

# X-cells in pseudotumors of yellowfin goby *Acanthogobius flavimanus*: a protistan organism distinct from that in flathead flounder *Hippoglossoides dubius*

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**ABSTRACT:** Yellowfin goby *Acanthogobius flavimanus* affected with X-cell pseudotumors were sampled from a river estuary in Tokyo Bay, Japan. We amplified the gene for small subunit ribosomal RNA (18S rRNA) of X-cells of the goby with PCR using universal primers. The gene that we obtained (DDBJ Accession no. AB451874) showed 91 % sequence identity to that of the X-cells of the flathead flounder *Hippoglossoides dubius*. With *in situ* hybridization, the probes specific for the gene that we obtained hybridized with the goby X-cells but not with the flounder X-cells, whereas probes for the 18S rRNA gene of flounder X-cells hybridized with the flounder X-cells but not with goby X-cells. These findings indicate that, although the X-cells found in the goby are closely related to the protist found in flounder, the two are clearly distinct organisms.

**KEY WORDS:** X-cell · Protistan · Goby · *Acanthogobius flavimanus* · 18S rRNA · *In situ* hybridization

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## INTRODUCTION

From autumn to early winter of 2007, tumor-like lesions were frequently noticed on the skin of the yellowfin goby *Acanthogobius flavimanus* caught by recreational anglers at the mouth of the Arakawa River flowing into Tokyo Bay, Japan. The lesions were found on about 30 % of the fish. We conducted histological investigations; these revealed the presence of numerous 'X-cells' in the lesions, indicating that the goby specimens were affected by X-cell disease. The disease is a tumor-like condition mainly affecting the body surface or branchial cavity of various bottom-dwelling fish species (Dawe 1981, McVicar et al. 1987, Franklin et al. 1993, Diamant et al. 1994, Dethlefsen et al. 1996), although lesions can also be formed in the internal organs (Diamant & McVicar 1987). The X-cells inside these tumor-like lesions are distinct from other cells of the affected fish; they are usually polygonal, with a large nucleus and a prominent nucleolus (Brooks et al. 1969). The size of X-cells may vary

greatly, from ca. 5 µm to more than 20 µm in diameter (Ito et al. 1976). The origin of these cells had long been a matter of debate. X-cells were once considered to be neoplastic cells of the host fish (Stich et al. 1976, Peters et al. 1981). However, subsequent circumstantial evidence suggested that X-cells are actually protistan parasites (Dawe 1981, Watermann & Dethlefsen 1982), and this hypothesis has recently been gaining popularity among researchers (Grizzle & Goodwin 1998).

In a previous paper (Miwa et al. 2004), we presented conclusive evidence that X-cells found in tumour-like lesions on the skin of flathead flounder *Hippoglossoides dubius* represent a protistan organism, based on the analysis of the small subunit ribosomal RNA gene (18S rDNA) of these cells. However, the taxonomic position of the organism is still to be ascertained. In the present study, we amplified 18S rDNA from goby X-cells and carried out phylogenetic analysis and *in situ* hybridization to clarify the relationship between goby X-cells and flounder X-cells.

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## MATERIALS AND METHODS

**Gobies and histopathology.** Affected specimens were sampled at Arakawa River close to the river mouth in Tokyo Bay, and used for the subsequent analyses. For histopathology, tumor-like lesions were excised from 3 affected fish and fixed in Davidson's fixative (330 ml 95% ethanol, 220 ml commercial formaldehyde solution containing 38% formaldehyde, 115 ml glacial acetic acid, and 335 ml distilled water) overnight. Then, the tissues were routinely embedded in paraffin, sectioned at 3  $\mu$ m, and stained with hematoxylin and eosin (H&E).

**PCR and phylogenetic analysis.** For gene extraction, tumor-like lesions were excised from affected fish and fixed in 100% ethanol. DNA was extracted from one of the ethanol-fixed lesions using Proteinase K and phenol-chloroform (Sambrook et al. 1989). Since we failed to amplify 18S rDNA of goby X-cells with primers designed for the X-cells of the flathead flounder, we used the universal primers for eukaryotes, 5'-18S (5'-CGA CAA CCT GGT TGA TCC TGC CAG T-3') and 3'-18Sr (5'-TTG ATC CTT CTG CAG GTT CAC CTA C-3'), to amplify the gene of goby X-cells. Cycling conditions for the PCR were 94°C (30 s), 55°C (30 s), and 72°C (1 min) for 30 cycles. The amplified fragment was purified and ligated into the plasmid vector pDrive (QIAGEN). The ligated plasmid was used to transform competent *Escherichia coli* (JM109, Nippon Gene). The plasmids containing the insert were isolated and applied to nucleotide sequencing. The first several clones sequenced were all identified as 18S rDNA of the host goby, and hence the insert of 90 clones were amplified with PCR and digested with *Hpa*II (Toyobo) to eliminate clones showing the same digestion pattern as that of the goby gene. Of the 90 clones, 87 were thus eliminated, and 3 clones showed a different pattern of digestion. The determined sequences of these 3 clones were identical. The obtained sequence and 18S rDNA sequences of various eukaryotes including the X-cell

organism found in the flathead flounder (Miwa et al. 2004) were aligned manually and analyzed by the Genetic software package (version 10.6, GENETYX). Evolutionary distances were calculated by the method of Kimura's 2-parameter model (Kimura 1980).

**Probes for *in situ* hybridization.** Three antisense oligonucleotide probes complementary to variable regions unique to the obtained sequence were synthesized so that they would hybridize with the 18S rRNA in the cytoplasm (Table 1). A further 3 oligonucleotide probes for the 18S rRNA of the X-cell organism from the flathead flounder were newly designed and synthesized (Table 1). An oligonucleotide probe was also synthesized for an identical sequence in the 18S rDNA of the yellowfin goby and flathead flounder and designated as shown in Table 1.

Probes were labeled with digoxigenin, using a commercial kit (DIG Oligonucleotide Tailing Kit, Roche) according to the manufacturer's instructions.

***In situ* hybridization.** Paraffin-embedded lesions sampled from the 3 specimens of yellowfin goby were sectioned at 3  $\mu$ m and subjected to *in situ* hybridization. In addition, sections of the X-cell lesions of the

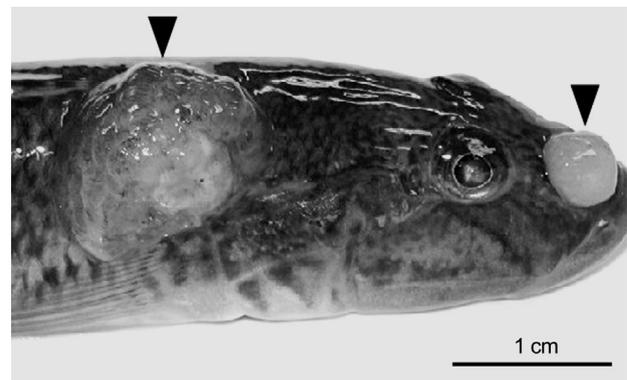


Fig. 1. *Acanthogobius flavimanus*. Yellowfin goby affected with X-cell disease. Note tumor-like lesions on the skin (arrowheads)

Table 1. Probes synthesized for *in situ* hybridization. Target sequences in **bold**

Probe	Sequence
<b>18S rRNA: obtained sequence from X-cells of yellowfin goby</b>	
HXC-217r	5'-CAGAACCGTGTGATCTGATAATAAGGTCTTCATTTCGAGAAGAAACATG-3'
HXC-1376r	5'-AAGTTGTAGACCGAATGTACAGAGGGAGGTCTTATCCCCTGAGAGAACAG-3'
HXC-1696r	5'-CGATTCACCAGTTCAGGCGAGTCTCTACTGAGCTTACCGGATCATCCAAT-3'
<b>18S rRNA: X-cells of flathead flounder</b>	
AXC-1376r	5'-AGCTGTTTCGCCGAAGGCTACGAGGAAGGTCTTATCTCTCTTAGAGAACAG-3'
AXC-1522r	5'-GTGAATTCAGGTTTGCAACGCCTGTCGGCGGAGCAACTCGTTATCCATG-3'
AXC-1696r	5'-CAGTGCACATAATTGAAGTGCCTTCTCCCGAGCTCACCGGACCATTCAAT-3'
<b>18S rDNA: identical sequence from yellowfin goby and flathead flounder</b>	
AH-769r	5'-CGGACCCCGCGGGACACTCAGCTAAGAGCATCGAGGGGGCGCCGAGAGGC-3'

flathead flounder (Miwa et al. 2004) were newly prepared from a stored paraffin block and also used for *in situ* hybridization. *In situ* hybridization was carried out according to the method described previously in Miwa et al. (2004). The 3 probes for the X-cell organism were used in combination for the *in situ* hybridization in order to intensify the expected signal.

## RESULTS

Fig. 1 shows the external appearance of an affected yellowfin goby. Under general histopathological examination, the lesions appeared like papillomas at a low magnification. However, they contained numerous X-cells, the largest of which (ca. 20  $\mu\text{m}$  in diameter) were observed in the hypertrophied epidermis of the lesions. The smallest cells (ca. 5  $\mu\text{m}$  in diameter) were located in the basal part of the epidermal growths and also in the granulation tissue formed under the epidermis. The general histopathological features, including the morphology of the X-cells, were almost identical to those of the X-cell lesions of the flathead flounder (Miwa et al. 2004).

The sequence of the obtained 18S rRNA gene (DDBJ Accession no. AB451874) had a 91% identity to that of the X-cells of the flathead flounder (Miwa et al. 2004) and both of them clearly formed a group in the phylogenetic tree based on the 18S rDNA sequences (Fig. 2). However, we could not find any other organisms that showed any possible phylogenetic relationships with X-cells, and hence the position of X-cell organisms in the tree is unstable. Although we employed the same organisms as we did in the previous study (Miwa et al. 2004) for the analysis to construct the tree in Fig. 2, the position of X-cell organisms in the tree can change depending on the organisms included in the analysis.

A strong hybridization signal was observed in the X-cells in the lesions of yellowfin goby by *in situ* hybridization with the 3 HXC probes (Fig. 3). The AH-769r probe hybridized only with the host goby cells (Fig. 3). No signal was obtained when the sections of the goby lesions were hybridized with the 3 AXC probes, which were designed for the flathead flounder X-cells (Fig. 3). On the other hand, when the sections of X-cell lesions of the flounder were hybridized with these probes, no signal was obtained with the HXC probes, whereas strong signals were obtained on the X-cells with AXC probes (data not shown).

## DISCUSSION

The results clearly indicate that the X-cells found in the yellowfin goby and in the flathead flounder (Miwa et al. 2004) are distinct organisms, although they are

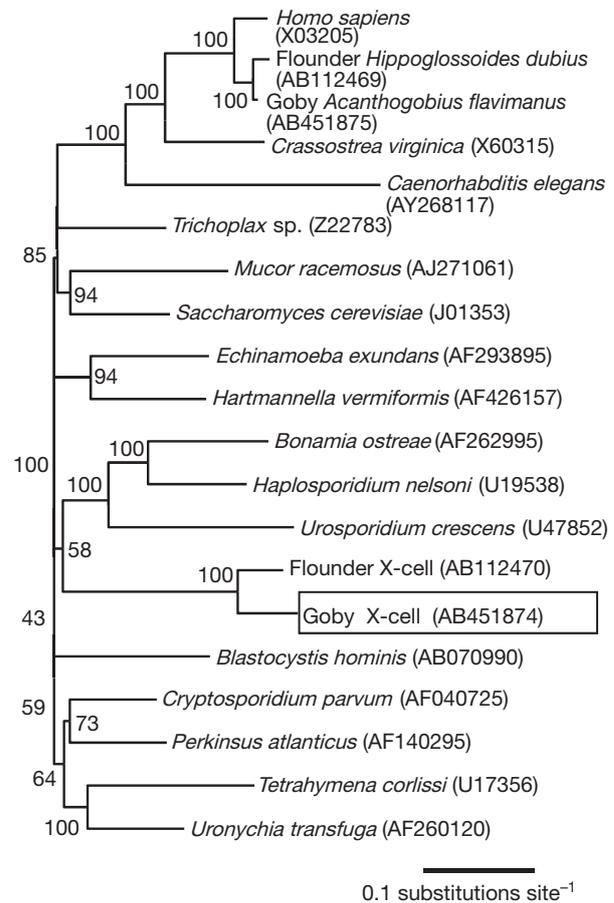


Fig. 2. Phylogenetic relationships of the X-cell organism from yellowfin goby among eukaryotes inferred from 18S rRNA gene sequences using the neighbor-joining method. This tree is unrooted. Bootstrap probabilities are shown on the internal branches (%)

certainly closely related to each other—as suggested by the phylogenetic analysis. The close resemblance of gross morphological and histological features of X-cell lesions of these 2 species was also described by Ito et al. (1976). At the time, they reported the lesions as papillomas, but the reported morphological features and a photomicrograph of the lesions in their paper indicate that they were actually X-cell pseudotumors. In the present study, the goby specimens were sampled from the tidal mouth of a river on the Pacific coast of Japan's main island, Honshu, whereas the flounder specimens were caught from offshore on the bottom in the Japan Sea in the previous study (Miwa et al. 2004). Thus, it is not certain, at present, whether the 2 X-cell organisms display host specificity or if they reflect different geographical distributions of the different X-cell species.

X-cell lesions are found in various fish species worldwide, but the morphology of the X-cells is very similar (McVicar et al. 1987), suggesting that the disease is caused by a similar, if not the same, organism. On the

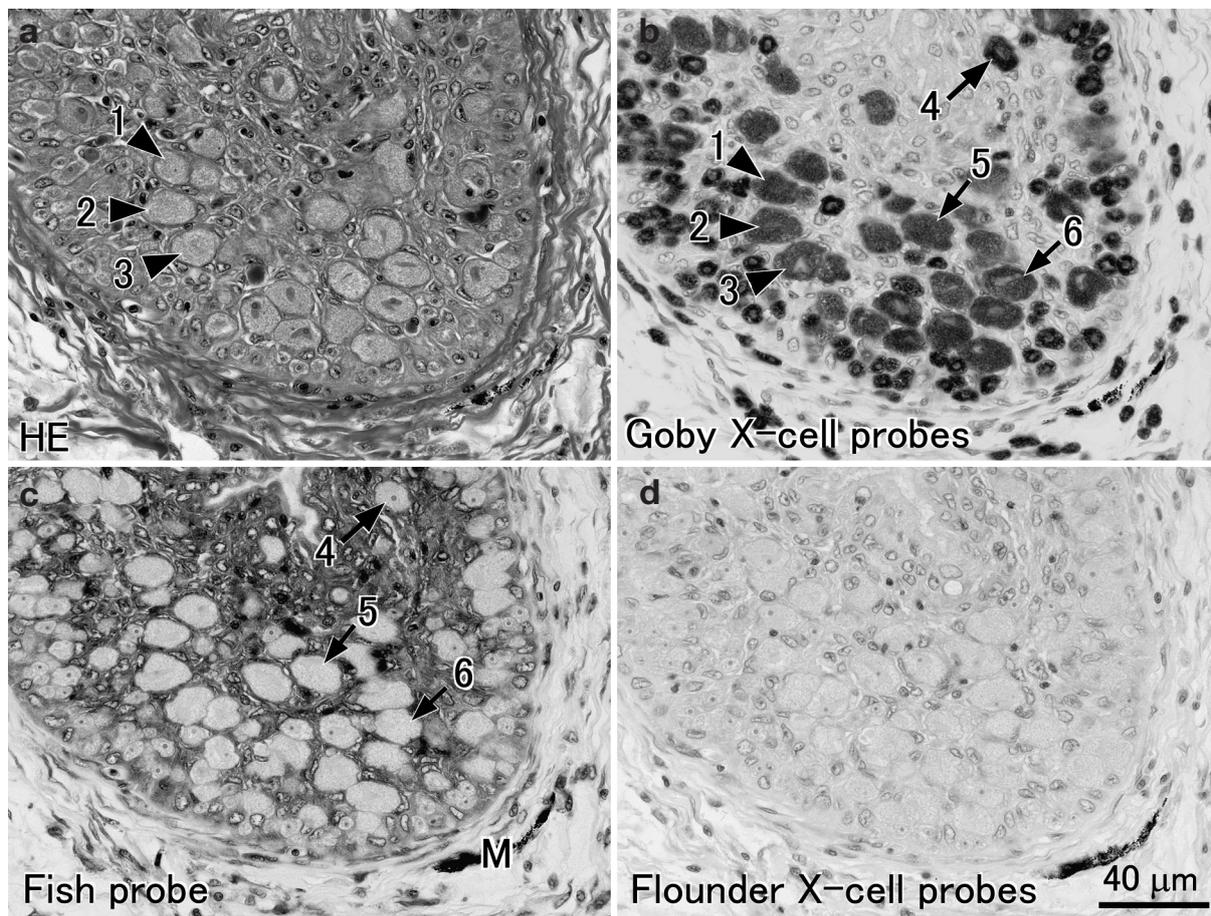


Fig. 3. *Acanthogobius flavimanus*. Four consecutive sections of an X-cell lesion from a yellowfin goby. (a) Section stained with hematoxylin and eosin (H&E). Note many X-cells of varying size. (b) Section hybridized with the combination of 3 oligoprobes (HXC-217r, HXC-1376r, HXC-1696r), which are specific to the sequence from a lesion of the goby. A strong hybridization signal is observed on X-cells. (c) Section hybridized with the probe (AH-769r), which is specific to the goby; the probe hybridized only with host goby cells but not with X-cells. (d) Section hybridized with the combination of 3 oligoprobes (AXC-1376r, AXC-1522r, AXC-1696r), which are specific to the X-cells of flathead flounder *Hippoglossoides dubius*; no hybridization signal is seen.

Arrows or arrowheads with the same numbers indicate the same X-cells. M: melanophore. See Table 1 for details of probes

other hand, the susceptible tissues are different among host species and also among geographical regions. In Pacific pleuronectids, as well as the goby in the present study, the lesions develop only on the body surface (Wellings et al. 1976, Katsura et al. 1984). In both Pacific and Atlantic gadoids, the lesions are found on the pseudobranchs (McCain et al. 1979, Dethlefsen et al. 1996) or on the wall of the oral cavity (Morrison et al. 1982). Furthermore, in dab in the North Sea (McVicar et al. 1987), and in a nototheniid fish in the Antarctic (Franklin et al. 1993), X-cell lesions are found mainly on the gills, and hence termed 'X-cell gill disease' (Møllergaard & Nielsen 1996). Watermann & Dethlefsen (1982) suggested that the X-cell diseases of Pacific flatfishes and gadoids are different, because mitotic activities of X-cells, which are well documented in the latter, were not observed in the former.

These differences in X-cell disease, together with the findings of the present study, indicate that various different species of X-cells probably exist. However, the phylogenetic affinity of X-cell organisms is still unknown. These organisms may constitute a completely new taxon, or they may be related to already known organisms for which 18S rDNA sequences have not yet been published. A different analytical method may be needed to find the phylogenetic affinity of X-cell organisms. The biology of X-cell organisms is also largely unknown. Many important questions remain to be answered, such as whether they have different life stages, or how they infect fish and how they leave the host. Since it is now clear that X-cells are parasites, future studies should address not only the taxonomical classification but also the life cycle of these yet enigmatic organisms.

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