IDENTIFICATION OF HOMOLOGUES OF THE *EMH-3* HOMEOBOX-CONTAINING GENE IN DEMOSPONGES

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ABSTRACT

In the present study, we have identified and characterized homologues of the homeobox-containing gene *EmH-3* from *Ephydatia muelleri* in three additional freshwater sponges: *Eunapius fragilis*, *Spongilla lacustris* and *Trochospongilla horrida*. They were designated *EmH-3Efr, EmH-3Sl* and *EmH-3Th*. They exhibited a great conservation of genomic and amino acid sequences, exon/intron structure and expression patterns with the *EmH-3* gene, which strongly suggested that they have the same function in sponge development. Phylogenetic analyses have shown a grouping of *E. muelleri* and *E. fluviatilis*, *E. fragilis* and *S. lacustris*, *T. horrida* being outside both groups. They also showed that *EmH-3* and its homologues were most closely related to the Tlx/Hox11 family of higher metazoans. They are probably representatives of this family as they display the family-specific signature and, as are the members of this family, are involved in cell multiplication and differentiation.

KEY WORDS

Demosponges, homeobox-containing genes, sequencing, expression, phylogeny.

INTRODUCTION

In recent years, efforts have been made to understand the molecular mechanisms underlying animal development in relation to different metazoans' body plans and developmental features. It has been shown that a family of homologue genes, the homeobox-containing genes play a key role in morphogenesis, particularly in the control of spatio-temporal organization, cell fate decisions and differentiation (MEYER, 1996; FINNERTY, 1998; GELLON & MCGINNIS, 1998; MARTINEZ *et al.,* 1999; GALLIOT & MILLER, 2000; MILLER & BALL, 2000; MORRIS, 2000). In this context the study of the structure and function of these genes in basal metazoans, *e.g.* Porifera has appeared to be fundamental to understanding the evolution of these genes throughout the animal kingdom (MARTINDALE & KOURAKIS, 1999; GAUCHAT *et al.,* 2000; MANUEL & LE PARCO, 2000; FINNERTY, 2001). Their study is particularly crucial as sponges and higher metazoan phyla share different metabolic pathways, structural elements and molecules suggesting that the basis of pluricellularity and the general regulation mechanisms have been established before the divergence of sponges and eumetazoans (EXPOSITO & GARRONE, 1990; DEGNAN *et al.,* 1993; MÜLLER & MÜLLER, 1999; BORCHIELLINI *et al.,* 2000; MÜLLER, 2001). In this context, we have investigated the expression of different homeobox-containing genes in freshwater sponges and determined the role the *EmH-3* gene plays in their morphogenesis. This gene, isolated from the genomic

library of the freshwater sponge *Ephydatia muelleri* (RICHELLE-MAURER *et al.,* 1998) presents a high similarity to *EfH-1/prox2* (98 %) isolated from the freshwater sponge *Ephydatia fluviatilis* (COUTINHO *et al.,* 1994; SEIMIYA *et al.,* 1994) and *Spox TA1* (96 %) isolated from the marine sponge *Tethya aurantium* (DEGNAN *et al.,* 1995). It is probably a representative of the bilaterian non-Hox Tlx/Hox11family (COUTINHO *et al.,* 1998; RICHELLE-MAURER *et al.,* 1998; GAUCHAT *et al.,* 2000). Expression analyses have shown that *EmH-3* expression is time and cell-specific. The levels of *EmH-3* expression increase from almost undetectable levels in resting gemmules to high levels at the moment of hatching and throughout the sponge's life (RICHELLE-MAURER *et al.,* 1998; RICHELLE-MAURER & VAN DE VYVER, 1999a). *EmH-3* is strongly expressed in the undifferentiated and pluripotent archaeocytes whereas in choanocytes or pinacocytes its expression is very weak, similar to that found in the resting gemmules (RICHELLE-MAURER & VAN DE VYVER, 1999b). In contrast, other genes such as the *E. fluviatilis* homeobox-containing gene *prox1* and the *E. muelleri* actin gene are expressed at almost the same level at all stages of development and in all cell fractions (RICHELLE-MAURER & VAN DE VYVER, 1999b). Regarding the function of *EmH-3*, the use of inhibitors of sponge development, such as retinoic acid, have shown that *EmH-3* is necessary for the differentiation of archaeocytes into choanocytes and hence for the completion of a functional sponge (NIKKO *et al.,* 2001). It is worth noting that the *EmH-3* promoter is functionally active in mammalian cells indicating that the regulatory programmes in Porifera are similar to those found in higher metazoans (COUTINHO *et al.,* 1998).

In the present work homologues of the *EmH-3* gene have been identified and characterized in three aditional freshwater sponge species, *Spongilla lacustris*, *Eunapius fragilis*, and *Trochospongilla horrida*, by PCR and RT-PCR with specific primers and sequencing.

MATERIALS AND METHODS

Sponge culture

Sponges were grown in laboratory conditions at 20° C, in Petri dishes containing mineral medium, from pure stocks of gemmules as described before (RICHELLE-MAURER *et al.,* 1998). Five species of freshwater sponges were used: *Ephydatia muelleri*, *Ephydatia fluviatilis*, *Eunapius fragilis, Spongilla lacustris*, and *Trochospongilla horrida.*

DNA extraction and PCR conditions

Genomic DNA was extracted from gemmules using DNAzol reagent (Invitrogen) following the manufacturer's instructions. The gemmules were ground in a Potter homogeniser in the presence of DNAzol, the empty shells were discarded and the homogenates centrifuged at 10000 x g for 10 min prior to DNA precipitation with ethanol. The quantity and quality of DNA were evaluated respectively by spectrometry and on a 0.7 % agarose gel.

PCR amplifications were performed in 50 µl using 45 µl of PCR SuperMix (Invitrogen), 400 nM primers and 10 ng DNA template. The typical reaction profile was 35 cycles (2 min 94° C, 1 min 55° C, 1 min 30 s 72° C) preceded by 1 min at 94° C and followed by 10 min at 72° C (Techne Unit Genius). However, in the case of *S. lacustris* and *T. horrida*, the annealing temperature had to be lowered from 55° C to 45° C to get amplification. The primers used were specific of the *EmH-3* gene, lying at the beginning of the first exon and in the homeobox (Fig. 2). About 10 % of the PCR products were electrophoresed on a 1.5 %

agarose gel. *E. muelleri* and *E. fluviatilis* were used as controls: their amplified PCR products were sequenced and compared to the published *EmH-3* and *prox2* sequences (RICHELLE-MAURER *et al.,* 1998, SEIMIYA *et al.,* 1994).

The sequences reported in this study have been deposited in the GenBank database under the following accession numbers AY300029 (*EmH-3Efr*), AY300030 (*EmH-3Sl*) and AY300031 (*EmH-3Th*).

RNA extraction and RT-PCR conditions

The expression of *EmH-3* homologue genes was analysed by RT-PCR using the same set of primers as those used for the PCR experiments. Total RNA extraction and PCR experiments were carried out as described elsewhere (RICHELLE-MAURER *et al.,* 1998, RICHELLE-MAURER & VAN DE VYVER, 1999a,b). Briefly total RNA was extracted at different stages of development from gemmules to the completion of functional sponges, using TRIzol reagent (Invitrogen) and was amplified using the Promega Access RT-PCR one tube System. The cycling conditions were those described in the manufacturer's protocol using 10 ng template and an annealing temperature of 55° C. Expressions patterns were compared to those obtained with *EmH-3* in *E. muelleri* and with the *EmA1* actin housekeeping gene in the different sponge species. Ten percent of the RT-PCR products were analysed on a 1.5 % agarose gel. Quantitative evaluation of the bands was made by using program ImageMaster ID Elite Version 3.0 for gel analysis (Amersham Pharmacia Biotech). The results are presented in the form of histograms.

Sequencing and Phylogenetic analysis

The (RT)-PCR products of the expected size were either directly purified on QIAquick columns (QIAGEN) or extracted from the agarose gel and purified on QIAquick extraction kit columns (QIAGEN). Sequencing was performed according to Sanger's method (SANGER *et al.,* 1977). PCR fragments were sequenced on both strands using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit, version 2.0 (Applied Biosystems 2000). Sequence chromatograph data were analysed and edited with the Sequencing analysis Program (ABI). The sequences of each strand were aligned with the Seq Pup program (GILBERT, 1996).

Multiple sequence alignments of nucleotide (nt) and amino acid (aa) sequences were constructed with the ClustalX program (THOMPSON *et al.,* 1997). They were optimized by testing various gap weights by the Soap Program (LÖYTYNOJA & MILINKOVITCH, 2001). The Paup software (SWOFFORD, 1998) was used for distance computation, Neighbour-joining (NJ) and Maximum Parsimony (MP) tree-buildings, and bootstrapping. The reliability of the nodes was assessed by using 500 bootstrap replicates (PAGE & HOLMES, 1998). Searches for related sequences in higher metazoans were performed in the GenBank databases by BLAST.

RESULTS

Gene structure and sequencing

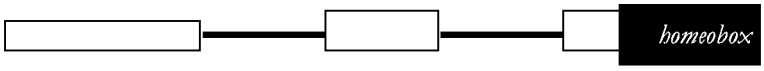
Amplified PCR products within the expected range size were recovered from *Spongilla lacustris*, *Eunapius fragilis* and *Trochospongilla horrida* with the *EmH-3* specific primers. Their size varied according to the species, from 736 bp (*E. fragilis*) to 905 bp (*T. horrida*) (Tab. I). The amplified products from *Ephydatia muelleri* and *Ephydatia fluviatilis* had the estimated size as deduced from the published sequences of *EmH-3* (RICHELLE-MAURER *et al.,* 1998) and *prox2* genes (SEIMIYA *et al.,* 1994) *i.e.* 753 bp and 738 bp respectively.

On the other hand all transcripts had the same size (444 bp) as *EmH-3* transcripts except *E. fragilis* (441 bp) (Tab. I). They were 54 bp (55 bp) longer than *E. fluviatilis* transcripts.

Tab. I. Size (bp) of the amplified PCR and RT-PCR products

	E. fragilis	*S. lacustris*	*T. horrida*	*E. muelleri*	*E. fluviatilis*
PCR	736	804	905	753	738
RT-PCR	441	444	444	444	390

The alignment of the genomic and cDNA sequences allowed the exons-introns boundaries to be distinguished. The genomic sequences presented the same structural organization in the three species, *i.e.* three exons separated by two introns, the homeobox lying in the third exon (Fig. 1). The exons had similar lengths in the three species, (Figs 1, 2) but the length of the introns varied, particularly the second one, from 232 bp in *E. fragilis* to 414 bp in *T. horrida*, accounting for the difference of length of the PCR products. Splicing sites were identical to those found in *E. muelleri* but differed from those found in *E. fluviatilis*, the first exon being 54 bp shorter in the latter species (Figs 1, 2). It has to be noted that the first codon of the third exon is formed by the last nucleotide of exon 2 and the two first nucleotides of exon 3.



	Exon 1		Intron 1	Exon 2	Intron 2	Exon 3	
Efr	**189 bp**	**63 aa**	63 bp		232 bp		
Sl	**192 bp**	**64 aa**	54 bp	**37 bp** **12 aa**	306 bp	**53 bp** **18 aa**	**162 bp** **54 aa**
Th	**192 bp**	**64 aa**	47 bp		414 bp		
Em	**192 bp**	**64 aa**	52 bp		257 bp		
Efl	**138 bp**	**46 aa**	83 bp		265 bp		

Fig. 1. Schematic representation of the exon-intron structure of the genomic sequences obtained from *Eunapius fragilis (Efr)*, *Spongilla lacustris (Sl)*, *Trochospongilla horrida (Th)* and from *Ephydatia muelleri (Em)* and *Ephydatia fluviatilis (Efl)* for comparison.

The genomic sequences obtained from *E. fragilis*, *S. lacustris*, and *T. horrida* displayed a high degree of identity with the *E. muelleri* sequence (Fig. 2). The identity was not restricted to the homeobox but extended to the three exons (Tab. II). Moreover, two thirds of the nucleotide substitutions were located at the level of the degenerated base of the codon and consequently had no influence on the deduced amino acid sequence. Identity was much lower in the untranslated regions (Tab. II).

It is worth noting that the genomic sequences obtained from *E. muelleri* and *E. fluviatilis* were identical to the published sequences from these species, respectively, *EmH-3* (RICHELLE-MAURER *et al.,* 1998) and *prox2* (SEIMIYA *et al.,* 1994) except that in the latter species two substitutions were observed at positions 194 and 487 in the introns (Fig. 2, highlighted).

Tab. II. Percentage identity between the nucleotide (nt) and deduced amino acid (aa) sequences from *Ephydatia muelleri* (*EmH-3)* and from *Eunapius fragilis*, *Spongilla lacustris* and *Trochospongilla horrida.*

	E. muelleri (*EmH-3*)		
	nt sequences Homeobox (exons)	**aa sequences** Homeodomain (exons)	Introns
E. fragilis	87.0 (84.9)	100.0 (87.6)	57.7
S. lacustris	87.0 (86.5)	98.2 (84.7)	52.7
T. horrida	91.4 (87.0)	100.0 (87.8)	56.0

The deduced amino acid sequences, presented in Fig. 3, showed a very high identity with the *EmH-3* sequence, up to 100 % in the homeodomain for *E. fragilis* and *T. horrida* (Tab. II). Identity remained very high outside the homeodomain (87.8 %), particularly at the level of the third exon (Fig. 3). Most of the differences were located in the last 18 amino acids of the first exon corresponding to the region, which was shorter in *E. fluviatilis*. Identity with *EmH-3* in this region dropped to 52.9 % (*S. lacustris*). All the homeodomains possessed 6 of the 7 conserved residues (R_1, R_{10}, K_{24}, S_{28}, K_{39}, and T_{47}) of the bilaterian Tlx/Hox11 family-specific signature (GAUCHAT *et al.,* 2000).

Expression analysis

Expression patterns were very similar in *E. fragilis*, *S. lacustris*, and *T. horrida* (only *E. fragilis* is shown in Fig. 4) and resembled those found in *E. muelleri*. When the *EmH-3* specific primers were used, transcripts were hardly discernible in the resting gemmules (Fig. 4a) but their level increased drastically at hatching and this high level was maintained in the functional sponges. On the contrary, with the *EmA1* actin primers, the levels of transcripts were almost the same in the gemmules and during sponge development (Fig. 4b).

Phylogenetic analyses

The Neighbour-joining (NJ) and Maximum Parsimony (MP) trees based on the genomic sequences and rooted with *Spox TA1* from the marine sponge *T. aurantium* presented the same topology: a grouping of *E. muelleri* and *E. fluviatilis* on one hand, of *S. lacustris* and *E. fragilis* on the other hand, with *T. horrida* being outside both groups (Fig. 5). Such grouping was supported by high bootstrap values. However, the position of *T. horrida* to the other species varied according to the method used. In the NJ tree, *T. horrida* was related to the *Sl-Efr* cluster (59 %) while in the MP tree this species was found to be related the *Em-Efl* cluster but this was weakly supported by a much lower bootstrap value (46 %, not shown).

Fig. 2. Sequences of PCR amplified products from *E. muelleri* (*Em), E. fragilis* (*Efr), S. lacustris* (*Sl*), *T. horrida (Th)* and *E. fluviatilis* (*Efl).* Sequences are aligned to *E. muelleri* (*Em)* allowing deletions/insertions (using the ClustalX software). The exons are in bold and the introns in italic; the homeobox region is boxed; nucleotide identities are indicated by hyphens; gaps by =; non-sequenced regions by dots, the primers used for (RT)-PCR are underlined; differences from the published *prox2* sequence (SEIMIYA *et al.,* 1994) are highlighted.

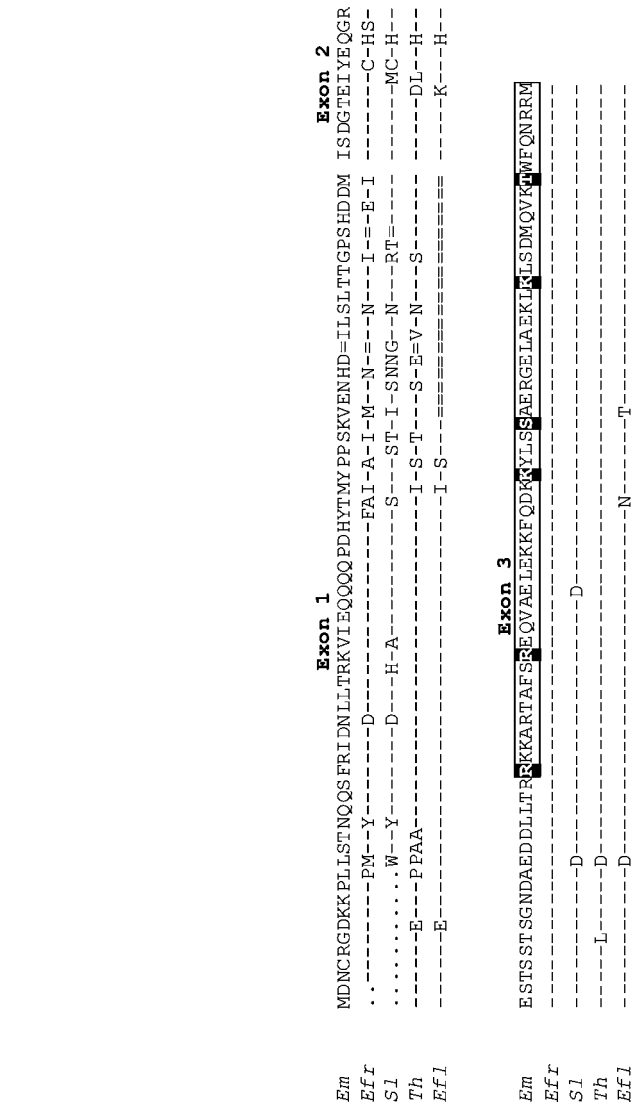


Fig. 3. Deduced amino acid sequences from *Ephydatia muelleri (Em)*, *Eunapius fragilis* (*Efr), Spongilla lacustris (Sl), Trochospongilla horrida (Th)* and *Ephydatia fluviatilis (Efl)*. Sequences are aligned to *E. muelleri (Em)* allowing deletions/insertions (using the ClustalX software). aa identities are indicated by hyphens, gaps by =; the homeodomain is boxed; the residues of the Tlx/Hox11 family-specific signature are highlighted (inverted colours); the boundaries between the tree exons are shown by a space.

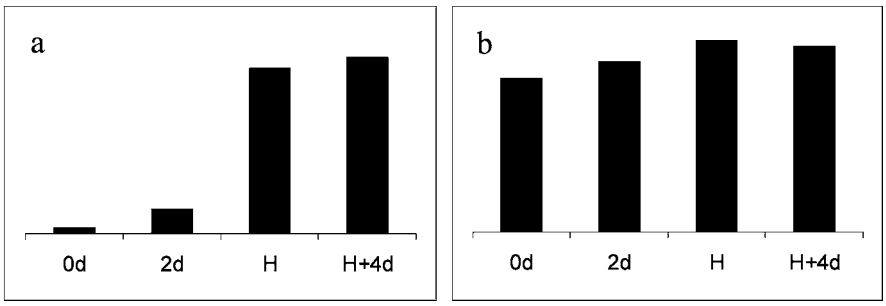


Fig. 4. Expression patterns in the course of development. Levels of the transcripts (pixel intensities in arbitrary units) in the three species (only *Eunapius fragilis* is shown as example) with the specific primers for *EmH-3* **(a)** and *EmA1* **(b)** at different stages of development: resting gemmules (0d); 2d incubation, hatching (H) and in functional sponges (H+4d). Incubation times are expressed in days (d) at 20° C in mineral medium.

Fig. 5. Rooted Neighbour-joining tree computed from the genomic sequences from *Ephydatia muelleri (Em)*, *Ephydatia fluviatilis (Efl), Eunapius fragilis* (*Efr), Spongilla lacustris (Sl)* and *Trochospongilla horrida (Th),* with Spox TA1 from the marine sponge *Tethya aurantium* (*Ta)* as an outgroup. The bootstrap percentages (500 replicates) are shown at the nodes.

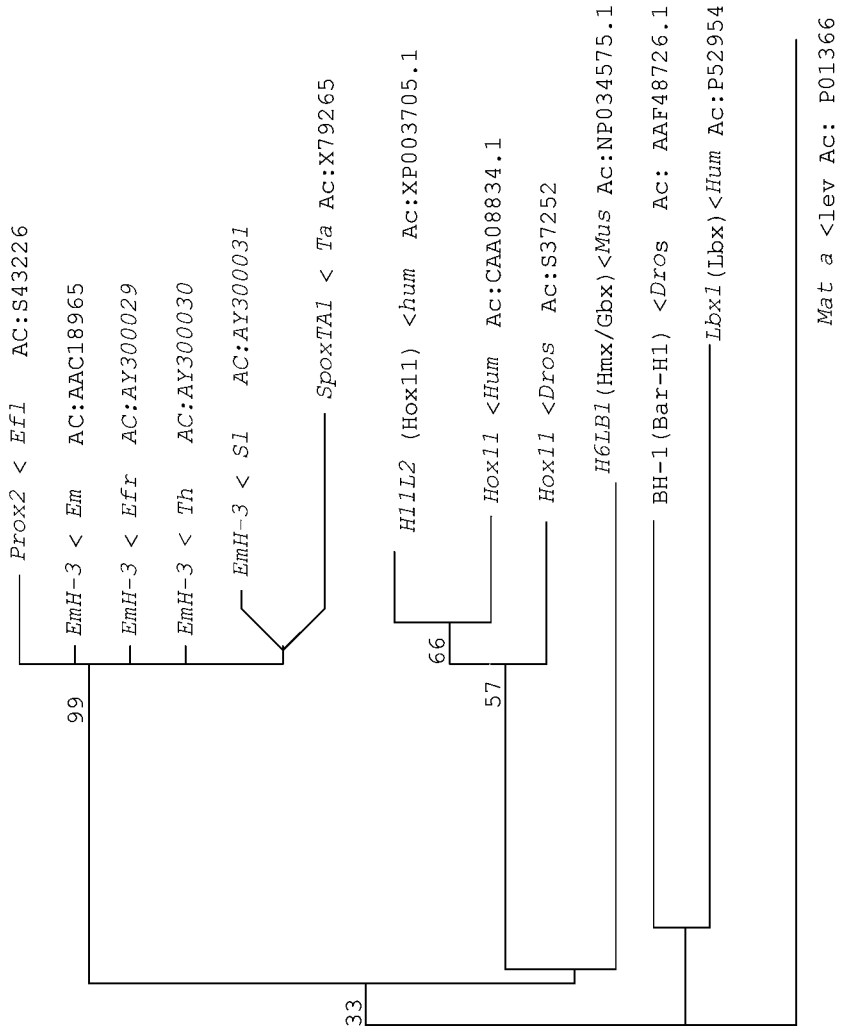


Fig. 6. Phylogenetic relationships between sponge homeodomains and the most closely related bilaterian homeodomains inferred by Neighbour-joining. The tree was computed from the alignment of the full-length homeodomain and was rooted with the divergent *Mat a* yeast homeodomain. The family to which the genes belong are indicated between parentheses, followed by the organisms from which the sequences were identified and the accession number in the protein databank. Porifera *Efl: Ephydatia fluviatilis*, *Efr: Eunapius fragilis, Em: Ephydatia muelleri, Sl: Spongilla lacustris, Ta: Tethya aurantium, Th: Trochospongilla horrida;* Arthropods *Dm: Drosophila melanogaster* (fly); Vertebrates *Gg: Gallus gallus* (Chick), *Hs: Homo sapiens*, *Mm: Mus musculus*; Yeast: lev: *Saccharomyces cerevisiae.* Numbers at the nodes indicate percentages of 500 bootstrap replicates; values under 50 % are omitted, except for significantly important nodes.

The evolutionary relationships between the homeodomains of freshwater sponges and bilaterian animals were assessed by homology searches by Blast in the GenBank database. They revealed that the freshwater sponge homeodomains displayed similarities with those of members of the non-Hox families, Tlx, Hox11, Gbx, Barx, and Lbx of higher metazoans. In particular, up to 77 % identity was found with members of the Tlx/Hox11 family. The NJ tree constructed from these homeodomain sequences showed that the sponge homeodomains were most closely related with members of the Tlx/Hox11 and Gbx families (Fig. 6) although this relationship was not well supported, the bootstrap values being less than 50 %.

DISCUSSION AND CONCLUSIONS

The genes identified and characterized in *Eunapius fragilis*, *Spongilla lacustris* and *Trochospongilla horrida* by PCR with the *EmH-3* specific primers can be considered as true homologues of the *EmH-3* homeobox-containing gene from *Ephydatia muelleri.* Indeed, they satisfy the criterions for homology *i.e.* a high identity of sequence, the same gene structure and expressions patterns (SCHIERWATER & KUHN, 1998). They were designated *EmH-3Efr, EmH-3Sl* and *EmH-3Th.* Sequence identity is not restricted to the conserved homeobox but extends outside the homeobox to the third exon and to a lesser extent to the other translated regions contrary to what is observed in other organisms where little or no similarity is found outside the homeobox (SCHIERWATER & KUHN, 1998). Identity is much lower in the untranslated regions, which showed also a greater variability in length. The three genes possess the same fundamental 3 exons-2 introns structure as the *EmH-3* gene. The position of the splicing sites confirmed those assumed in *EmH-3* (RICHELLE-MAURER *et al.,* 1998). Interestingly, of the five freshwater sponge species studied, only *Ephydatia fluviatilis* displayed an alternative splicing site, which resulted in a difference of length of the first exon.

As to their expression, *EmH-3Efr, EmH-3Sl* and *EmH-3Th* are differentially expressed during sponge development, from almost undetectable levels in gemmules to high levels at hatching and in functional sponges, which strongly suggests a role in cell multiplication and differentiation during sponge development as demonstrated for *EmH-3* (NIKKO *et al.,* 2001).

The Neighbour-joining tree rooted with *T. aurantium*, showed a grouping of *E. muelleri* with *E. fluviatilis*, and *E. fragilis* with *S. lacustris*, *T. horrida* being outside both groups. These are in accordance with morphological data (BERGQUIST, 1978), southern blot analyses (RICHELLE-MAURER *et al.,* 1996) and 18S rDNA phylogenetic trees (ITSKOVITCH *et al.,* 1999; BORCHIELLINI *et al.,* 2000). However, the position of *T. horrida* remained to be ascertained as NJ and MP trees gave a different relationship of this species to the other species.

Phylogenetic analyses showed that sponge homeodomains were found to be most closely related to the non-Hox Tlx/Hox11 family in agreement with previous research (COUTINHO *et al.,* 1998; RICHELLE-MAURER *et al.,* 1998; GAUCHAT *et al.,* 2000; MANUEL, 2001; COUTINHO *et al.,* 2003). Although this relationship is only weakly supported by low bootstrap values, *EmH-3, EmH-3Efr, EmH-3Sl* and *EmH-3Th* are probably representatives of the Tlx/Hox11 family. In fact, they display the family-specific signature and as determined for *EmH-3* they are involved in cell

multiplication and differentiation like the members of the Tlx/Hox11 family (KENNEDY *et al.,* 1991; LU *et al.,* 1991)

It is interesting to note that homeodomains from different taxonomic groups of animals are found in this family. This corresponds to the evolutionary scenario of homeodomains suggesting an ancient origin of the non-Hox families, the Hox/para Hox families being considered as more recent (KAPPEN *et al.,* 1993; VALENTINE *et al.,* 1996; BROOKE *et al.,* 1998; GAUCHAT *et al.,* 2000).

Work is in progress to identify *EmH-3* homologues in marine sponges and to determine if, in these sponges, the same conservation of sequence and structure is observed as in freshwater sponges. As mentioned before the presence of an *EmH-3*-like gene in a primitive marine sponge *T. aurantium* (DEGNAN *et al.,* 1995) suggests that the *EmH-3* gene may be widespread among Porifera and may constitute one of the metazoan stem homeobox-containing genes.

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