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## *sine oculis* in basal Metazoa

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**Abstract** We report the recovery of homologs of *Six1/2/sine oculis* (*so*), a homeodomain-containing member of the *Six*-gene family, from a diverse set of basal Metazoa, including representatives of the poriferan classes Demospongia, Calcarea and Hexactinellida, the cnidarian classes Hydrozoa, Scyphozoa and Anthozoa, as well as a ctenophore. *so* sequences were also recovered from a platyhelminth, an echiurid and two bivalve molluscs, members of the super-phyletic group Lophotrochozoa. In the case of the platyhelminth, multiple distinct *so* sequences were recovered, as well as a member of the related group *Six4/5/D-Six4*. Extended sequences of the *so* gene were recovered from the demosponge, *Halicionasp.*, and the scyphozoan *Aurelia aurita* via PCR, and 3' RACE. The affinities of all recovered sequences were assessed using a parsimony analysis based on both nucleic and amino acid sequence and using successive character

weighting. Our results indicate that *so* is highly conserved across the animal kingdom. Preliminary expression data for *Aurelia* reveal that transcripts of the *so* homolog are present in the manubrium as well as in the rhopalia, which contain the statocyst and eyes, in the free-swimming ephyra and juvenile stages of these jellyfish.

**Keywords** *Six1/2/sine oculis* · Porifera · Cnidaria · Lophotrochozoa

### Introduction

Although metazoan sensory organs operate in different sensory modalities such as sight and hearing, their shared attributes point toward a common origin. Most sensory cells in sense organs employ some form of transmembrane G-protein in their signal transduction, and sensory cells generally possess apical hairs and microvilli, albeit modified for specific sensory function. In addition, regulatory genes involved in sense organ development are conserved. Members of the PAX gene family appear to play specific conserved roles in the development of certain classes of sense organs such as eyes or ears in divergent members of the Bilateria (e.g. Tomarev et al. 1997; McCauley and Bronner-Fraser 2002). Other genes have common functions in different types of sense organs further attesting to shared evolutionary history of sense organs. The *atonal* gene of *Drosophila*, a proneural gene, plays a specific role in the proximal spacing of sensory cells in sense organs of all types (Jarman et al. 1995; Gopfert et al. 2002; Chen et al. 2002). Several other genes, such as the POU-domain-containing genes *Acj6 /Brain 3* (e.g. O'Brien and Degnan 2002), and *Dachshund* (Hammond et al. 2002), regulate the development of multiple types of sense organs in a variety of bilaterian systems. In this work we focus on the deep evolutionary history of *sine oculis* (*so*), another gene known to function broadly in bilaterian sense organ development. We report the recovery of *so* in taxa basal to the Bilateria, as well as members of the bilaterian group Lophotrochozoa (Fig. 1).

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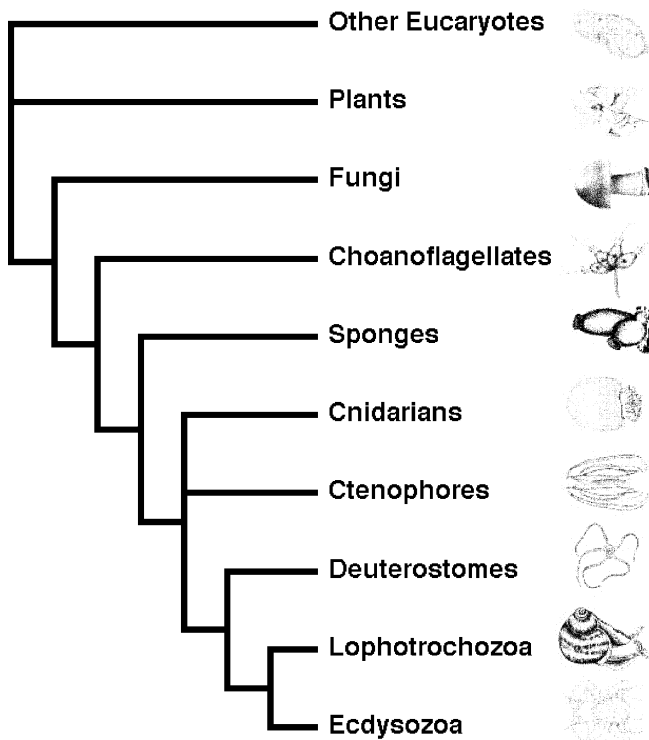
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**Fig. 1** The generally accepted scheme of basal branches of the metazoan phylogeny

The rationale for exploring *so*, a member of the *Six*-gene family, in basal Metazoa includes the importance and conservation of this gene and gene family in bilaterian sense organ development as discussed in detail later. In addition, phylogenetic analysis of homeodomain-containing genes generated a basal position for *so* (Agosti et al. 1996) suggesting antiquity of the gene and predicting its presence in taxa basal to the Bilateria. Here we assay expression of *so* in *Aurelia aurita* a scyphozoan cnidarian with discrete sensory structures. Although some work has been done on PAX genes in the model cnidarian *Hydra vulgaris* (e.g. Sun et al. 1997), *Hydra* lacks the discretely differentiated sense organs found in medusoid cnidarians hence our choice of *Aurelia* for initial experimental investigation of sensory structures. Other basal Metazoa exhibit evidence of sensory structure or process. Ctenophores have an apical statocyst and a group of posterior sensory cells respond to light in some sponge larvae (e.g. Leys et al. 2002). This evidence of basal metazoan sensory structures, our initial positive results from *Aurelia*, and the presence of the sense-organ-related POU/homeodomain gene *Brain3* throughout the basal Metazoa (Jacobs and Gates 2003; Gates and Jacobs, unpublished data), inspired our PCR survey for *so* in additional basal metazoan taxa.

Members of the *Six*-gene family are involved in a variety of fundamentally important developmental processes and contain two conserved domains, the *Six*-type homeodomain, and the *Six* domain located just 5' of the homeodomain. These domains are involved in DNA binding and protein-protein interactions, respectively (for review see Kawakami et al. 2000; Pignoni et al. 1997).

There are three *Six* genes in *Drosophila melanogaster*, *so*, *optix* and *D-Six4*, also referred to as *myotonix* (Seo et al. 1999). The nine vertebrate homologs are classified based on their similarity to the *Drosophila* gene, as follows: *Six1/2/so*, *Six3/6/7/9/optix*, and *Six4/5/8/D-Six4*. It should be noted that *Six3* was initially considered to be the vertebrate homolog of *so*; however, it is now clear that this gene is more closely related to *optix*, the member of the *Six*-family most recently recovered from *Drosophila* (Seimiya and Gehring 2000; Toy et al. 1998).

Of the three groups of *Six*-genes, the *Six1/2/so* and *Six3/6/7/9/optix* groups are implicated in eye and sensory structure development. Much of the research effort directed at this gene family in the context of sensory structure development has focused on the *Six1/2/so* group in bilaterian model taxa such as *Drosophila* (Cheyette et al. 1994; Serikaku and O'Tousa 1994), *Caenorhabditis elegans* (Dozier et al. 2001), *Homo sapien* (Boucher et al. 1996), *Mus musculus* (Oliver et al. 1995a, b; Zheng et al. 2003), *Gallus gallus* (Bovolenta et al. 1998) and *Xenopus laevis* (Loosli et al. 1999). This work has revealed that, in *Drosophila*, *so* is associated with the eye imaginal disc and Bolwig's organ, and expression of *so* in the unpatterned epithelium is required for eye morphogenesis and development of the entire visual system (Cheyette et al. 1994). A mutation in the *so* homeobox disrupts the larval visual system and abolishes the larvae's response to light (Hassan et al. 2000). *so* expression is regulated by *eyes absent* (*eya*) and the expression of both *so* and *eya* is required to induce ectopic eyes (Halder et al. 1998; Pignoni et al. 1997). *Six1/2/so* gene homologs have now been recovered from other invertebrates. In a polychaete (Arendt et al. 2002) and a planarian (Pineda et al. 2000) *so* has been demonstrated to play a critical role in eye development.

Although *so* is best known for its role in eye development in invertebrates, the vertebrate homologs of this gene (*Six1* and *Six2*) serve in a broad array of developmental processes, including sense organ development, but do not appear to play a key role in the development of eyes per se. However, *Six1* is localized in the otic and olfactory placodes (Laclef et al. 2003b) and is critical for proper development of the sensory structures of the nose and auditory system (Laclef et al. 2003b; Zheng et al. 2003). Additionally, *Six1* expression in Rathkes' pouch, a structure that later develops into the glandular part of the pituitary gland (Oliver et al. 1995a), is of interest as the vertebrate pituitary likely evolved from a sensory organ (Gorbman 1995). *Six1* and *Six2* are also expressed in head mesoderm and are involved in murine mesodermal patterning and limb tendon development (Oliver et al. 1995a) as well as kidney development. Mice deficient in *Six1* confirm that this gene plays a critical role in primary myogenesis as well (Laclef et al. 2003a). Similarly, in *Xenopus*, *Six1* and *Six2* are both expressed in the anlagen of the sensory placodes, as well as head mesenchyme and mesenchyme associated with the stomach and pronephros (Ghanbari et al. 2001).

As in *Six1/2/so*, the other two gene groups in the *Six* family of genes, *Six3/6/optix* and *Six4/5/D-Six4*, also share

some expression patterns between invertebrates and vertebrates. In *Drosophila*, *optix* is expressed in the cephalic region of the embryo (Toy et al. 1998). Similarly, the mouse homolog *Six3* is expressed in the developing brain, including Rathkes' pouch and nasal placodes (Oliver et al. 1995b). *Drosophila D-Six4* expression is linked to head, muscle, ventral nerve chord and gonad development (Kirby et al. 2001; Seo et al. 1999) and *Six4* and *Six5* in vertebrates have been localized to skeletal muscle and sensory and neural tissues, among others (Heath et al. 1997; Ohto et al. 1998). To date, *Six* genes have been explored in ecdysozoan and vertebrate bilaterian model systems. With the notable exception of the work in a polychaete (Arendt et al. 2002) and in a planarian (Pineda et al. 2000), *so* is poorly understood in lophotrochozoans and completely unstudied in basal metazoans. To begin a more comprehensive examination of this developmentally important gene group, we focus on one member of the group, *so*, and attempt to recover homologs of this gene from members of the basal phyla Cnidaria, Ctenophora and Porifera and representatives of the bilaterian lophotrochozoan phyla Platyhelminthes, Mollusca and Echiura (Fig. 1). We examine the relationship between our newly recovered sequences and published *Six*-gene sequences using phylogenetic analyses, and report the results of a preliminary RT-PCR expression study aimed at exploring the possible function of this gene in the medusoid cnidarian *Aurelia*.

## Materials and methods

### Organisms

Taxonomic and collection information for the organisms used in this study is presented in Table 1.

### DNA and RNA extraction

All animals were starved for at least 48 h prior to nucleic acid extraction to prevent the contamination of organismic DNAs with food items. Genomic DNA was extracted using either a classic C-Tab protocol (Wilson 1987) or the DNeasy Kit (Qiagen) following the manufacturers instructions. RNA was isolated from *Aurelia* and *Haliclona* using TRIzol reagent (Life Technologies) using the manufacturer's protocol, with the exception that *Aurelia* tissues were frozen in liquid nitrogen and ground with a pestle and mortar prior to the addition of the TRIzol reagent.

### Primer design

Degenerate primers were designed to the aligned nucleic acid sequences for the *Six*-type homeodomain and *Six* domain of the following published *Six1/2/so* sequences: *Drosophila so* (NG 000163; Q27350), *Giardia tigrina so* (AJ251660), *Platynereis dumerilii Six1/2* (AJ316542), *Mus Six1* (X80339), *Mus Six2* (X80338), and *Homo Six1* (X91868). One forward and one reverse primer was designed for the *Six*-type homeodomain and corresponded to the conserved amino acid sequences WDGEETSY and VSNWFKNRRQ, named SO-F2 and SO-R1 respectively (Table 2). We subsequently designed an additional forward degenerate primer, SixDomain-F1 (Table 2), corresponding to the conserved amino acid sequence QVACVC of the 5' end of the *Six* domain using the following sequences: *Drosophila so* (Q27350), *Giardia so* (AJ251660), *Platynereis dumerilii Six1/2* (AJ316542), *Mus Six1* (X80339), *Mus Six2* (X80338), *Homo Six1* (X91868), and *Homo Six2* (NM\_016932). In designing the primers, care was taken such that the annealing temperatures of the forward and reverse primers were compatible.

### PCR and cloning

We initially used the SO-F2/SO-R1 combination of primers on all species and then the SixDomain-F1/SO-R1 combination of primers on *Aurelia* and *Haliclona*. These primer combinations were used in separate 50- $\mu$ l PCR reactions consisting of 1 U Taq (Applied Biosystems) or 1.5 U HotMaster Taq (Eppendorf), 1  $\mu$ l genomic DNA, 0.5  $\mu$ M (each) primer, 2 mM dNTP mix (Amersham Pharmacia Biotech), 2 mM MgCl<sub>2</sub> (omitted when using HotMaster Taq), 5  $\mu$ l DMSO and 5  $\mu$ l 10 $\times$  PCR enzyme appropriate PCR

**Table 1** List of species, their current classification and collection details

Species name	Phylum	Class	Supplier	Location of collection
<i>Leucosolenia eleanor</i>	Porifera	Calcarea	Marinus Inc.	Long Beach, Calif.
<i>Haliclona</i> sp.	Porifera	Deemospongiae	Jacobs Lab	Los Angeles, Calif.
<i>Rhabdocalyptus dawsoni</i>	Porifera	Hexactinellida	S. Leys	Bamfield, B.C., Canada
<i>Bougainvillia bougainvillei</i>	Cnidaria	Hydrozoa	Cabrillo Aquarium	Long Beach, Calif.
<i>Aurelia aurita</i> <sup>a</sup>	Cnidaria	Scyphozoa	Cabrillo Aquarium	Long Beach, Calif.
<i>Pelagia colorata</i>	Cnidaria	Scyphozoa	Cabrillo Aquarium	Long Beach, Calif.
<i>Cyanea capillata</i>	Cnidaria	Hydrozoa	Cabrillo Aquarium	Long Beach, Calif.
<i>Anthopleura elegantissima</i>	Cnidaria	Anthozoa	Jacobs Lab	Los Angeles, Calif.
<i>Porites astreoides</i>	Cnidaria	Anthozoa	R. Gates	Florida Keys, Fla.
<i>Mnemiopsis leidyi</i>	Ctenophora	Tentaculata	M. Martindale	Woods Hole, Mass.
<i>Nutricula tantilla</i>	Mollusca	Bivalvia	W. Borgeson/S. Lee	Bodega Harbor, Calif.
<i>Crassostrea gigas</i>	Mollusca	Bivalvia	W. Borgeson/S. Lee	Bodega Harbor, Calif.
<i>Urechis caupo</i>	Echiura	Echiurida	Jacobs Lab	Venice, Calif.
<i>Macrostomum</i> sp.	Platyhelminthes	Turbellaria	Peter Ladurner	Lagune of Ligano, Italy

<sup>a</sup>*Aurelia aurita* appears to be a cryptic species complex. Material used in this study was assigned to *Aurelia* sp.1 *sensu* Dawson and Jacobs (2001) based on the COI sequence

**Table 2** List of all primers

Primer name	Primer type	Primer sequence 5'-3'	Temp °C
SO-F2	Degenerate	TGG-GAY-GGN-GAR-GAR-ACS-AGY-TA	65.5
SO-R1	Degenerate	TGY-CKK-CKG-TTY-TTR-AAC-CAR-TTG-SWV-AC	66.0
SixDomain-F1	Degenerate	CAR-GKB-GCB-WGY-GTB-TGY-GA	64.5
AureliaSO-F1	Specific	TCG-AGC-TAT-CCT-CAG-AGA-GAC-TGG	64.5
AureliaSO-F2	Specific	CGT-ACC-CAT-CAC-CGA-GAG-AAA-AG	64.5
AureliaSO-R1	Specific	GTG-TGG-TGG-ATA-GAC-CCG-TTC-C	66.5
AureliaSORT-PCR-F1	Specific	CGA-GCT-ATC-CTC-AGA-GAC-TG	62.5
AureliaSORT-PCR-R1	Specific	ATC-TCT-TTG-CCG-TCT-GTT-C	58.0
Mae-T17	Adapter	AAG-CAG-TGG-TAA-CAA-CGC-AGA-GTA-CT(17)	64.0
BamH1-T17	Adapter	GAG-AGA-GAG-AGG-ATC-CAA-TAC-TGG-AGA-GT(17)	66.5
Mae-alone	Adapter	AAG-CAG-TGG-TAA-CAA-CGC-AGA-GTA-C	64.5
BamH1-alone	Adapter	GAG-AGA-GAG-AGG-ATC-CAA-TAC-TGG-AGA-G	67.5
HaliclonaSO-F1	Specific	TGC-TTC-AAG-GAG-AAA-TCT-AGG-GTA-G	63.0
HaliclonaSO-F2	Specific	CGA-CAG-TGG-TAC-ACC-AAG-AAT-CCA	64.5
HaliclonaSO-R1	Specific	TGA-GAC-CAG-TTT-GTT-CTG-CCA-AT	61.0
Actin-F1	Degenerate	GAY-AAY-GGW-TCH-GGW-ATG-TGY-YAA-A	61.0
Actin-R1	Degenerate	GYT-CRT-TGT-ARA-ARG-TRT-GAT-GCC-A	62.0
Actin-R2	Degenerate	CYT-GWC-CRT-CDG-GHA-RTT-CRT-A	62.0
Actin-R3	Degenerate	GAR-ATC-CAC-ATY-TGY-TGG-AAR-G	61.0
AureliaActin-F1	Specific	TGC-CAT-CCA-AGC-TGT-CCT	60.0
AureliaActin-R1	Specific	TCG-AAG-TCG-AGT-GCG-ACA	60.0
AureliaActinRT-PCR-F1	Specific	TGG-TAT-GGG-ACA-GAA-AGA-CTC	60.5
AureliaActinRT-PCR-R1	Specific	CAG-TTT-GTG-ACA-ATT-CCG-TG	58.0
AureliaActinRT-PCR-R2	Specific	TTG-AGC-TGG-TTG-ATG-CTG	57.5

Buffer. The amplification protocol consisted of an enzyme activation step of 94°C for 12 min (2 min for HotMaster Taq), followed by 35 cycles of 30 s at 94°C (20 s for HotMaster Taq), 30 s at 52°C and 1 min at 72°C (68°C for HotMaster Taq), and a final extension of 7 min at 72°C (68°C for HotMaster Taq), performed on a DNA Engine Gradient PTC-200 (MJ Research). For *Porites astreoides*, *Pelagia colorata* and *Bougainvillea bougainvillei* a secondary round of PCR was performed using 1 µl primary PCR product as template, including the negative control, and the same conditions to those described above except with an annealing temperature of 58°C or 60°C.

The resulting PCR products were visualized using agarose gel electrophoresis and stained with ethidium bromide. Bands corresponding to the expected size were identified by comparison with a 1-kb ladder (Invitrogen), cut from the gel and purified using the Ultra Clean 15 Kit (Mo. Bio Labs). The resulting purified products were A-tailed with Taq polymerase before cloning into pCRII-TOPO vector using the TOPO TA cloning Dual Promotor kit (Invitrogen). Plasmids were isolated with the Wizard Plus SV Minipreps DNA Purification System (Promega) and screened for *so* using chain termination manual sequencing (Sanger et al. 1977). The sequences of at least three so-positive clones per species were confirmed in both directions by automated sequencing (ABI Prism 377 XL).

#### cDNA synthesis and 3' RACE

*Aurelia* and *Haliclona so* fragments were extended using 3' RACE. First strand cDNA was synthesized from total RNAs using Super Script II reverse transcriptase (Invitrogen) and the oligo (dT) adapted primers Mae-T<sub>17</sub> or BamH1-T<sub>17</sub>(Table 2). 3' RACE was achieved through two rounds of PCR using gene-specific primers designed to the *Aurelia* and *Haliclona so* gene fragments isolated above. In the primary amplification the cDNAs were amplified with AureliaSO-F1 (*Aurelia*) and HaliSO-F1 (*Haliclona*) (Table 2) and

Mae or BamH1 using the conditions previously described for HotMaster Taq (Eppendorf), but with an annealing temperature of 54°C, and omitting the DMSO. A second round of PCR was performed using the nested primers AureliaSO-F2 (*Aurelia*) and HaliSO-F2 (*Haliclona*; Table 2) and Mae or BamH1. PCR conditions were the same as for the primary reaction except the annealing temperature was increased to 60°C. Products were cloned and sequenced in both directions as described above.

#### Actin control

To provide a control for our expression work we isolated actin from *Aurelia* using degenerate primers designed against the consensus sequence of the following aligned actin sequences: *Hydra attenuata* (M32364), *Podocoryne carnea* (X69059), *Oikopleura longicauda* (AB034209), *Dictyostelium discoideum* (X03282), *Molgula oculatam* (AF076516) and *Caenorhabditis* (NM\_073417). The primer sequences for Actin-F1, Actin-R1, Actin-R2 and Actin-R3 are listed in Table 2. Each pair was used in one round of PCR performed as described earlier for the HotMaster Taq (Eppendorf) with an annealing temperature of 52°C. The amplicons were cloned and sequenced in both directions using the ABI Prism 377 XL. Three gene-specific primers were designed against the resulting *Aurelia* actin sequence, AureliaActin-F1, AureliaActin-R1, and AureliaActin-F2 (Table 2) and these primers used as internal controls for the RT-PCR and 3' RACE experiments described below.

#### Phylogenetic analysis

Published *Six* gene nucleic acid sequences were aligned in MacClade (Maddison and Maddison 1999) using the display-amino acid translation function and the phylogenetic analysis was done using PAUP\*4.0 b10 (Swafford 1998). We performed a

successive weighting analysis comparable to that described by Lee et al. (2003). This type of analysis permits the application of information from more complete sequences to the phylogenetic assessment of smaller gene fragments. The initial step consisted of a heuristic search run on the best-aligned complete cDNA sequences (sequences with accession numbers in Fig. 3 as well as sequences for *Aurelia* and *Haliclona* reported here). Aligned positions included the first 30 amino acids adjacent to the start codon of the longest sequence, 17 positions before the *Six* domain, the entire *Six* and homeodomain and 164 positions 3' to the homeodomain. The data matrix consisted of the amino acids and corresponding nucleotides alignment minus third positions for a total of 1,017 characters. One hundred repetitions of the heuristic search were conducted using the tree bisection/reconnection branch-swapping algorithm.

The resulting resolved tree topology was then used to reweight the characters in the matrix using the rescaled consistency index. Our gene fragments (those that do not contain accession numbers in the name on Fig. 3) were added to the reweighted data set, third base-pair positions were removed for the nucleic acid section of the combined database and the data was subjected to a bootstrap analysis consisting of 500 replicates of 10 heuristic searches each. *Caenorhabditis Six* genes were not included in the analysis due to difficulty in aligning the sequences in the 5' region. Although we are publishing a *so* sequence obtained from *Mnemiopsis leidy*, we did not include it in the phylogenetic analysis because it did not group with either of the clades consistently.

cDNA synthesis for RT-PCR and RT-PCR protocol

*Aurelia* ephyrae were relaxed in a 1:1 solution of seawater and 0.37 M MgCl<sub>2</sub> before the manubrium, lappets and rhopalia were dissected from each individual using a sterile scalpel under a Leica MZ12 microscope. Several experiments were conducted to determine the ideal amount of each tissue to be used and the resulting amounts were 12 rhopalia, 4 manubria and 8 lappets for each sample. Tissue was kept in seawater on ice and used directly for cDNA synthesis using the Cells-to-cDNA kit (Ambion). The reverse transcription was conducted on the tissue lysates using both the reverse transcriptase provided with the kit and iScript from (BioRad), an enzyme specifically designed for reverse transcribing low copy genes. RT-PCR was done on the resulting cDNAs using one pair of *Aurelia*-specific *so* primers, AureliaSORT-PCR-F1 and AureliaSORT-PCR-R1, and two pairs of actin primers, AureliaActinRT-PCR-F1 with AureliaActinRT-PCR-R1 and AureliaActinRT-

PCR-R2 (Table 2). PCR conditions were as described for HotMaster *Taq*, except that 3 µl of cDNA was used as a template in each reaction, DMSO was omitted and the annealing temperature was 55°C.

Our cDNA synthesis included a DNase I step prior to reverse transcription; however, to ensure that our products contained no contaminating genomic DNA templates that could potentially serve as templates in PCR, we performed a control that was treated identically to those described earlier except that water was added to the reaction instead of the reverse transcriptases. We also controlled for contamination by foreign templates throughout the reverse transcription and subsequent experimental steps by including a negative that contained no tissue lysate.

RT-PCR was also performed on cDNAs reverse transcribed in a more traditional way from the RNA of *Aurelia* juveniles and ephyrae medusa as described in the section cDNA synthesis and 3' RACE and the amplified *so* from these cDNAs using the *so* and actin primers detailed in this section and earlier.

Results

PCR and cloning

Using PCR and degenerate primers we amplified a *so* homolog from 14 invertebrate species (Fig. 2a, b) representing four phyla (Table 1). Of the four primers designed to the *Six*-type homeodomain, only one pair, SOF2/R1, amplified effectively and produced the expected 177-bp fragment (including primer sequence). The primary PCR yielded the target band in all species except *Porites*, *Pelagia* and *Bougainvillia*, all of which required a second round of PCR using the same primers but at a slightly higher annealing temperature before the correct band size was visible on the gel.

Homeodomain sequences we identified as *so* exhibit from 71% to 89% amino acid identity to *Drosophila so* and contain consistent patterns of amino acid change among the phyla investigated (Fig. 2a, Table 1). For example, all the cnidarian sequences have an isoleucine at

**Fig. 2 a** Comparison of the homeodomain sequences from 12 species representing four different metazoan phyla. Percent identity (% Ident) to *Drosophila so* (accession no. S77459) is shown at the end of each row. **b** *Aurelia* and *Haliclona* PCR products extended using 3' RACE aligned with *Drosophila so* sequence. Question marks indicate missing data and dashes indicate identical residues

Species	Amino Acid Sequence				% Ident
	H 1	H 2	H 3		
<i>Drosophila so</i>	CFRKEKSRVLRDWSHNPYSPREKRDLAETGLTTQ				100
<i>Rhabdocalyptus so</i>	-----GL-----LK-----E--DL-----				82
<i>Leucosolenia so</i>	-----AR--E--T-----K-----Q-----				84
<i>Bougainvillia so</i>	-----AI-----TR-----K--DG-----				82
<i>Pelagia so</i>	-----AI-----TR-----K--DG--S---				79
<i>Cyanea so</i>	-----AI--E--VR-----K--DG--S---				76
<i>Anthopleura so</i>	-----NI--E-----E--N-----				87
<i>Porites so</i>	-----NI--E-----E--N-----				89
<i>Mnemiopsis so</i>	-----AI--KE--K-----E--NTA--A-Q				71
<i>Nutricola so</i>	-----T--E--A-----E-S-G-----				84
<i>Crassostrea so</i>	-----TI--E--Q-----E-----				87
<i>Urechis so</i>	-----T--E--A-----E--G-----				86
<i>Macrostomum so1</i>	-----Q--E--A-----KE--E-----				84
<i>Macrostomum so2</i>	-----Q--E--A-----KE--E-----				84
<i>Macrostomum so3</i>	-----N--E--T-----E-----				89
<i>Macrostomum Six4</i>	-----QA-KEC-KQNR--TPE--KQ--SS--M--				55

Species	Amino Acid Sequence				% Ident
	H 1	H 2	H 3	H 4	
<i>Drosophila so</i>	FTQEQVACVCEVLQQAGNIERLGRFLWSLPQCDKQLQNESVLKAKAVVAFHRGQYKELRYLL				
<i>Aurelia so</i>	?????????-----G-S-D--A-----N-EISN-----H-NFQ--NI-				
<i>Haliclona so</i>	?????????????-----S-----A-----A-EQI-K-----LI--Q-NFP----I				

**Six-domain**

Species	Amino Acid Sequence				% Ident
	H 1	H 2	H 3	H 4	
<i>Drosophila so</i>	EHHSFSAQNHAKLQALWLKHAHYEAEKLRGRPLGAVGKYRVRKFPPLPRTIWDGEETS YCF				
<i>Aurelia so</i>	-N-N--ISS-V--S-----I--I-----				
<i>Haliclona so</i>	-LNS-TPES-P-M-Q--Q--I--R-K-----I-----				

**Homeodomain**

Species	Amino Acid Sequence				% Ident
	H 1	H 2	H 3	H 4	
<i>Drosophila so</i>	GEETS YCF KEKSRVLRDWSHNPYSPREKRDLAETGLTTQVSNWFKNRRQRDRAAE				
<i>Aurelia so</i>	-----AI-----TR-----K--DG--S-----R-				
<i>Haliclona so</i>	-----V--P--TK-----Q--Q-----S-				

**3' Region**

Species	Amino Acid Sequence				% Ident
	H 1	H 2	H 3	H 4	
<i>Drosophila so</i>	HKDGDSTDKQHLDDSSDSEMEGSMPLPSQSAHQHQHQHQHQHSPGNSSGNNGL				
<i>Aurelia so</i>	A--RENFE-KIMKYQ--SDSKDGIKATHPGL--PVMNMNMCVHSPVLDPLS-				
<i>Haliclona so</i>	T-R??				

position 15 and both mollusc sequences contain a threonine at position 14, a position that is highly variable among the *Six* family of genes. Also, among the cnidarian sequences, the Hydrozoa and Scyphozoa share common amino acids distinct from those in the Anthozoa at positions 14, 21, 22, 32, 36, 37 and 41. Furthermore, comparison of our mollusc and echiuran sequences with the published *Platymereis Six1/2/so* sequence indicates that they are 95% and 97% identical, respectively.

### 3' RACE

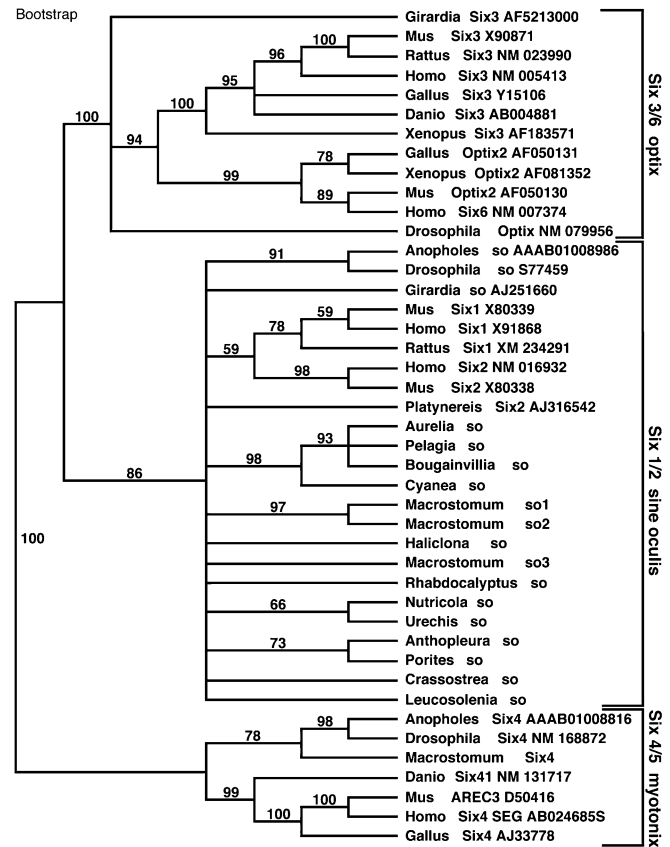
We extended the *Aurelia* and *Haliclona so* fragments using gene-specific primers and 3' RACE. In both cases the resulting products were truncated; however, we were able to elongate the *Aurelia* and *Haliclona* fragments by an additional 67 and 19 nucleic acids, respectively, and these extended fragments show 79% and 75% similarity to *Drosophila so* on an amino acid level (Fig. 2b). Both sequences lack an arginine at position 5 and a glutamine at position 12 in helix 1. The absence of these amino acids at these positions is considered a defining trait of the *Six*-type homeodomain (Kawakami et al. 2000).

### Phylogenetic analysis

Our phylogenetic analysis consisted of an initial heuristic search using the alignable regions of published *Six* genes to create a topology which took best advantage of the variable characters and limitations of the dataset. This tree (not shown) consisted of three distinct clades representing *Six1/2/sine oculis*, *Six3/6/optix* and *Six4/5/D-Six4*. After reweighting the characters of this topology we added our recovered sequences and performed a bootstrap analysis (Fig. 3). Each of the three groups of *Six* genes, *Six3/6/optix*, *Six1/2/sine oculis* and *Six4/5/D-Six4*, are well supported and bootstrap values indicate that our sequences belong to the *Six1/2/sine oculis* clade. There is high support for the cnidarian clade and good support for the insect clade. Most of the vertebrate topologies in the other *Six* gene groups are also well supported with high bootstrap values (Fig. 3).

### RT-PCR

We used RT-PCR to explore the expression of *Aurelia so* in different life history stages of *Aurelia* and in the different tissues of the ephyrae. *so* transcripts were detected in both the ephyrae and juvenile *Aurelia* (Fig. 4b) in multiple experiments. To investigate the expression pattern of *so* in more detail, we examined three tissues in *Aurelia* ephyrae, the manubrium, rhopalia and lappets. In four separate RT-PCR experiments, a total of 4 samples out of 28 were positive in the rhopalia set. In five separate experiments, a total of 8 samples out of 13 were positive in the manubrium set. Lastly, in seven separate

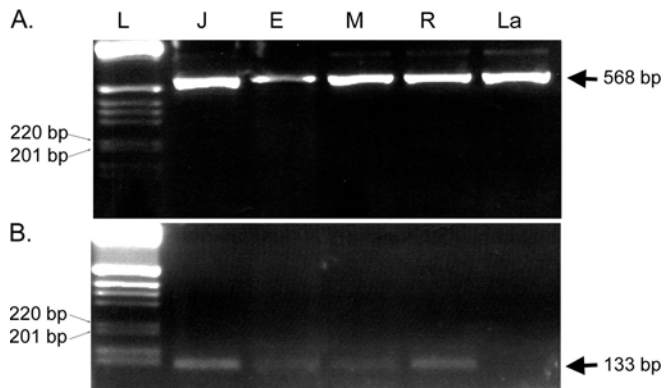


**Fig. 3** Phylogenetic tree representing the three groups of *Six* genes and placement of our sequences in the *Six1/2/so* group. Bootstrap analysis of 500 replicates of 10 heuristic searches each was performed after successive weighting (see text for tree construction methods). Note high support for the *Six1/2/so*, *Six3/6/Optix* and *Six4/5/D-Six4*, as well as high support for the medusoid and anthozoan cnidarian clades and the insects within the *Six1/2/so* group. Previously published sequences are followed by GenBank accession number

experiments, 1 sample out of 37 was positive in the lappet set. Based on these findings we conclude that the gene was expressed in the manubrium and rhopalia, but transcripts are not regularly detected in the lappets (Fig. 4b). *Aurelia actin* was detected in 36 out of 38 samples in the RT-PCR reactions containing tissue lysates including the lappets indicating that the reverse transcription was successful and that the absence of *so* transcripts in the lappet tissue was not due to a failure in the protocol (Fig. 4a). None of our negative controls yielded products, demonstrating that our samples were free of genomic DNA and contaminating templates (data not shown).

### Discussion

Previous studies of the *so* gene have concentrated on model systems and have exclusively examined and compared bilaterian taxa. Aspects of expression in sensory structures in a number of taxa suggest that this gene may have functioned in the development of sense organs in taxa basal to the Bilateria. In addition, basal placement of *so*



**Fig. 4a, b** Results of RT-PCR. **a** *Aurelia actin* expression, expected size 568 bp (*L* ladder, *J* juvenile, *E* ephyra, *M* manubrium, *R* rhopalia, *La* lappets). **b** *Aurelia so* expression, expected size 133 bp. Samples in gel **a** were run with xylene phenol dye, which appears as a dark band across the gel. Samples in gel **b** were run with bromo phenol dye, which is not visible in this portion of the gel

and the *Six*-gene family in gene trees (e.g. Agosti et al. 1996) suggest a greater antiquity of this group of genes relative to other homeodomains. On the basis of this information we recovered, via PCR, sequences of the *so* gene from a range of taxa not previously investigated, including a suite of taxa basal to the Bilateria, and conducted a preliminary expression assay of the gene via RT-PCR in the sensory tissues of the medusa phase of the jellyfish *Aurelia*.

Phylogenetic analysis (Fig. 3) confirms membership in the *Six1/2/so* subgroup of the *Six*-gene family for sequences recovered from three classes of cnidarians and three classes of sponges. These results document the presence of *so* homologues throughout the basal Metazoa. Our recovery of *so* from the bilaterian Lophotrochozoa (bivalve molluscs, *Nutricula* and *Crassostrea*, echiurid *Urechis* and flatworm *Macrostomum*) is expected given the previous recovery of *so* from bilaterians and the polychaete *Platynereis* (Arendt et al. 2002). Nevertheless, work on molecular aspects of molluscs is modest (e.g. Nederbragt et al. 2002) and such data in Echiura is extremely limited (Lee et al. 2003). Such little studied groups clearly merit more attention.

*Macrostomum* has three copies of the *so* gene, a result consistent with the polyploidy of many rhabdocoel flatworms (e.g. Gregory et al. 2000). A single copy of *D-Six4* was also recovered from *Macrostomum* (Fig. 3). This is the only non *Six1/2/so* subfamily member of the *Six*-gene family recovered in this study. The prevalence of recovery of *Six1/2/so* genes is consistent with primers designed specifically to recover this subfamily. Thus whether other *Six*-gene subfamilies are present in the basal Metazoa awaits further investigation.

The presence of *so* in sponges combined with their lack of obvious sensory structures would appear to raise as many questions as it answers. However, this conflict may be more apparent than real. Although not much is known about photoreceptors in sponges, the larvae of the haplosclerid sponge, *Reniera*, display directionality in swimming apparently controlled by large ciliated cells on

the posterior of the larva (Woollacott 1993) and analysis of the action spectrum indicates maximal sensitivity to blue light, suggesting that the photoreceptor may be a pigment which is likely to be a flavin or carotenoid (Leys et al. 2002). Furthermore, it has been observed that some sponges can contract their oscula in response to light (Reiswig 1971), and there is evidence that the hexactinellid sponge *Rhabdocalyptus* conducts electrical impulses (Leys et al. 1999). Sponges respond to stimuli by stopping feeding current (Lawn et al. 1981) suggesting that choanocytes and groups of choanocytes integrate and communicate sensory inputs.

A variety of homeobox genes have been recognized in sponges (Degnan et al. 1995; Kruse et al. 1994; Lee et al. 2003; Manuel and Le Parco 2000; Nikko et al. 2001; Richelle-Maurer and Van de Vyver 1999; Seimiya et al. 1994, 1997). Given the limited study of sponge developmental genetics to date, it is surprising that organisms that appear so simple should have such a complex and comprehensive regulatory apparatus. Evidently this morphological simplicity conceals an underlying complexity. Understanding this regulatory complexity is likely to reveal new insights into the evolution of metazoan development.

Cnidarians branch basal to bilaterians but above the sponges (Fig. 1). Considerable work has been done on the regeneration system in *Hydra* where work tends to focus on axial and head development and the expression of a range of molecular markers has been studied. Similarly there has been much focus on HOX genes and axial organization of anthozoans such as *Nematostella* where the study of development proper is more tractable (e.g. Finnerty et al. 2003). In addition there has been study of the muscle development of the medusoid phase of the hydrozoan *Podocoryne* (e.g. Spring et al. 2002). However, the genes involved in sense organ development in Cnidaria have not been well characterized, even though the medusoid life history stage of cnidarians bears the most basal well-defined sense organs on the tree of life. Phylogenetic methods do not clarify whether these features share ancestry with the sense organs of Bilateria (Jacobs and Gates 2003), thus other information is necessary to assess the evolutionary history of these structures.

We chose to initiate *so* expression analysis in *Aurelia*, a prevalent marine Scyphozoan. The life cycle of *Aurelia* consists of an alternation of sexual and asexual stages. The medusa reproduces sexually and releases fertilized eggs which develop into ciliated planulae larvae and settle on substrate. These develop into scyphistoma, or polyps. Polyps release ephyrae, the earliest swimming medusoid phase, in a process referred to as strobilation (e.g., Arai 1997). The *Aurelia* medusa has distinct sensory structures called rhopalia. The ephyrae typically bear eight rhopalia consisting of a statocyst and ocelli located between, and ventral to, a pair of flap like lappets. The eight rhopalia dominate the margin of the ephyra. As is often the case with sensory structures, the rhopalia do not increase much in size during growth. Thus the rhopalia appear small and

isolated on the margin of a larger medusa (Spangenberg 1991).

Rhopalia carry information to the nerve net through the adjacent node of a ring or eight such nodes spaced evenly around the bell (e.g., Arai 1997). Rhopalia have been shown to be important in orientation and sensing gravity (Passano 1982; Schwab 1977; Spangenberg 1968, 1991) and aid in recovery of tilting during swimming (Passano 1982; Schwab 1977). In ephyrae excision of the rhopalia prevents it from pulsing rhythmically (Spangenberg et al. 1989). Additionally, the ability to respond to on and off light stimuli corresponds to ocelli development (Yoshida and Yoshino 1980) and electrical potentials have been recorded from the vicinity of the ocelli (Irisawa et al. 1956; Yamashita 1957).

In addition to the rhopalia, the region around the mouth bearing the oral arms, referred to as the manubrium, is likely to have tactile and olfactory sensory functions. The oral arms function in reproduction, storing the gametes and planula larvae and coordinating their release. Previous observations (Jacobs and Gates 2003; and unpublished data) indicate that these structures express the pituitary-related gene *Pit1*. Given the origin of the pituitary as a sense organ (Gorbman 1995) and the association of *Six 1* expression with the pituitary (Oliver et al. 1995a, b), we anticipated the possibility of expression of *Aurelia so* in the manubrium as well as the sense organ bearing structures of the rhopalia. As a control we used the lappets located adjacent to the sense organs of the rhopalia. The lappets are not thought to be sensory, but they do contain a typical compliment of ectodermal epithelial cells including cnidae, the stinging cells of Cnidaria.

Our preliminary RT-PCR results revealed the presence of the *so* transcript in the rhopalia sensory structures, and in the manubrium of the ephyra, but expression was not detectable in the lappets. Our interpretation of these limited results is informed by previous observations of the expression of POU homeodomain genes. The *Aurelia* homolog of the vertebrate sense-organ-specific gene *Brain3* is expressed in the rhopalia, and the homolog of the vertebrate pituitary gene *Pit1* is expressed in the manubrium. Neither of these genes was observed to be expressed in the lappets (Jacobs and Gates 2003; Gates and Jacobs, unpublished results). In this context, expression in the rhopalia is consistent with a possible sensory role for *so* during the development of the statocyst and eye. Expression in the manubrium is consistent with a sensory or pituitary-like reproductive signaling role. *Six* genes have also been associated with organ development in the vertebrate gut (e.g. Oliver et al. 1995a). Thus, still other explanations for the manubrial expression of *so* are possible.

The association of expression of *so* with sensory structures in Cnidaria and the presence of this gene in sponges argues for an evolutionary history of genes and related structures antecedent to the evolution of bilaterian sensory structures. Clearly a broad range of additional

work will have to be undertaken to understand the intricacies of this evolution.

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## References

- Agosti D, Jacobs DK, DeSalle R (1996) On combining protein and nucleic acid sequences in phylogenetic analysis: the homeobox protein case. *Cladistics* 12:65–82
- Arai MN (1997) A functional biology of the Scyphozoa. Chapman and Hall, London, pp 30–78
- Arendt D, Tessmar K, de Campos-Baptista MM, Dorresteijn A, Wittbrodt J (2002) Development of pigment-cup eyes in the polychaete *Platynereis dumerilii* and evolutionary conservation of larval eyes in Bilateria. *Development* 129:1143–1154
- Boucher CA, Carey N, Edwards YH, Siciliano MJ, Johnson KJ (1996) Cloning of the human *Six1* gene and its assignment to chromosome 14. *Genomics* 33:140–142
- Bovolenta P, Mallamaci A, Puelles L, Boncinelli E (1998) Expression pattern of *cSix3*, a member of the *Six/sine oculis* family of transcription factors. *Mech Dev* 70:201–203
- Chen P, Johnson JE, Zoghbi HY, Segil N (2002) The role of *Math1* in inner ear development: uncoupling the establishment of the sensory primordium from hair cell fate determination. *Development* 129:2495–2505
- Cheyette BNR, Green PJ, Martin K, Garren H, Hartenstein V (1994) The *Drosophila sine oculis* locus encodes a homeodomain-containing protein required for the development of the entire visual system. *Neuron* 12:977–996
- Dawson MN, Jacobs DK (2001) Molecular evidence for cryptic species of *Aurelia aurita* (Cnidaria, Scyphozoa). *Biol Bull* 200:92–96
- Degnan BM, Degnan SM, Giusti A, Morse DE (1995) A *hox/hom* homeobox gene in sponges. *Gene* 155:175–177
- Dozier C, Kagoshima H, Niklaus G, Cassata G, Burglin TR (2001) The *Caenorhabditis elegans Six/sine oculis* class homeobox gene *ceh-32* is required for head morphogenesis. *Dev Biol* 236:289–303
- Finnerty JR, Pang K, Burton P, Martindale MQ (2003) The evolution of key bilaterian traits: insights into axial patterning and mesoderm formation from the sea anemone *Nematostella*, a non-bilaterian animal. *Dev Biol* 259(2):716
- Ghanbari H, Seo HC, Fjose A, Brandli AW (2001) Molecular cloning and embryonic expression of *Xenopus Six* homeobox genes. *Mech Dev* 101(1–2):271–277
- Gopfert MC, Stocker H, Robert D (2002) *Atonal* is required for exoskeletal joint formation in the *Drosophila* auditory system. *Dev Dyn* 225(1):106–110
- Gorbman A (1995) Olfactory origins and evolution of the brain-pituitary endocrine system: facts and speculation. *Gen Comp Endocrinol* 97:171–178
- Gregory TR, Hebert PDN, Kolasa J (2000) Evolutionary implications of the relationship between genome size and body size in flatworms and copepods. *Heridity* 84:201–208
- Halder G, Callerts P, Flister S, Walldorf U, Kloter U, Gehring W (1998) *Eyeless* initiates the expression of both *sine oculis* and *eyes absent* during *Drosophila* compound eye development. *Development* 125:2181–2191

- Hammond KL, Hill RE, Whitfield TT, Currie PD (2002) Isolation of three zebrafish *dachshund* homologues and their expression in sensory organs, the central nervous system and pectoral fin buds. *Mech Dev* 112(1–2):183–189
- Hassan J, Busto M, Iyengar B, Campos AR (2000) Behavioral characterization and genetic analysis of the *Drosophila melanogaster* larval response to light as revealed by a novel individual assay. *Behav Genet* 30(1):59–69
- Heath SK, Carne S, Hoyle C, Johnson KJ, Wells DJ (1997) Characterization of expression of *mDMAHP*, a homeodomain-containing gene at the murine DM locus. *Hum Mol Genet* 6:651–657
- Irisawa H, Irisawa T, Nishida Y (1956) Responses of the receptor of a medusa *Aurelia auritata* light stimuli (in Japanese). *Kagaku Nan'yo* 26:312–313
- Jacobs DK, Gates RD (2003) Developmental genes and reconstruction of Metazoan evolution—implications of evolutionary loss, limits of inference of ancestry and type 2 errors. *Integr Comp Biol* 43:11–18
- Jarman AP, Sun Y, Jan LY, Jan YN (1995) Role of the proneural gene, *atonal*, in formation of *Drosophila* chordotonal organs and photoreceptors. *Development* 121:2019–2030
- Kawakami K, Sato S, Ozaki H, Ikeda K (2000) *Six* family genes—structure and function as transcription factors and their roles in development. *Bioessays* 22:616–626
- Kirby RJ, Hamilton GM, Finnegan DJ, Johnson KJ, Jarman AP (2001) *Drosophila* homolog of the myotonic dystrophy-associated gene, *SIX5*, is required for muscle and gonad development. *Curr Biol* 11:1044–1049
- Kruse M, Mikoc A, Cetkovic H, Gamulin V, Rinkevich B, Müller IM, Müller WEG (1994) Molecular evidence for the presence of a developmental gene in the lowest animal: identification of a homeobox-like gene in the sponge *Geodia cydonium*. *Mech Ageing Dev* 77(1):43–54
- Laclef C, Hamard G, Demignon J, Souil E, Houbron C, Maire P (2003a) Altered myogenesis in *Six1*-deficient mice. *Development* 130:2239–2252
- Laclef C, Souil E, Demignon J, Maire P (2003b) Thymus, kidney and craniofacial abnormalities in *Six1* deficient mice. *Mech Dev* 120:669–679
- Lawn ID, Mackie GO, Silver G (1981) Conduction system in a sponge. *Science* 211(4487):1169–1171
- Lee SE, Gates RD, Jacobs DK (2003) Gene fishing: the use of a simple protocol to isolated multiple homeodomain classes from diverse invertebrate taxa. *J Mol Evol* 56:509–516
- Leys SP, Mackie GO, Meech RW (1999) Impulse conduction in a sponge. *J Exp Biol* 202:1139–1150
- Leys SP, Cronin TW, Degnan BM, Marshall JN (2002) Spectral sensitivity in a sponge larva. *J Comp Physiol A* 188(3):199–202
- Loosli F, Winkler S, Wittbrodt J (1999) *Six3* overexpression initiates the formation of ectopic retina. *Genes Dev* 13:649–654
- Maddison WP, Maddison DR (1999) MacClade: analysis of phylogeny and character evolution. Version 3.08. Sinauer, Sunderland, Mass.
- Manuel M, Le Parco Y (2000) Homeobox gene diversification in the calcareous sponge *Sycon raphanus*. *Mol Phylogenet Evol* 17:97–107
- McCauley DW, Bronner-Fraser M (2002) Conservation of *Pax* gene expression in ectodermal placodes of the lamprey. *Gene* 287(1–2):129–139
- Nederbragt AJ, van Loon AE, Dictus WJAG (2002) Expression of *Patella vulgata* orthologs of *engrailed* and *dpp-BMP2/3* in adjacent domains during molluscan shell development suggests a conserved compartment boundary mechanism. *Dev Biol* 246:341–355
- Nikko E, Van de Vyver G, Richelle-Maurer E (2001) Retinoic acid down-regulates the expression of *EmH-3* homeobox-containing gene in the freshwater sponge *Ephydatia muelleri*. *Mech Ageing Dev* 122:779–794
- O'Brien EK, Degnan BM (2002) Developmental expression of a class IVPOU gene in the gastropod *Haliothis asinina* supports a conserved role in sensory cell development in bilaterians. *Dev Genes Evol* 212:394–398
- Ohto H, Takizawa T, Saito T, Kobayashi M, Ikeda K, Kawakami K (1998) Tissue and developmental distribution of *Six* family gene products. *Int J Dev Biol* 42:141–148
- Oliver G, Wehr R, Jenkins N, Copeland NG, Chayette BN, Hartenstein V, Zipursky SL, Gruss P (1995a) Homeobox genes and connective tissue patterning. *Development* 121:693–705
- Oliver G, Mailhos A, Wehr R, Copeland N, Jenkins N, Gruss P (1995b) *Six3*, a murine homologue of the *sine oculis* gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. *Development* 121:4045–4055
- Passano LM (1982) Electrical conduction and behaviour in “simple” invertebrates. Scyphozoa and Cubozoa. In: Shelton GAB (ed) Oxford University Press, Oxford, pp 149–202
- Pignoni F, Hu B, Zavitz KH, Xiao J, Garrity PA, Zipursky SL (1997) The eye-specification proteins *so* and *eya* form a complex and regulate multiple steps in *Drosophila* development. *Cell* 91:881–891
- Pineda D, Gonzales J, Callerts P, Ikeo K, Gehring WJ, Salo E (2000) Searching for the prototypic eye genetic network: *sine oculis* is essential for eye regeneration in planarians. *Proc Natl Acad Sci USA* 97(9):4525–4529
- Reiswig HM (1971) In situ pumping activities of tropical Demospongiae. *Mar Biol* 9:38–50
- Richelle-Maurer E, Van de Vyver G (1999) Temporal and spatial expression of *EmH-3*, a homeobox-containing gene isolated from the freshwater sponge *Ephydatia muelleri*. *Mech Ageing Dev* 109:203–219
- Sanger F, Nicklen S, Coulson RE (1977) DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* 74:5463–5467
- Schwab WE (1977) The ontogeny of swimming behavior in the Scyphozoan, *Aurelia aurita*. 1. Electrophysiological analysis. *Biol Bull* 152:233–250
- Seimiya M, Gehring WJ (2000) The *Drosophila* homeobox gene *optix* is capable of inducing ectopic eyes by an *eyeless*-independent mechanism. *Development* 127:1879–1886
- Seimiya M, Ishiguro H, Miura K, Watanabe Y, Kurosawa Y (1994) Homeobox-containing genes in the most primitive Metazoa, the sponges. *Eur J Biochem* 221:219–225
- Seimiya M, Watanabe Y, Kurosawa Y (1997) Identification of POU-class homeobox genes in a freshwater sponge and the specific expression of these genes during differentiation. *Eur J Biochem* 243(1–2):27–31
- Seo H, Curtiss J, Mlodzik M, Fjose A (1999) *Six* class homeobox genes in *Drosophila* belong to three distinct families and are involved in head development. *Mech Dev* 83:127–139
- Serikaku MA, O'Tousa JE (1994) *sine oculis* is a homeobox gene required for *Drosophila* visual system development. *Genetics* 138:1137–1150
- Spangenberg D (1968) Statolith differentiation in *Aurelia aurita*. *J Exp Zool* 178:183–194
- Spangenberg D (1991) Rhopalium development in *Aurelia aurita* ephyrae. *Arobiologia* 216/217:45–49
- Spangenberg D, Phillips R, Kostas A (1989) Effect of gravireceptor excision in *Aurelia* ephyra swimming/pulsing behavior at 1 G and during flight. *Am Soc Grav Space Biol* 2:24
- Spring J, Yanze N, Jösch C, Middel AM, Winninger B, Schmid V (2002) Conservation of Brachyury, Mef2, and Snail in the myogenic lineage of jellyfish: a connection to the mesoderm of Bilateria. *Dev Biol* 244 (2):372–384
- Sun H, Rodin A, Zhou Y, Dickinson DP, Harper DE, Hewett-Emmett D, Li WH (1997) Evolution of paired domains: isolation and sequencing of jellyfish and hydra *Pax* genes related to *Pax-5* and *Pax-6*. *Proc Natl Acad Sci USA* 94:5156–5161

- Swafford DL (1998) PAUP\*. Phylogenetic analysis using parsimony (\*and other methods). Version 4. Sinauer, Sunderland, Mass.
- Tomarev SI, Callaerts P, Kos L, Zinovieva R, Halder G, Gehring W, Piatigorsky J (1997) Squid Pax-6 and eye development. Proc Natl Acad Sci USA 94:2421–2426
- Toy J, Yang J-M, Leppert GS, Sundin O (1998) The *Optx2* homeobox gene is expressed in early precursors of the eye and activates retina-specific genes. Proc Natl Acad Sci USA 95:10643–10648
- Wilson K (1987) Current protocols in molecular biology. Wiley, New York
- Woollacott RM (1993) Structure and swimming behavior of the larva of *Haliclona tubifera* (Porifera: Domospongiae). J Morphol 218:301–321
- Yamashita T (1957) Das Aktionspotential der Sinneskörper (Randkörper) der Meduse *Aurelia aurita*. Z Biol 109:116–122
- Yoshida M, Yoshino Y (1980) Differentiation of ocelli in ephyrae of *Aurelia aurita*. In: Tardent P, Tardent T (eds) Developmental and cellular biology of Coelenterates. Elsevier/North Holland, Amsterdam, pp 343–346
- Zheng W, Huang L, Wei Z, Silviu D, Tang B, Xu P (2003) The role of *Six1* in mammalian auditory system development. Development 130:3989–4000