

# Immunocytological evidence supports the hypotheses that *Xenoturbella bocki* (Westblad 1949), phylum uncertain, is a deuterostome and that Ambulacraria is monophyletic

Thomas Stach<sup>1,3</sup>, Samuel Dupont<sup>1</sup>, Olle Israelson<sup>2</sup>, Geraldine Fauville<sup>1</sup>, Hiroaki Nakano<sup>1</sup>, Mike Thorndyke<sup>1</sup>

Addresses: <sup>1</sup> Kristineberg Marine Research Station, 45034 Fiskebäckskil, Sweden; <sup>2</sup> Museum of Evolution, Uppsala University, Norbyv. 16, S-752 36 Uppsala, Sweden; <sup>3</sup> Freie Universität Berlin, Fachbereich Biologie / Zoologie, Arbeitsgruppe Systematik und Evolution der Tiere, Königin Luise Str. 1-3, 14195 Berlin, [tstach@zoosyst-berlin.de](mailto:tstach@zoosyst-berlin.de)

## Abstract

The phylogenetic position of *Xenoturbella* spp. has been uncertain since their first discovery in 1949. It has been recently suggested that worms in this taxon 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 was primarily based on the analysis of DNA sequence data. We tested both hypotheses using antibodies raised against SALMFamide 1 and 2 (S1, S2), neuropeptides isolated from echinoderms, on *X. bocki* and the enteropneust *Harrimania kupferi*, as well as numerous marine invertebrates from different high ranking taxonomic groups. While immunoreactivity against S1 was widespread indicating an early evolutionary origin, immunoreactivity against S2 was restricted to nervous structures in the taxa traditionally considered non-chordate deuterostomes plus *X. bocki*. This finding supports the Ambulacraria-hypothesis and suggests a close phylogenetic relationship of *X. bocki* to Ambulacraria.

## Introduction

*Xenoturbella* has been classified as flatworms, as the sister group to Bilateria, as holothurians, as relatives of enteropneusts, or as modified bivalves (see Israelsson, 1999). Recently, molecular data were interpreted as evidence that *X. sp.* was a deuterostome (Bourlat et al., 2003). The exact phylogenetic position of *X. bocki* in this analysis depended on analysis method and choice of outgroup.

SALMFamides are structurally related neuropeptides isolated from echinoderms (Thorndyke et al., 1994). We used immunoreactivity with two SALMFamides (S1, S2) on *X. bocki* and the enteropneust *Harrimania kupferi* in order to test the phylogenetic hypotheses of a close interrelationship of *X. bocki* to Ambulacraria and the Ambulacraria-hypothesis.

## RESULTS

*Xenoturbella bocki* and *Harrimania kupferi*, possess a basoepithelial nerve net ( Figs. 1 & 2). In *H. kupferi*, local concentrations of nervous tissue are found but not in *X. bocki*.

In *H. kupferi*, the basoepithelial nerve net showed positive immunoreactivity with antibodies raised against S1 (Fig. 1A, B) and S2 (Fig. 2A, B). Positive staining was revealed in the cytoplasm and in axons.

In *Xenoturbella bocki*, the epidermis consists of tall columnar, multiciliated cells, mucous cells, and a basal net-like layer of neural cells (Figs. 1 & 2) and S2 (Figs. 2 C & D). Only the most basally situated part of the neural net was stained positively.

Table 1 summarizes results of experiments with S1 and S2 antibodies in different taxa (bold underlined – own experiments, regular font – literature data).

## DISCUSSION

Nervous systems can be compared at many levels in phylogenetic studies (e.g., Lowe et al., 2003; Stach, 2005).

### Support for the "Ambulacraria-hypothesis"

Molecular analyses suggest that Hemichordata and Echinodermata form a monophylum (e.g., Peterson, 2004). Morphological analyses often support the hypothesis that Enteropneusta are more closely related to Chordata (e.g., Nielsen, 2001; see Fig. 3).

S1 immunoreactivity is widespread (Table 1) and probably a common feature in Bilateria. The distribution of S2 is more informative. If enteropneusts are more closely related to Chordata (Fig. 3A), either the independent origin of S2 immunoreactivity in *H. kupferi* and echinoderms or the origin of S2 immunoreactivity in Deuterostomia and subsequent loss of S2 immunoreactivity in Chordata has to be assumed. The Ambulacraria-hypothesis requires only the occurrence of S2 immunoreactivity in the line leading to Ambulacraria. S2-immunoreactivity data thus support the hypothesis that Enteropneusta are closer related to Echinodermata than to Chordata (Fig. 3).

### Phylogenetic position of *Xenoturbella sp.*

Immunopositive reactions with antibodies against S2 are found only in Echinodermata and *H. Kupferi* and *Xenoturbella bocki* (Table 1), it can be concluded *X. sp.* is either a sister taxon to Enteropneusta, to Echinodermata, or to Ambulacraria (Figure 3B, grey stippled line).

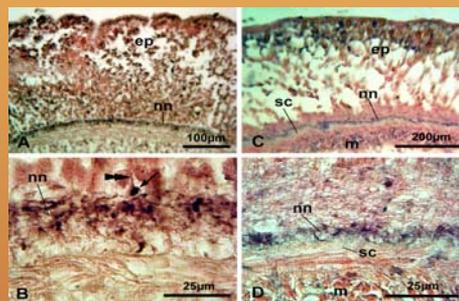


Figure 1 - Light micrograph showing immunoreactivity against S1-antibodies. A&B *Harrimania kupferi*. C&D *Xenoturbella bocki*.

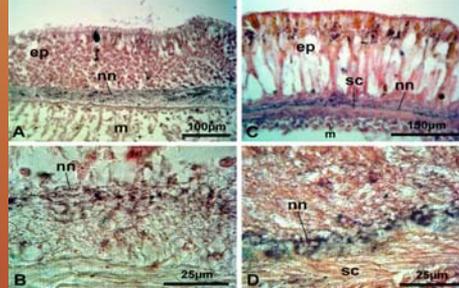


Figure 2 - Light micrograph showing immunoreactivity against S2-antibodies. A&B *Harrimania kupferi*. C&D *Xenoturbella bocki*.

Abbreviations used in Figures 1 & 2: ep - epidermis, m - musculature, nn - basoepithelial nerve net, sc - subepidermal membrane complex, arrow: positively stained cell body, double arrowhead: positively stained axon

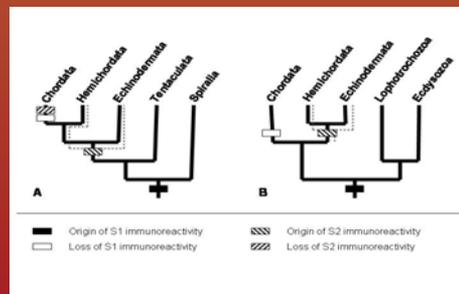


Figure 3 - Simplified cladograms. A traditional view. B Ambulacraria hypothesis. Immunoreactivity data are mapped on the cladograms. Grey stippled line marks branches to which *Xenoturbella sp.* could plausibly be connected (see text for details).

## MATERIALS AND METHODS

Individuals were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (6µm) were incubated with polyclonal antibodies in PBS (1:2000), incubated for 1h in biotinylated antiserum 1:200 and processed using ABC reagent (Vector labs), according to instructions. Immunolabelling was visualized with 0.04% DAB and 0.01% H<sub>2</sub>O<sub>2</sub>.

Table 1 - List of species for which immunoreactivity against S1& S2-antibodies was determined. + positive, - negative, ? Not tested, (?) not reported, bold and underlined: species tested by present authors

Species	Higher taxon	S1	S2
<i>Ciona intestinalis</i>	Chordata	-	-
<i>Antedon mediterranea</i>	Echinodermata	+	+
<b><u>Gorgonocephalus capulmedusae</u></b>	Echinodermata	+	+
<i>Ophiocoma nigra</i>	Echinodermata	?	+
<i>Ophiotrix fragilis</i>	Echinodermata	?	+
<i>Ophiura ophiura</i>	Echinodermata	?	+
<b><u>Amphiura chiajei</u></b>	Echinodermata	?	+
<i>Asterias rubens</i>	Echinodermata	+	+
<i>Asterina pectinifera</i>	Echinodermata	+	(?)
<i>Pisaster ochraceus</i>	Echinodermata	+	(?)
<i>Patriella regularis</i>	Echinodermata	+	(?)
<i>Amphipholis squamata</i>	Echinodermata	+	+
<i>Ophiactis resiliens</i>	Echinodermata	+	(?)
<i>Ophiura ophiura</i>	Echinodermata	+	(?)
<i>Dendroaster excentricus</i>	Echinodermata	+	(?)
<i>Psammochinus miliaris</i>	Echinodermata	+	+
<b><u>Xenoturbella bocki</u></b>	incertae sedis	+	+
<b><u>Harrimania kupferi</u></b>	Enteropneusta	+	+
<i>Argyrotheca cordata</i>	Brachiopoda	+	(?)
<i>Argyrotheca cuneata</i>	Brachiopoda	+	(?)
<i>Cerebratulus sp.</i>	Nemertini	+	-
<i>Glycera alba</i>	Annelida	+	-
<i>Melina cristata</i>	Annelida	+	-
<b><u>Meganetyphantes norvegica</u></b>	Crustacea	+	-
<i>Ascaris suum</i>	Nematoda	+	(?)
<i>Cystidicola farionis</i>	Nematoda	+	(?)
<i>Didiphophora merlangi</i>	Platyhelminthes	+	(?)
<i>Diphyllobothium dendriticum</i>	Platyhelminthes	+	(?)
<i>Grillonia erinaceus</i>	Platyhelminthes	+	(?)
<i>Sanguinicola inermis</i>	Platyhelminthes	+	-
<i>Schistosoma mansoni</i>	Platyhelminthes	+	(?)
<i>Stenostomum leucops</i>	Platyhelminthes	+	(?)
<b><u>Pennatula phosphorea</u></b>	Cnidaria	-	-

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