig. 7A).

**Comparison of ciliated cells of larva and juvenile.** The gross morphology of the ciliated epithelial cell of the larva and of the choanocyte of the juvenile (e.g., cell and nuclei shape, and size) was very different (Table 1; Fig. 8). The vacuoles

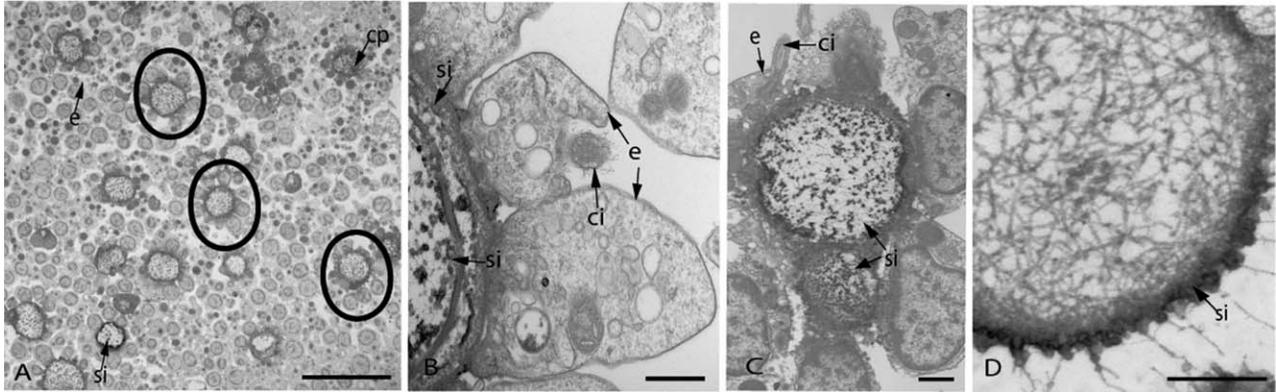


Fig. 5. *Haliclona indistincta* (A) Longitudinal section of an epithelial layer of the larva of *H. indistincta* with cells containing spherical inclusions, associated with some epithelial cells. (B,C) Longitudinal sections of epithelial layer with cells containing spherical inclusions associated with some epithelial cells. (D) Close-up of a spherical inclusion. bb, basal body; ci, cilia; si, spherical inclusion; e, epithelia cell. Scale bars: A = 10  $\mu$ m, B-D = 500 nm.

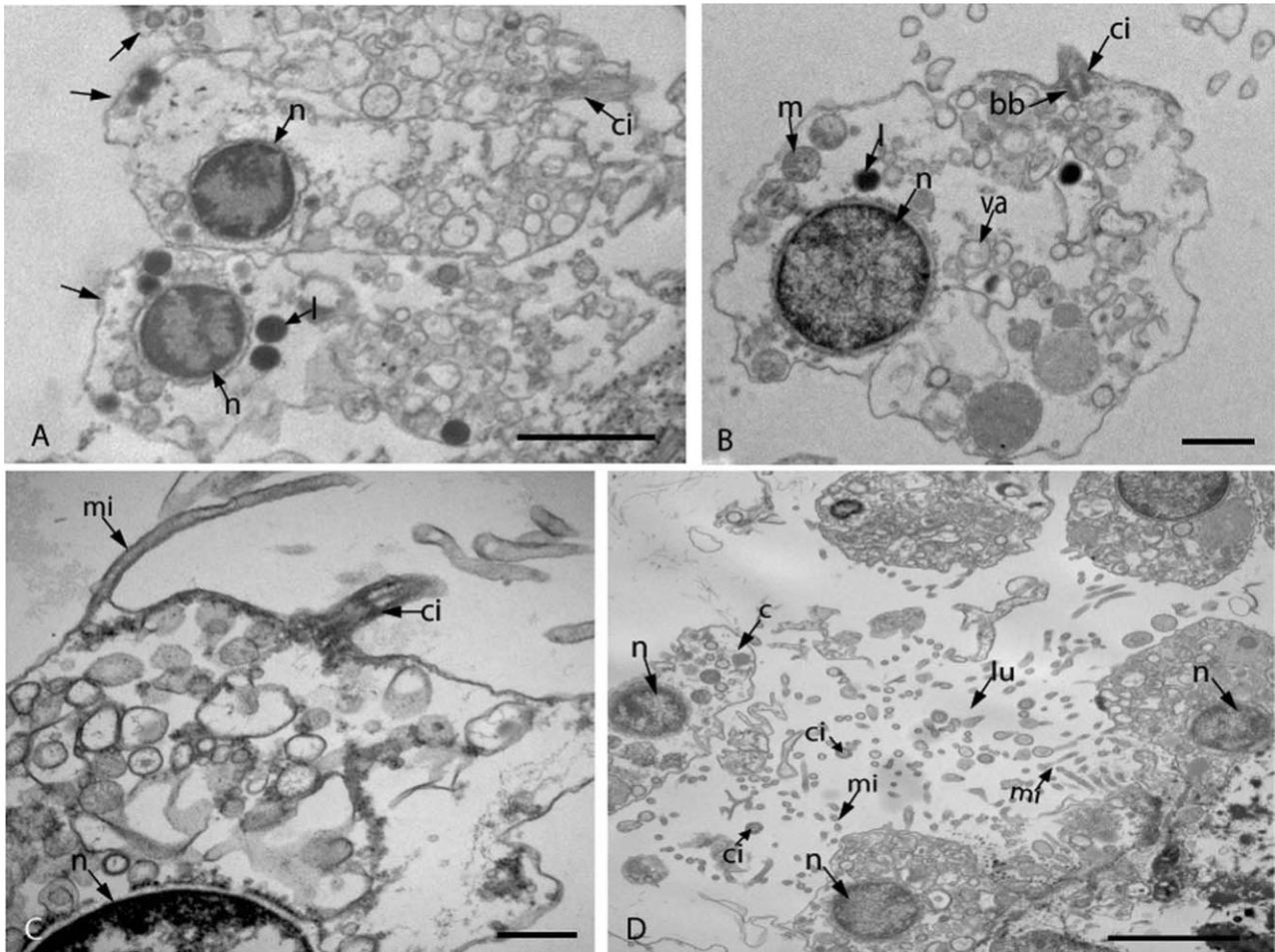


Fig. 6. The ciliated cells of *Haliclona indistincta* juveniles. (A) Longitudinal section of a pseudocylindrically shaped choanocyte. (Section from juvenile settled 8 days). (B) Longitudinal section of an ovate shaped choanocyte. (Section from juvenile settled 32 days). (C) Longitudinal section of an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

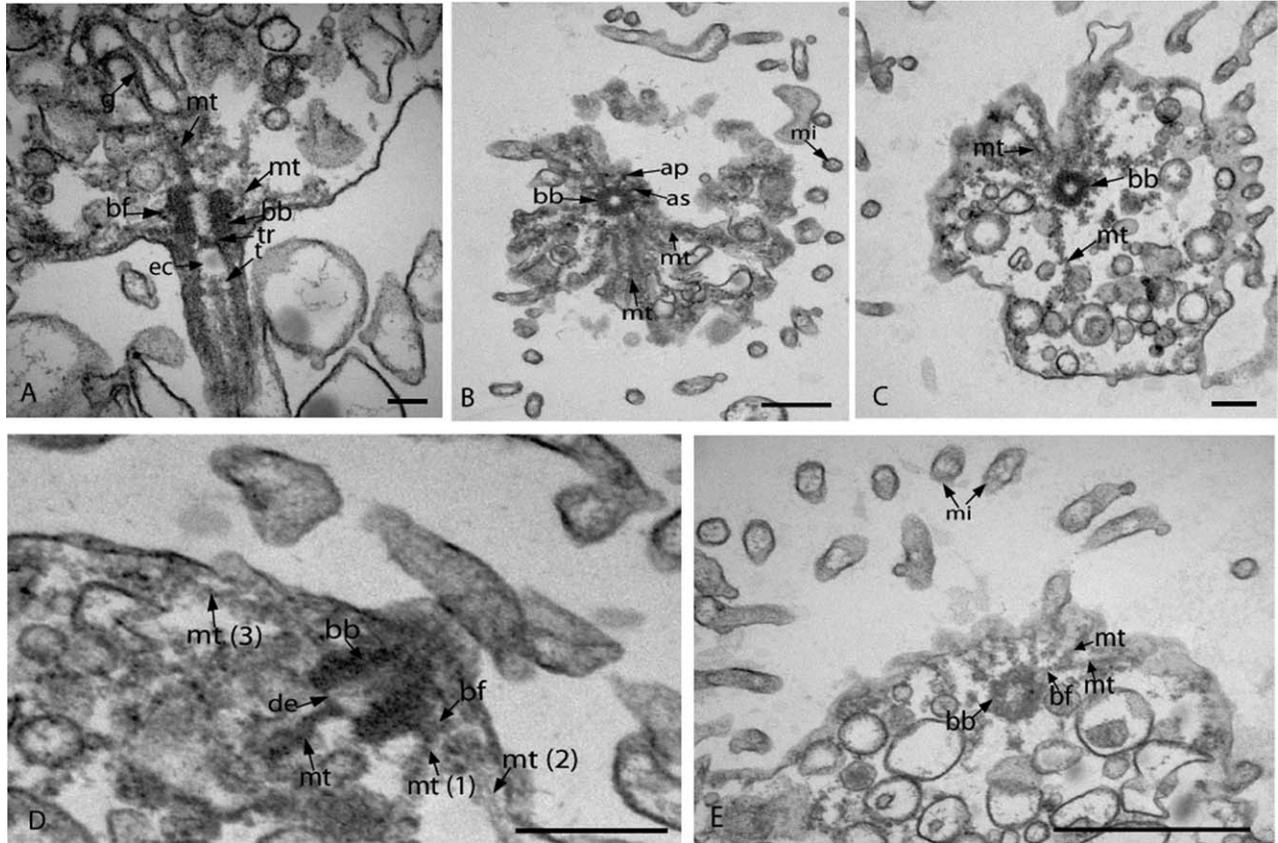


Fig. 7. Choanocyte of young *Haliclona indistincta* sponge. (A) Longitudinal section including microtubule protruding from basal body. (B,C) Cross-sections of apical part of the cell, showing the transition from the alar sheets attached to microtubules that radiate from the basal body in a spiral-like configuration. (D) Longitudinal section of apical part of the cell including microtubules protruding from tip of the basal foot (mt 1 and mt 2) and basal body (mt 3). Also showing the distal end of the basal body and a typical example of the transitional region 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 = 100 nm, B–E = 500 nm.

were much more numerous, the nucleus was more compact and mitochondria were more regular in shape in the choanocytes, than in the ciliated larval cell. While both cell types unsurprisingly shared the same cilium axoneme configuration (9+2) and shape and size of the basal foot and basal body, the basal apparatus of the choanocyte had a noticeably more elaborate microtubular cytoskeleton than that of the larva (Table 1; Fig. 8). The choanocyte had a transverse cytoskeleton comprised of a collar of microtubules that extended from the basal body in a spiral formation, 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. By comparison, 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 toward 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, 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 of the juvenile, with a skirt of parallel fibres as well as microtubule strands, while only a bundle of microtubules were attached to the base of the basal apparatus of the choanocyte (Table 2).

## DISCUSSION

Our initial hypothesis was that, through comparison of the cells that formed the ciliated epithelial layer of the larvae and the cells that formed the choanoderm of the juvenile, we would be able to show a direct relationship between the two cell types, thus tracing the derivation of choanocytes from epithelial cells. This hypothesis was based on the relationship between larval and juvenile ciliated cells observed in other sponges (e.g., Maldonado,

TABLE 1. Comparison of ciliated cells of larvae and juvenile of *Haliclona indistincta*: gross morphology of cell types, and ciliary basal apparatus. Mt = microtubules

	Cell characteristics	Larval ciliated epithelial cell	Juvenile choanocyte	
Gross morphology of cell	Cell shape	Flask-like	Pseudocylindrical Ovate to spherical	
	Cell length and width	5.4–8 $\mu\text{m}$ $\times$ 1–2 $\mu\text{m}$ ( $n = 5$ )	4–6 $\mu\text{m}$ $\times$ 1.6–3 $\mu\text{m}$ ( $n = 5$ ) 2.8–3.1 $\mu\text{m}$ $\times$ 3–4.5 $\mu\text{m}$ ( $n = 5$ )	
	Glycocalyx	Abundant	Sparse	
	Nuclei shape	Pear-shaped	Spherical	
	Mitochondria shape	Spherical	Spherical	
	Mitochondria length and width	325–475 nm ( $n = 5$ ) 585–760 nm $\times$ 350–490 nm ( $n = 5$ )	Ovate	335–440 nm ( $n = 5$ )
	Golgi Apparatus	Numerous	Few	
Cilium and basal apparatus	Vacuoles	Few	Numerous	
	Cilia axonema configuration	9+2	9+2	
	Electron-free area at base of cilium	41–81 nm $\times$ 97–114 nm	102–173 nm $\times$ 61–123 nm	
	Basal foot shape	Trapezoid	Trapezoid	
	Transverse cytoskeleton: microtubules (mt)	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	
	Longitudinal cytoskeleton	Two components: 1) some 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) few mt located opposite side of basal foot; 3) grouping of mt attached to basal end of basal body (lacking fine parallel fibers)	

2004; Leys and Ereskovsky, 2006; Ereskovsky, 2007, 2010; Ereskovsky et al., 2007, 2009). Although the cell types were sufficiently different to prevent a direct link between the cells of the two developmental stages (i.e., free-swimming and sessile), changes in the positioning of larval ciliated epithelial cells throughout metamorphosis, suggested two possible fates for the ciliated, epithelial cells. Larval ciliated cells were observed intact in an internal position throughout the three presettlement stages. Migration of the larval epithelial cells inward has been described in some *Calcinea* species (Borojevic, 1969) and in the demosponge *Halisarca dujardini* (Gonoboleva and Ereskovsky, 2004). The first fate of these migrating cells is suggested by the reabsorption of cilia, which was evident in the cytoplasm of some cells. This process, we suggest, is part of the transformation of the epithelial cells of *H. indistincta* into choanocytes, 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), the freshwater sponges *Eunapius fragilis*, *Ephydatia muelleri*, and *Spongilla lacustris* (Ivanova, 1997a,b) as well as other demosponges (Borojevic and Lévi, 1965; Gonoboleva and Ereskovsky, 2004). Second, phagosomes with remnants of cilium 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 between a type of spherulous cell and/or their inclusions, and larval epithelial cells, as has

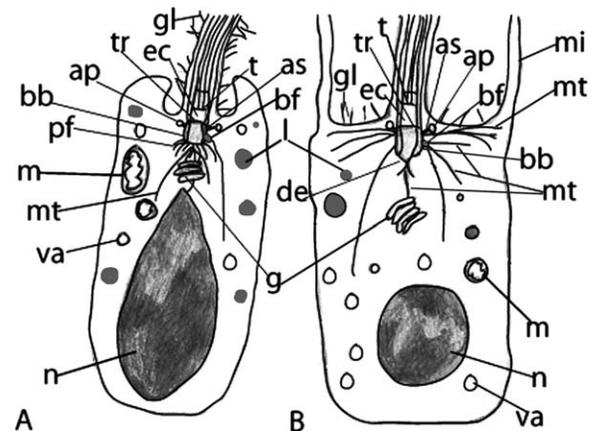


Fig. 8. A Schematic drawing showing the ciliary basal apparatus and relevant organelles observed in the longitudinal sections of the ciliated epithelial cell of the larva of *H. indistincta* (A), and choanocyte of the juvenile sponge (B) of *H. indistincta*. ap, anchoring point; as, alar sheet; bb, basal 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 2. Comparison of ciliary basal apparatus in different larval types found in demosponges

Order Species	HAP (M)										HAP (FW)	HAL		POE		DEN <i>Dendrilla</i> <i>cactus</i> ( <i>Aplysilla</i> sp.)	DIC <i>Ircinia</i> <i>oros</i>	AST <i>Thoosa</i> <i>mismaloli</i>	CHO <i>Chondrilla</i> <i>australensis</i>	HALI <i>Halisarca</i> <i>diuardini</i>
	<i>Haliclona</i> <i>indistincta</i>	<i>Haliclona</i> <i>tubifera</i>	<i>Haliclona</i> <i>permollis</i>	<i>Sigmadocia</i> <i>caerulea</i>	<i>Eumapius</i> <i>fragilis</i>	<i>Ephydatia</i> <i>muelleri</i>	<i>Spongilla</i> <i>lacustris</i>	<i>Halichondria</i> <i>melanadocia</i>	<i>Mycale</i> <i>ceclia</i>	<i>Mycale</i> <i>syrtax</i>		PA	PA	PA	PA					
Larval type	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA	PA
Basal body Type	2	2	2	2	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Basal foot	Y	Y	?	Y	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Basal foot shape	T	M	?	S	?	?	M	?	A	A	A	A	A	A	A	A	A	A	A	A
Accessory centriole	N	N	?	N	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N	N	N
Transverse cytoskeleton	N	Y	?	N	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N	N	N
Cross-striated rootlet(s)	N	N	?	N	?	?	N	?	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Lateral arms	N	Y	?	N	?	?	N	?	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Basal foot microtubule(s)	Y	Y	?	Y	?	?	Y	?	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Longitudinal cytoskeleton	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Fibrous rootlet(s)	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N	N	N
Laminar sheets	N	N	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Golgi associated with rootlet(s)	Y	N	N	N	Y(?)	?	Y	?	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
References	Stephens et al. (this work)	Woollacott, '93; Woollacott and Pinto, '95;	Amano and Hori, '96	Maldonado et al., 2003	Ivanova, '97a; '97b	Ivanova, '97a; '97b;	Ivanova, '97a; '97b;	Woollacott and Pinto, '95	Woollacott and Pinto, '95	Woollacott and Pinto, '95	Wilson, '35	Woollacott and Hadfield, '89; Woollacott and Pinto, '95;	Ereskovsky and Tokina '04	Bautista-Guerrero et al., '10;	Usher and Ereskovsky, '05	Gonobleva and Maldonado, '09	Ereskovsky, 2004; Gonobleva and Maldonado, '09	Usher and Ereskovsky, '05	Gonobleva and Maldonado, '09	Ereskovsky, 2004; Gonobleva and Maldonado, '09

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

been observed in this study. The inclusions grew to a comparatively large size (expanding to over 3 times their original size), and we have not yet determined if they remain inside the spherulous cell (obscuring other cell components), or have been released when they attain this size. Not all ciliated epithelial cells were associated with these spherulous cell/inclusions. When it occurred, the association involved the attachment of the epithelial cells to the spherulous cell/inclusions by the basal end of the epithelial cell. The spherulous cell may have a role in the positioning and fate of larval cells. These cells were concentrated beneath the layer of epithelial cells of the larvae. This is the same location as the layer of an unidentified material described in Stephens et al. (2012) that separated the epithelial layer of the embryos from the ICM. However, throughout the presettlement and postsettlement stages, the spherulous cells (with enlarged inclusions) were visible deep within the ICM of the larvae that were not observed in the embryos examined using histology. The larvae of *H. indistincta* were sticky. This study suggests that the sticky nature of the larvae, and the adult of this species, [as described by Lévi (1956)], may be used as a distinguishing taxonomic feature.

Multiple ciliar axonemes were commonly observed within single cytoplasm, which is a feature that has been reported in several other sponge larval studies (e.g., Lévi, 1964; Boury-Esnault and Vacelet, 1994; Boury-Esnault et al., 1999; Maldonado et al., 2003; Maldonado, 2004). *Haliclona indistincta* parenchymella differ from the larvae of other haplosclerid species described by lacking spicules, a trait which is shared by some species of the demosponge orders: Halisarcida, Chondrosida, Dictyoceratida, Dendroceratida, and Halichondrida (Woollacott and Hadfield, 1989; Woollacott and Pinto, 1995; Leys and Degnan, 2001; Maldonado et al., 2003; Ereskovsky and Tokina 2004; Gonoboleva and Ereskovsky, 2004; Usher and Ereskovsky, 2005; Gonoboleva, 2007; Ereskovsky, 2010). Given the variation in the appearance of spicules across sponges in the literature (e.g., Ivanova, 1997a,b), the timing of spicule production is likely to be variable, and not of phylogenetic importance. *Haliclona indistincta* larvae also differ from other haplosclerid species described, in the ciliation pattern, being uniformly ciliated and lacking longer cilia at the posterior pole.

The positioning of the choanocyte chambers in *H. indistincta* adults (being directly in contact with the mesohyl) is more similar to poecilosclerids than to other haplosclerid species (i.e., *H. oculata*, *H. rosea*, *H. simulans*, *H. fistulosa*, *H. elegans*, and *Niphates digitalis*), whose chambers are separated from the mesohyl by pinacocytes and the walls of incurant canals. This is a feature that led Langenbruch and Jones (1990) to suggest that *H. indistincta* is of poecilosclerid, and not haplosclerid,

origin. Ciliar basal apparatus 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*, which lacked the lateral arms observed in *H. tubifera* (Woollacott and Pinto, 1995). 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 (Stephens PhD thesis) but only distantly related to the type *Haliclona* species *H. oculata*.

Lévi (1956) and Bergquist et al. (1979) suggested larval morphology to be an informative character for sponge taxonomy. The following features are characteristic of the larvae of marine haplosclerida: the absence of flagellated cells in the posterior pole, which is fringed by a circle of cells with longer flagella; the presence of a skeleton representing a dense bundle of oxeads located in the posterior portion of the larva; and the concentration of pigment in the cells of the posterior pole devoid of flagellum (Ereskovsky, 1999, 2010). These characters have diagnostic value in the systematics of sponges (Bergquist et al., 1979; Simpson, 1984; Wapstra and van Soest, 1987; Woollacott, 1993; Fromont, 1994; Ereskovsky, 1999). The ciliation pattern of *Haliclona tubifera* larvae is considered typical of *Haliclona* species (Woollacott, 1993; Woollacott and Pinto, 1995). *Haliclona oculata* larvae also share this ciliation pattern (Wapstra and van Soest, 1987). *H. indistincta* however, does not, which is consistent with the fact that they belong to a different clade than *H. oculata* and *H. tubifera*, being more closely related to members of *Niphates*, than to other *Haliclona* (Stephens unpublished PhD thesis). However, Ilan and Loya (1988) and Ilan et al. (2004), described *Niphates rowi* also as having longer cilia forming a ring around the posterior pole. Until more larvae of different species are investigated with both morphological and molecular data, 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 ciliar apparatus characters can be useful in identifying closely related species, as congruence was found among the poecilosclerid species studied (i.e., *Mycale cecilia*, *M. contarenii*, and *Hamigera hamigera*) and within the halichondrids, (i.e., *Halichondria melanadocia*, *H. coerulea*, and *H. heliophila*), using basal ciliar apparatus morphology. These authors also compared the haplosclerid *H. tubifera* to two additional *Haliclona* spp., which lacked a transverse cytoskeleton as also observed in *H. indistincta* (Woollacott and Pinto, 1995). Despite the differences between the larval and juvenile ciliated cells in *H. indistincta*, the overall

shape of the cells was similar to that of other sponge orders and freshwater sponges [i.e., *E. fragilis*, *E. muelleri*, and *S. lacustris* (Ivanova, 1997a,b)] (Table 2). Seravin (1986) suggest that microtubules, like those that projected from the basal body in a spiral-like arrangement of the choanocyte of *H. indistincta*, are a typical feature of sponge choanocytes, e.g., *Spongilla lacustris* (Fjerdingstad, 1961), *Ephydatia fluviatilis* (Feige, 1969), *H. rosea* (Garrone, 1969), *Baikalospongia bacillifera* (Efremova et al., 1988), and *Halisarca dujardini* (Gonobobleva and Maldonado, 2009). *H. indistincta*, however also possesses a bundle of microtubules attached to the base of the basal body that is uncommon in other sponge choanocytes (Woollacott and Pinto, 1995; Gonobobleva and Maldonado, 2009). A common structure present in ciliated eukaryote epithelial cells is the accessory centriole, which is involved during cell division as microtubule organizing centers (Nielsen, 1987; Gonobobleva, 2007). However, accessory centrioles were not observed in ciliated larval cells and in the choanocytes of *H. indistincta*. They are also absent in *H. tubifera*, and *M. cecilia* (Woollacott and Pinto, 1995) but are otherwise commonly found in sponge larva (e.g., Homoscleromorpha, Dictyoceratida and Halisarcida; Table 2; Maldonado et al., 2003; Boury-Esnault et al., 2003; Ereskovsky and Tokina 2004; Gonobobleva and Ereskovsky, 2004; Ereskovsky et al., 2007, 2009; Gonobobleva and Maldonado, 2009).

Unfortunately, the level of detail included in this and Woollacott and Pinto's (1995) study is not yet available for many sponges 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). Therefore, additional comparative studies targeting the basal apparatus of putatively closely related species of *H. indistincta* (e.g., *H. viscosa* and *Niphates* spp.) and additional specimens of this species, are necessary for determining the level that these, and other characters, are informative. However, in addition to shedding some light on the origin of the choanocytes in this species, this study provides valuable information for such comparative morphological analyses in the future, which will be necessary to further understand evolutionary relationships in this important group of animals.

## ACKNOWLEDGMENTS

This project has been funded through a National University of Ireland Galway PhD fellowship, and the Thomas Crawford Hayes Trust Fund Scheme (NUI Galway) awarded to Kelly Stephens. Authors

would like to thank the two anonymous reviewers for their suggestions, which have improved the manuscript.

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