J. Mar. Biol. Ass. U.K. (2005), **85**, 1519–1524
Printed in the United Kingdom

Nerve cells of *Xenoturbella bocki* (phylum uncertain) and *Harrimania kupfferi* (Enteropneusta) are positively immunoreactive to antibodies raised against echinoderm neuropeptides

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The phylogenetic position of *Xenoturbella* spp. has been uncertain since their discovery in 1949. It has been recently suggested that they could be related to Ambulacraria within Deuterostomia. Ambulacraria is a taxon that has been suggested to consist of Hemichordata and Echinodermata. The hypothesis that *X. bocki* was related to Ambulacraria as well as the hypothesis of a monophyletic Ambulacraria is primarily based on the analysis of DNA sequence data. We tested both phylogenetic hypotheses using antibodies raised against SALMFamide 1 and 2 (S1, S2), neuropeptides isolated from echinoderms, on *X. bocki* and the enteropneust *Harrimania kupfferi*. Both species showed distinct positive immunoreactivity against S1 and S2. This finding supports the Ambulacraria-hypothesis and suggests a close phylogenetic relationship of *X. bocki* to Ambulacraria. In particular, the presence of immunoreactivity against S2 can be interpreted as a synapomorphy of Enteropneusta, Echinodermata, and *Xenoturbella* spp.

INTRODUCTION

Ciliated worms of the genus *Xenoturbella* have puzzled researchers interested in the phylogeny of animals since they were discovered on the Swedish west coast and described by Westblad in 1949. With their simple 'Bauplan' these animals have been variously classified by morphologists as primitive flatworms (Westblad, 1949), acoelomorph flatworms (Lundin, 1998), as the sister group to the rest of the Bilateria (Ehlers & Sopott-Ehlers, 1997), as derived holothurians or relatives of enteropneust worms (Reisinger, 1960), or as extremely modified bivalves (Israelsson, 1997). Molecular systematists originally supported the latter hypothesis, suggesting that *Xenoturbella* is a relative of nuculid bivalves (Noren & Jondelius, 1997). Recently, new molecular analyses served as evidence that *Xenoturbella* was a deuterostome animal possibly related to Ambulacraria and that the originally reported sequences similar to bivalves stemmed from contamination, possibly from prey eaten by *Xenoturbella* (Bourlat et al., 2003). However, the exact phylogenetic position of *Xenuturbella* in the recent analysis of molecular data depended on analysis method and choice of outgroup (Bourlat et al., 2003 supplementary information). Given the difficulties with DNA contamination and the still uncertain phylogenetic position of these enigmatic animals, immunocytological evidence regarding specific peptides can contribute to solve these problems.

SALMFamides are two structurally related neuropeptides isolated from echinoderms (Elphick et al., 1991a,b). SALMFamide 1 (S1) is an octapeptide (Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH_2) and SALMFamide 2 (S2) is a dodecapeptide (Ser-Gly-Pro-Tyr-Ser-Phe-Asn-Ser-Gly-Leu-Thr-Phe-NH_2). The widespread occurrence of these neuropeptides in the nervous systems of various developmental stages of echinoderms suggests important and diverse physiological roles for these substances (see Thorndyke & Candia-Carnevali, 2001). S1-like immunoreactivity has been found in diverse taxa (Echinodermata, Brachiopoda, Nemertini, Annelida, Crustacea, Platyhelminthes; see Table 1) and the early evolutionary occurrence of this character seems likely. Interestingly, it has not been found in chordate taxa, despite attempts with several methods in ascidians (Elphick, 1991a) and a basal craniate (*Myxine glutinosa*, M. Thorndyke, unpublished data). However, since it has also been suggested that *Xenoturbella* spp. could comprise the sister taxon to Bilateria (Ehlers & Sopott-Ehlers, 1997), it is still of phylogenetic interest to know whether S1-like immunoreactivity is present or absent in this taxon. On the other hand, S2-like immunoreactivity is presently known only from echinoderms (see Table 1) and may therefore be an informative character in phylogenetic argumentation. In combination, the presence or absence of the immunoreactivity against the two neuropeptides in *Xenoturbella bocki* and the enteropneust worm *Harrimania kupfferi* could therefore be used to test the phylogenetic hypothesis of a close interrelationship

Table 1. *Distribution of immunoreactivity against antibodies raised against two neuropeptides, SALMFamide 1 (S1) and SALMFamide 2 (S2), isolated from echinoderms.*

Species	Higher taxon	S1	S2	References
Ciona intestinalis	Chordata	−	−	Elphick, M.R., 1991. PhD thesis, University of London, UK
Antedon mediterranea	Echinodermata	+	+	Bonasoro et al., 1995. In *Echinoderms*, pp. 237–243. Rotterdam: Balkema
Gorgonocephalus caputmedusae	Echinodermata	+	+	Dupont et al., unpublished
Ophiocomina nigra	Echinodermata	?	+	Dupont et al., unpublished
Ophiothrix fragilis	Echinodermata	?	+	Dupont et al., unpublished
Ophiura ophiura	Echinodermata	?	+	Dupont et al., unpublished
Amphiura chiajei	Echinodermata	?	+	Dupont et al., unpublished
Asterias rubens	Echinodermata	+	+	*Journal of Experimental Biology*, **198**, 2519–2525
Asterina pectinifera	Echinodermata	+	(?)	*Zoological Science*, **21**, 299–303
Pisaster ochraceus	Echinodermata	+	(?)	*Journal of the Marine Biological Association of the United Kingdom*, **74**, 61–71
Patiriella regularis	Echinodermata	+	(?)	*Journal of Comparative Neurology*, **451**, 101–114
Amphipholis squamata	Echinodermata	+	+	*Proceedings of the Royal Society*, B, **264**, 667–674
Ophiactis resiliens	Echinodermata	+	(?)	*Invertebrate Biology*, **122**, 177–185
Ophiura ophiura	Echinodermata	+	(?)	*Philosophical Transactions of the Royal Society*, B, **346**, 433–444
Dendraster excentricus	Echinodermata	+	(?)	*Acta Zoologica*, **73**, 207–212
Psammechinus miliaris	Echinodermata	+	+	*Biological Bulletin, Marine Biological Laboratory, Woods Hole*, **200**, 268–280
Xenoturbella bocki	incertae sedis	+	+	Present study
Harrimania kupffferi	Enteropneusta	+	+	Present study
Argyrotheca cordata	Brachiopoda	+	(?)	*Journal of Submicroscopic Cytology and Pathology*, **27**, 543–556
Argyrotheca cuneata	Brachiopoda	+	(?)	*Journal of Submicroscopic Cytology and Pathology*, **27**, 543–556
Cerebratulus sp.	Nemertini	+	−	Dupont et al., unpublished
Glycera alba	Annelida	+	−	Dupont et al., unpublished
Melinna cristata	Annelida	+	−	Dupont et al., unpublished
Meganyctiphanes norvegica	Crustacea	+	−	Dupont et al., unpublished
Ascaris suum	Nematoda	+	(?)	*Acta Biologica Hungarica*, **46**, 205–209
Cystidicola farionis	Nematoda	+	(?)	*Acta Biologica Hungarica*, **44**, 133–136
Diclidophora merlangi	Platyhelminthes	+	(?)	*Acta Biologica Hungarica*, **46**, 205–209
Diphyllobothrium dendriticum	Platyhelminthes	+	(?)	*Acta Academia Aboensis*, **50**, 45–52
Grillotia erinaceus	Platyhelminthes	+	(?)	*Acta Biologica Hungarica*, **46**, 205–209
Sanguinilicola inermis	Platyhelminthes	+	−	*Journal of Helminthology*, **70**, 309–317
Schistosoma mansoni	Platyhelminthes	+	(?)	*Parasitology*, **110**, 143–153
Stenostomum leucops	Platyhelminthes	+	(?)	*Acta Academia Aboensis*, **50**, 45–52
Pennatula phosphorea	Cnidaria	−	−	Dupont et al., unpublished

+, immunoreactivity detected; −, no immunoreactivity detected; ?, not tested; (?), not reported whether tested.

of *X. bocki* to Ambulacraria and the Ambulacraria-hypothesis itself, both of which had been derived from gene sequence data.

MATERIALS AND METHODS

Sampling

Xenoturbella bocki was isolated from muddy sediment collected with a Waren sledge in the vicinity of Kristineberg Marine Research Station at the mouth of Gullmarsfjord on the west coast of Sweden. Sediment was sieved through a 1 mm sieve and the retained material spread out into several bowls. Individuals of *X. bocki* were picked out of these bowls over the course of 12 h after sieving. *Harrimania kupffferi* was dredged off Göteborg on the west coast of Sweden during the cruise of the RV 'Skagerak' in March 2003 and fixed on board.

Immunohistochemistry

Individuals were fixed in 4% paraformaldehyde in filtered (0.45 μm) seawater for 4 h at room temperature, then subsequently dehydrated in a series of ethanol and embedded in paraffin. Sections (6 μm) were mounted on poly-L-lysine coated slides, dewaxed, and re-hydrated to phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked by a 15 min rinse in 2% H_2O_2 in PBS. Sections were immersed for 1 h in blocking buffer (PBS and 5% normal goat serum) before incubation with primary antisera diluted in PBS (1:2000 for S1 and S2) for 2 h at room temperature. Polyclonal antibodies were raised in rabbits and have a high affinity for either S1 (Elphick et al., 1991a,b) or S2 (Newman et al., 1995), with negligible cross-reactivity.

After three washes in PBS the sections were incubated for 1 h in biotinylated goat anti-rabbit antiserum 1:200 in PBS and processed using the ABC reagent (Vector Laboratories), according to the manufacturer's instruction. The immunolabelling was visualized with 0.04% diaminobenzidine (DAB) and 0.01% hydrogen peroxide in Tris–HCl at pH 7.6; development time was 5–9 min. Controls were carried out by preabsorption of primary antisera at working dilution (1:2000) or by replacing the primary antibodies with PBS.

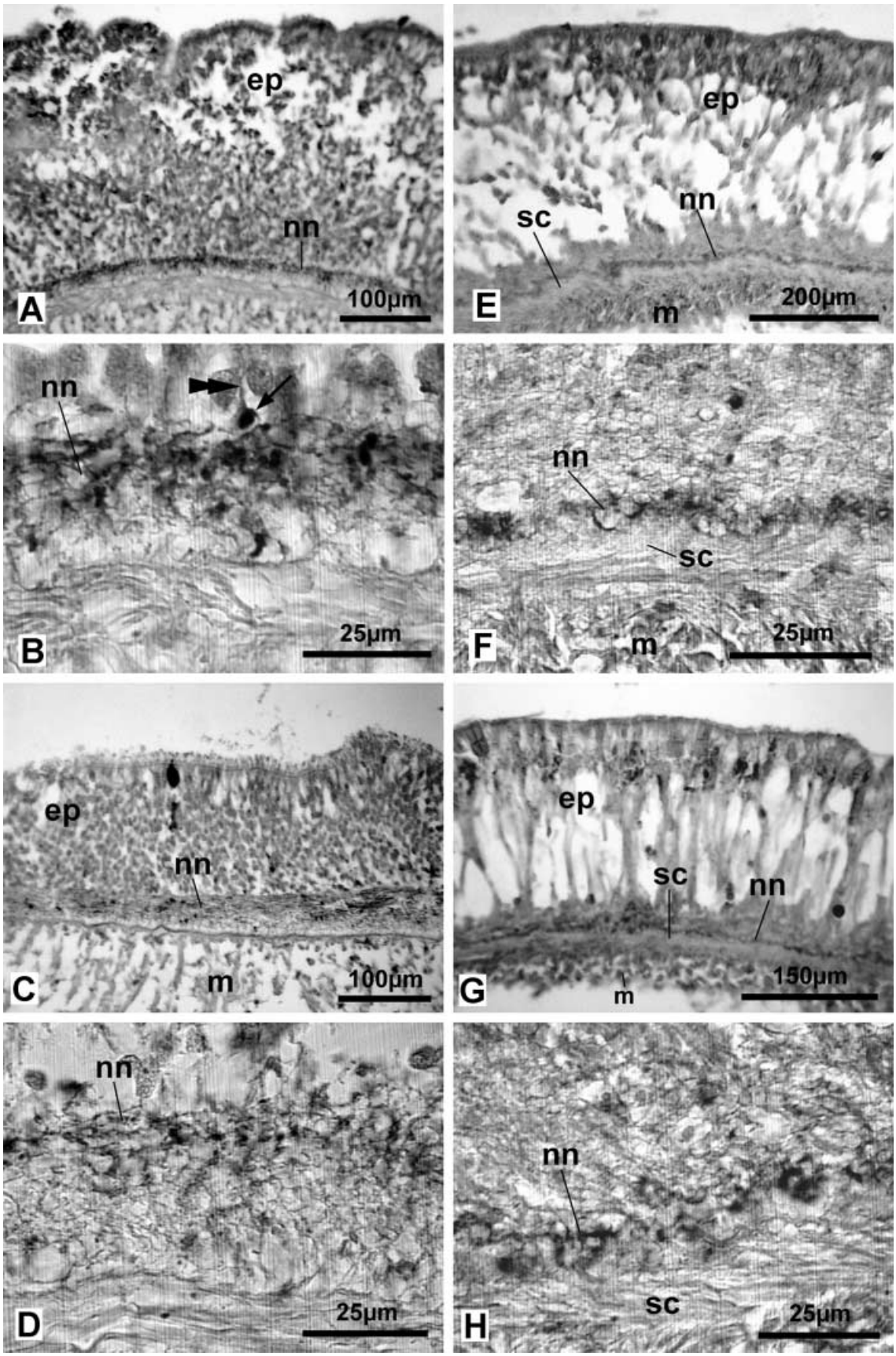

Figure 1. Immunohistochemical staining with antibodies against the neuropeptide SALMFamides 1 and 2 (S1 and S2 respectively). Peroxidase conjugated antibodies using DAB as substrate, eosin counterstaining. (A&B) S1-like immunoreactivity in the epidermis of the proboscis of the enteropneust *Harrimania kupfferi*; (C&D) S2-like immunoreactivity in the epidermis of the proboscis of the enteropneust *Harrimania kupfferi*; (E&F) S2-like immunoreactivity in the epidermis *Xenoturbella bocki*; (G&H) S2 immunoreactivity in the epidermis of *Xenoturbella bocki*. ep, epidermis; m, musculature; nn, basoepithelial nerve net; sc, subepidermal membrane complex; arrow, positively stained cell body; double arrowhead, positively stained axon.

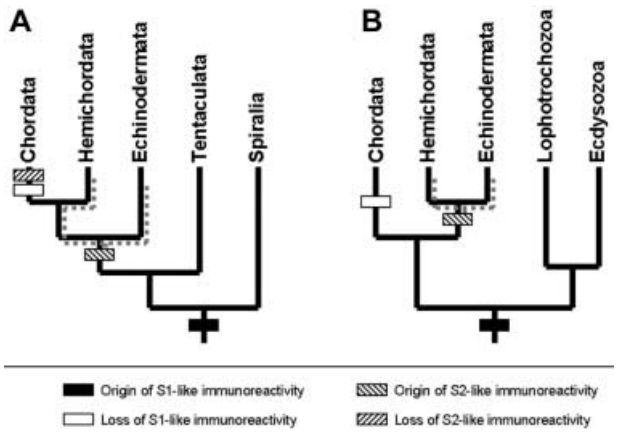

Figure 2. Two contrasting phylogenetic hypotheses for the interrelationship of deuterostome taxa. Evolutionary events of gain and loss of immunoreactivity with SALMFamide antibodies are mapped on the respective cladograms. The dashed grey line indicates the possible phylogenetic position of *Xenoturbella* spp. Both, evolutionary events and the potential phylogenetic position of *Xenoturbella* spp., are inferred from the currently known distribution of immunoreactivity in the animal kingdom, summarized in Table 1. (A) Traditional hypothesis based mainly on morphological characters (note that there is currently no consensus regarding the monophyly of Hemichordata and Tentaculata); and (B) 'Ambulacraria-hypothesis', based mainly on evidence derived from DNA sequences.

RESULTS

Both *Xenoturbella bocki* and *Harrimania kupfferi* are equipped with a nerve net that lies in the basal part of the epidermis between the epithelial cells (Figure 1). In *H. kupfferi*, local concentrations of nervous tissue are found in the dorsal side of the mesosome and along the dorsal and ventral sides of the metasome. No such concentrations of nervous tissue could be seen in *X. bocki*.

Immunoreactivity to SALMFamide 1 (S1)

Harrimania kupfferi. In the enteropneust *H. kupfferi*, the basoepithelial nerve net in the epidermis showed positive immunoreactivity with antibodies raised against S1 (Figure 1A,B). Specifically, positive staining was revealed in the cytoplasm surrounding the prominent nuclei as well as in the extended fibres surrounding the base of the epithelial cells (Figure 1B). Labelling is clearly evident in fine branches of axons. Staining with the S1 antibody revealed that the perikarya of the basoepithelial nerve cells were mainly situated within, and only rarely above, the fibrous basal layer (Figure 1A,B).

Xenoturbella bocki. The epithelially organized epidermis in *X. bocki* consisted mainly of extremely tall columnar multiciliated cells, mucous cells, and a basal net-like layer of neural cells (Figure 1). Only part of the fibrous neuronal net, the most basally situated part was stained positively with antibodies against S1 (Figure 1E,F). The perinuclear areas of the nerve cells were situated within the fibre net and were also positively labelled.

Immunoreactivity to SALMFamide 2 (S2)

Harrimania kupfferi. Nerve cells in the basoepithelial nerve net of *H. kupfferi* were positively stained with antibodies raised against S2 (Figure 1C,D). Labelling could be revealed in the perinuclear area as well as in thin fibre-like processes in the nerve net layer. Again, staining with the S2 antibody demonstrated that the perikarya of the basoepithelial nerve cells were situated predominantly in the distal part of the fibrous basal layer.

Xenoturbella bocki. The basoepithelial nerve net in the epidermis in *X. bocki* reacted positively with antibodies against S2 (Figure 1G,H). Perinuclear areas of the nerve cells as well as fibrous thin projections, probably axons were stained (Figure 1D). The cell bodies of these nerve cells are situated within the fibrous area.

DISCUSSION

In the present study, we demonstrated the presence of positive immunoreactivity with antibodies raised against two echinoderm neuropeptides, the SALMFamides S1 and S2, in the intraepithelial nerve nets of the enteropneust *Harrimania kupfferi* and the enigmatic worm *Xenoturbella bocki*.

Support for the 'Ambulacraria-hypothesis'

Many molecular analyses support the hypothesis that Hemichordata and Echinodermata form a monophylum (e.g. Furlong & Holland, 2002; Peterson, 2004). This clade is sometimes named Ambulacraria. On the other hand, morphological analyses often support the hypothesis that Hemichordata, or Enteropneusta are more closely related to Chordata than to other deuterostome taxa, though different morphology-based hypotheses are not consistent in all aspects of phylogenetic relationship (e.g. Hay-Schmidt, 2000; Nielsen, 2001). The two contrasting views are depicted in somewhat simplified manner in Figure 2. Tracing the inferred evolutionary events of origin and loss of immunoreactivity towards S1 and S2 antibodies on the respective trees, leads to the conclusion that S1-like immunoreactivity originated early during evolution. Because S1-like immunoreactivity is reported from nemertines, nematodes, flatworms, brachiopods, molluscs, annelids, crustaceans and echinoderms (see Table 1), it can be concluded that it is a feature of all bilateria. However, physiological experiments have shown that S1 functions as a general muscle relaxant in echinoderms (Elphick & Melarange, 2001), but not in the gastropod *Achatina fulica* (Araki et al., 1995). This could indicate that the antibodies raised against S1 from echinoderms detect a related neuropeptide in lophotrochozoans. The distribution of S2-like immunoreactivity, on the other hand, is more restricted and therefore more informative for phylogenetic conclusions. If enteropneusts are more closely related to Chordata than to other deuterostomes (Figure 2A), either the independent origin of S2-like immunoreactivity in *Harrimania kupfferi* and echinoderms or the loss of S2-like immunoreactivity in Chordata has to be assumed. Alternatively, the 'Ambulacraria-hypothesis' (Figure 2B) requires only one evolutionary step. In this scenario S2-like immunoreactivity would occur only once

in the stem line leading to Ambulacraria. The S2-like-immunoreactivity data therefore lend support to the hypothesis that Enteropneusta are more closely related to Echinodermata than to Chordata, because the latter hypothesis requires less ad hoc assumptions (Figure 2).

Phylogenetic position of Xenoturbella *spp.*

Worms in the genus *Xenoturbella* have few morphological characters of low complexity, so that their relation to other taxa remains obscure despite numerous attempts to resolve their phylogenetic position based on morphological studies (e.g. Ehlers & Sopott-Ehlers, 1997; Israelsson, 1997; Lundin, 1998). Recent molecular data were interpreted as evidence that the genus might be included in the high ranking taxon Deuterostomia (Bourlat et al., 2003). These authors also convincingly demonstrated that previous sequences isolated from *Xenoturbella* could stem from contaminating DNA in the endoderm. Nervous systems of animals have been compared at many levels over a wide range of taxa in phylogenetic studies (e.g. Hay-Schmidt, 2000; Lowe et al., 2003; Stach, 2005). Immunohistochemical analyses have contributed invaluable data in these investigations. S1-like immunoreactivity is present in *Xenoturbella bocki*. Because immunoreactivity with antibodies against S1 has probably originated early in evolution (see above; Figure 2), it is a plesiomorphic character at the level of Deuterostomia. As such, S1-like immunoreactivity in *X. bocki* does not allow us to suggest a closer relationship with specific bilaterian taxa. Because immunopositive reactions with antibodies against S2 are found only in Echinodermata and the enteropneust *Harrimania kupfferi* (see Table 1), the presence of such immunoreactivity in *X. bocki* is more informative. It can be concluded that based on this immunoreactivity *Xenoturbella* spp. are either a sister taxon to Enteropneusta, to Echinodermata, or to both, i.e. to Ambulacraria (Figure 2B, grey stippled line).

Future research

Immunoreactivity with antibodies is a strong indication, but not definitive proof for the presence of the epitopes under consideration. It is noteworthy that the primary antibodies have been used at low concentrations in the present study. This can be regarded as an indication of their specificity (Newman et al., 1995). In order to confirm that the two neuropeptides or related substances are present in *Xenoturbella bocki* and the enteropneust *Harrimania kupfferi*, they needed to be isolated and sequenced. This has so far only been accomplished in the sea stars *Asterias rubens*, *A. forbesi*, *Pycnopodia helianthoides* (Elphick et al., 1991), and *Marthasterias glacialis* (Yun, 1999). In addition, similar neuropeptides were characterized in *M. glacialis* (M. Thorndyke, unpublished data), *Ophiotrix fragilis* (Yun, 1999) and in the sea cucumber *Holothuria glaberrima* (Diaz-Miranda et al., 1992). However, the amount of tissue needed in this approach is prohibitive for the small and rarely caught *Xenoturbella* spp. and probably also for the small *Harrimania kupfferi*. As an alternative research strategy, it could be feasible to design primers against the nucleotide sequence inferred from the amino acid sequences and perform a polymerase chain reaction on cDNA isolated from tissue of *Xenoturbella* spp. and enteropneusts. In addition, in the course of the present study it became evident that more taxa need to be studied in respect of their immunoreactivity against S1 and especially S2, in order to achieve a more complete evolutionary picture. Therefore we started a survey of different taxa for which such data were lacking (S. Dupont et al., unpublished; results included in Table 1). Details will be published elsewhere.

CONCLUSIONS

The present study demonstrated that the enigmatic *Xenoturbella bocki* and the enteropneust *Harrimania kupfferi* showed immunoreactivity to antibodies raised against the echinoderm SALMFamides S1 and S2. This is interpreted as support for the phylogenetic hypotheses that *X. bocki* belongs to the non-chordate Deuterostomia and that Enteropneusta are closely related to Echinodermata. However, a study of more taxa, especially with antibodies against S2 and the use of molecular genetic and sequencing techniques complementing immunohistochemical investigations are warranted.

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Submitted 21 October 2004. Accepted 28 September 2005.