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# Identification of the Nramp Gene in TV-1 Fibroblasts from Turbot *Psetta maxima*, Formerly *Scophthalmus maximus* L. 1758 (Pisces: Scophthalmidae)

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## Abstract

***The correct identification of the species of origin in cell culture procedures as well as the detection of eventual cross-contamination by other cells are of essential importance to ensure the correctness of all experimental trials. In this connection, PCR is a fundamental technique to identify the occurrence of genetic information belonging to a defined species. In this paper the correspondence between the known sequences for turbot Psetta maxima (formerly Scophthalmus maximus) and those obtained from TV-1 cells from juvenile Psetta maxima was searched by PCR by analysing the Nramp (Natural Resistance Associated Macrophage Protein) gene, which is known to occur in several tissues of turbot and in a turbot embrional cell line. The obtained results showed an high degree of homology of the amplified fragment of TV-1 cell line with the Nramp coding region of Teleosts, and a high degree of probability of the occurrence of mRNA belonging to Psetta maxima; so it could be excluded at a greater extent a contamination by genetic material of other organisms and the occurrence of information coming from Teleosts in TV-1 cells was confirmed.***

## Introduction

Turbot *Psetta maxima*, formerly known as *Scophthalmus maximus*, is a plateless, benthic, demersal, euryaline marine flatfish living on sandy, pebbled and muddy bottoms up to 100 metres depth [1]; its distribution spreads out from the Mediterranean and the Black Sea up along the Atlantic European coasts to the Baltic Sea and the Norwegian coast beyond along the British Islands and the Faeroe Island [2, 3]. Particularly, this flatfish is abundant in the eastern North Atlantic, in the Mediterranean Sea [4] and in the Baltic Sea [3, 5]. *Psetta maxima* is a commercially valuable species of high concern in aquaculture [6, 7] whose farming in Europe is to date expanded [8]. As increasing fish production several new viral diseases arise [9], the usefulness of new research methods able to counter virus-induced pathologies, such as the utilization of fish cell cultures, has been emphasized [10].

The first Teleost cell line was reported in the literature in early 60's [11] and the first attempts to establish marine fish cell cultures were made in late '70s and early '80s of the last century [12, 13]. Overall, early fish cell culture were developed mainly for applied biology purposes taking into account the economic value of fishes as food [14]. Subsequently, they have had a number of applications, for example to evaluate the cytotoxicity and the genotoxicity of compounds of environmental concern [15-17], the sensitivity to different chemicals and to surfactants [17-20], the toxicity of PAHs and phenols, the induction of P450 cytochrome and the estrogenic activity of chemicals and environmental samples [21], and to develop and to analyse drugs occurring in aquatic systems [22]. Furthermore, fish cell cultures have been used to detect the expression of particular cell functions induced by specific pollutants [23]. In this connection, to know the exact origin of cultured cells and to make sure that they really belong to the species of interest is an important aspect in order to avoid misinterpretations of results obtained in the course of experimentation; for this purpose PCR is a valuable tool being able to recognize through molecular genetics methods if cultured cells belong to a defined species [24-25]. In order to identify the occurrence of genetic information belonging to *Psetta maxima* in a turbot cell line (TV-1) and to verify by PCR the correspondence between the known sequences for turbot and those obtained from TV-1 cells, Nramp (Natural Resistance Associated Macrophage Protein) gene, which was recently cloned and sequenced and was seen to be expressed in several tissues and in an embryonic cell line of turbot [26, 27], was considered.

Therefore, aim of this work was to recognize and verify through the identification of Nramp gene by biomolecular methods, if TV-1 cells cultured in our laboratory belong to the species *Psetta maxima* in order to exclude the contamination by cells from other organisms.

## Materials and methods

*Cells:*

Fibroblasts of the continuous cell line TV-1 (Turbot Vigo - 1) have been obtained from juvenile *Psetta maxima* and have been established at the Instituto de Investigaciones Marinas CSIC at Vigo (Spain) [10, 28, 29]; cells were kindly placed at our disposal by the Searchers of CSIC.
In our laboratory, cells were maintained in Eagle's MEM medium (EuroClone, Pero, Milano, Italy) supplemented with 10% foetal serum, 1% l-glutamine and 1% penicillin-streptomycin (EuroClone, Pero, Milano, Italy), at 20°C in humidified incubator and handled upon vertical laminar flow.

*RT-PCR:*

RNA was extracted from TV-1 cell line according to standard techniques [30]. Considering that in PCR assays the presence of a control is required because several factors can make PCR ineffectual, RNA extracted from human peripheral blood was used as a control of Nramp amplified fragment.
cDNA synthesis was performed at 37°C for 1h in a 25 µl reaction volume containing 5 µg total cellular RNA, 1 µg random hexamers (Gibco-BRL), 20 U RNAsin (Promega), 4 mM dNTPs and 200 U Superscript II reverse transcriptase (Gibco-BRL). cDNA was used as a template for PCR, with specific primers for a fragment of the codifying region of the Nramp gene. The Nramp gene cDNA was amplified using the following primer pair: 5'- -3' (sense primer) and 5'-CTGCCCGGAGTAAGTGCCTGTC-3' (reverse primer). Primer sequences were designed by the Amplify-PCR (Version 2.53) and Oligo-PCR (Version 4.0, MedProbe A.S.) software programs. PCR reaction required 2.5 µl of the cDNA reaction mixture to be added to the PCR mixture containing 1x PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 5 pmol of each primer and 1 U Taq polymerase, in a total volume of 25 µl. Agarose gel 2% was used to detect the PCR product.

*Sequencing:*

Direct DNA sequencing of the amplified product was carried out using a dRhodamine Terminator Cycle Sequencing Kit (PE Applied Biosystem, Foster City) and an ABI-PRISM 310 Genetic Analyzer (PE Applied Biosystem, Foster City), according to the manufacturer's instructions. Forward and reverse sequences were analysed using Sequence Navigator Program (PE Applied Biosystem, Foster City). The bioinformatic analysis of sequence alignment with known sequences was performed using the database BLAST [31, 32].

## Results

In the present work we performed RNA extraction that proceeded with the amplification of the segment of interest by PCR, the direct sequencing of the amplified fragment and finally the alignment of the obtained sequence with known sequences on a database. The amplification and the direct sequencing of human Nramp fragment, used as a control of the PCR system, was useful to verify the specificity of the amplified fragments obtained through a single primers pair. Primers pair was designed to amplify a fragment of 407 bp (c.829-c.1236 bp) of the Nramp coding region (Fig. 1).

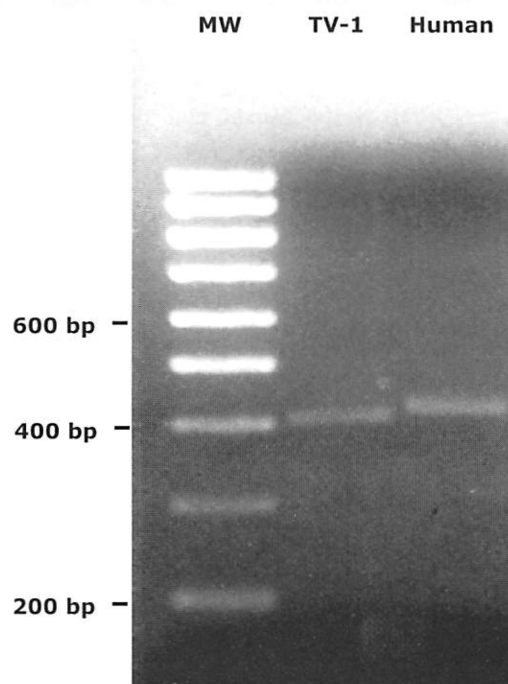


*Fig. 1 - Detection of the Nramp amplified product from TV-1 cell line and human cDNA. MW: 100 bp ladder*

Primers pair was chosen to be homologous to regions highly similar between species encompassing a Nramp cDNA region, corresponding to the space between the residues 254 and 389 of the protein, which allows to show some differences between species. The electropherograms obtained from the direct sequences of the amplified segments, both from human and TV-1 cDNAs, showed an unique sequence with no heterozygous peaks and, without ambiguous interpretation, suggesting the presence of an unique cDNA product without traces of any other polluting DNA both in the fragment amplified from human and from TV-1 cDNAs. Sequence alignments using the BLAST program allowed us to classify the obtained sequences as a part of the Nramp coding region. We used the BLAST alignment comparing both the obtained nucleotide sequence and the corresponding translated sequence with those in the database. The fragment obtained from TV-1 cell line showed the highest degree of homology with the sequences in the database related to the Teleosts, to which *Psetta maxima* belongs (Fig. 2).
The fragment obtained from human DNA amplification resulted also highly specific and showed homology with Primate Nramp sequences at the highest degree with Hominidae sequences (data not shown).

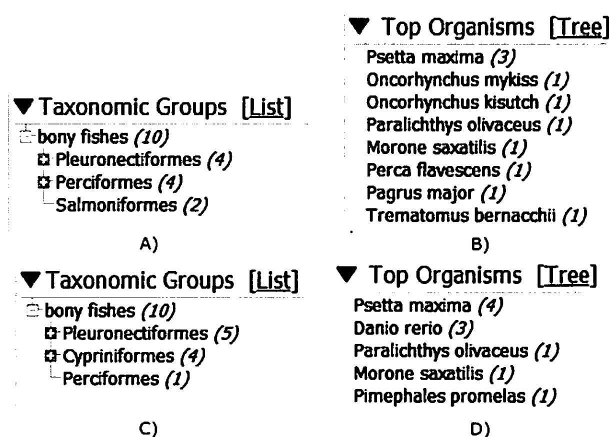


*Fig. 2 - Tree (A) and list (B) of the highest homologous nucleotide Nramp sequences obtained comparing amplified fragments with sequences in BLAST database. Tree (C) and list (D) of the highest homologous translated Nramp sequences obtained comparing amplified fragments with protein sequences in BLAST database.*

## Discussion

In the framework of the development of modern and sustainable research methods, one of the main items both in toxicology and in ecotoxicology is the replacement of *in vivo* experiments with *in vitro* procedures. It is known that the bulk of the available continuous cell lines are derived from mammals, but other important cell lines, used chiefly in ecotoxicology, come from heterotherm vertebrates [33], mainly from freshwater fish [34, 35]. On the whole, in the last two decades the efforts to develop marine fish cell culture has increased greatly and several cell lines derived from marine fish of commercial concern have been reported [10, 28, 29, 35-37]. In this connection, cell misidentification and cross-contamination are major problems in cell cultures and can affect the reproducibility of data so making unreliable the obtained results; furthermore, unlike contamination by bacteria and fungi, cell cross-contamination is not readily detectable, and as a rule does not modify the morphology and the behaviour of cultured cells [24]. Overall, the contamination or overgrowth of unrelated cells is a potential and recurring problem [38] both for mammalian and non-mammalian cell cultures. PCR is a flexible and sensitive method which has a lot of applications in the basic research and in several biomedical fields such as in genetics, microbiology, oncology, legal medicine and in clinical follow-up procedures. In the environmental field it is useful for palaeontological and evolution studies and to recognize species; for the latter aspect, the identification of a species needs to amplify a known gene, or a part of it, in order to directly sequence the PCR product, which then can be compared with a reference one, so obtaining information about the genetic status. Nramp gene has been identified in several mammal species such as human, mouse, bovine, sheep and pig and it was shown to have a various degree of homology in the different domains of the protein; furthermore, it seems one of the major candidates to control the natural resistance and/or the susceptibility to intracellular pathogens in vertebrates [27]. Nramp was recently cloned, characterized and analysed in turbot observing that it has got an homology ranging from 60 to 92% with the same gene of other 13 vertebrate species [27]. In this research primer pairs, located in highly conserved Nramp coding region, were used to amplify the cDNA region corresponding to the residues 254 to 389 of the Nramp protein, which show some differences between species, in order to co-amplify and identify the presence of different fragments. In TV-1 cell line the highest degree of homology of the amplified fragment was found with the Nramp coding region of Teleosts, so we can exclude a substantial contamination with cells of other organisms. In conclusion, the utilized method allowed to verify the occurrence of mRNA belonging, with a high degree of probability, to *Psetta maxima* and more generally to Teleosts, in the examined cell line TV-1, excluding at a greater extent the contamination by genetic material belonging to other organisms.

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