A novel rod-like opsin isolated from the extra-retinal photoreceptors of teleost fish

Alisdair R. Philp[a,1], James Bellingham[a,1,*], Josè-M. Garcia-Fernandez[b], Russell G. Foster[a]

[a] *Department of Biology, Sir Alexander Fleming Building, Imperial College of Science, Technology and Medicine, London SW7 2AZ, UK*
[b] *Departamento de Morfologia y Biologia Celular, Universidad de Oviedo, 33071 Oviedo, Spain*

Received 3 December 1999; received in revised form 26 January 2000

Edited by Matti Saraste

Abstract We have isolated a novel opsin from the pineal complex of Atlantic salmon (*Salmo salar*) and from the brain of the puffer fish (*Fugu rubripes*). These extra-retinal opsins share approximately 74% identity at the nucleotide and amino acid level with rod-opsins from the retina of these species. By PCR, we have determined that the novel rod-like opsin is not expressed in the salmon retina, and the retinal rod-opsin is not expressed in the salmon pineal. Phylogenetic analysis suggests that the rod-like opsins arose from a gene duplication event approximately 205 million years ago, a time of considerable adaptive radiation of the bony fish. In view of the large differences in the coding sequences of the pineal/brain rod-like opsins, their extra-retinal sites of expression, and phylogenetic position we have termed these novel opsins 'extra-retinal rod-like opsins' (ERrod-like opsins). We speculate that the differences between retinal rod-opsins and ERrod-like opsins have arisen from their differing photosensory roles and/or genetic drift after the gene duplication event in the Triassic.

Key words: Evolution; Extra-retinal photoreceptor; Opsin; Pineal; Teleost fish; Vertebrate photoreception

1. Introduction

Non-mammalian vertebrates possess multiple photoreceptor organs that develop from the embryonic forebrain. These are classified as: (1) an intracranial pineal organ or pineal body (epiphysis cerebri) which contains photoreceptors in all non-mammalian vertebrates; (2) an intracranial parapineal organ, found in many bony fish and lampreys; (3) an extracranial 'third eye', variously called a frontal organ (frogs) or parietal eye or parietal body (lizards); (4) deep brain photoreceptors, located in several sites in the brain and found in all non-mammalian vertebrates; and (5) lateral eyes, which contain photoreceptors in all the vertebrate classes. Traditionally these diverse photoreceptor organs have been associated with two broad photosensory tasks. The lateral eyes, employing rod and cone photoreceptors, mediate image detection (vision), whilst the extra-retinal photoreceptors are thought to use environmental irradiance cues for tasks which include: the regulation (entrainment) of circadian rhythms; behavioural orientation; the regulation of body pigmentation and colouration; and the regulation of pupil size [1].

*Corresponding author: Fax: (44)-207-594 5449.
E-mail: j.bellingham@ic.ac.uk

[1] These authors contributed equally to this work.

The photoreceptors of all animals appear to utilise photopigments with broadly conserved characteristics; all consisting of a form of opsin protein coupled to a chromophore derived from an 11-*cis* form of vitamin A retinaldehyde [2]. Photoisomerisation of 11-*cis* retinal to the all-*trans*-state induces conformational changes in the opsin, which in-turn, activate a G-protein (transducin) phototransduction cascade. The identification of such photopigments in extra-retinal photoreceptors has, until recently, relied upon the use of antibodies raised against purified retinal rod- and cone-opsins. By employing such antibodies, a large number of studies have succeeded in labelling a number of different extra-retinal photoreceptor populations e.g. [1,3]. These findings led to two conclusions: (1) that extra-retinal photopigments are opsin based, and (2) that identical photopigments are shared by the visual and extra-retinal photoreceptor system [1]. The second of these two conclusions forms the central focus of the current paper.

Because of their differing photosensory tasks, light environment, development and evolutionary histories, we decided to examine the assumption that the photopigment genes expressed in retinal and extra-retinal photoreceptors are the same. To this end we have: (a) used immunocytochemical approaches to demonstrate the presence of rod- and cone-like opsin in the retina and pineal of the Atlantic salmon (*Salmo salar*); (b) compared the rod-opsin cDNA sequences isolated from the pineal and retina of the Atlantic salmon; (c) compared rod-opsin cDNA sequences from cDNA libraries constructed from the eye and brain of a second teleost species, the puffer fish *Fugu rubripes*; (d) examined the phylogenetic relationship of the opsins isolated from the retina and pineal/brain. Our results show that the rod-like opsins from retinal and extra-retinal photoreceptors are encoded by different genes in these teleost fish.

2. Materials and methods

2.1. Opsin immunocytochemistry

Salmon were anaesthetised with tricaine methanesulphonate (MS-222, Sigma) and killed by intracardiac perfusion with Bouin's fixative. Brains were removed and placed in the same fixative for 48 h prior to dehydration, paraffin embedding and sectioning. Transverse and sagittal sections (8 μm thick) were collected on gelatin-coated slides and hydrated in 0.1 M PBS containing 0.2% Triton X-100 (PBS-T) for 30 min. Endogenous peroxidase activity was blocked by incubation for 10 min. in 0.1 M PBS (pH 7.4) containing 1% methanol and 0.3% H_2O_2, followed by three washes in PBS-T. Background staining was blocked by incubating the section for 20–30 min. in blocking serum (diluted 1/30 in PBS-T). Sections were then transferred into one of two primary antisera (72 h in a humid chamber at 4°C): (1) CERN 874 antisera raised in rabbits against purified undifferentiated chicken

PII: S0014-5793(00)01217-5

cone opsins. This antibody is specific for vertebrate cone opsins and will not cross-react with rod-opsins at the dilutions used in this study (1:4000 in PBS-T) [4]; (2) CERN 858 antisera was raised in rabbits against purified lipid free bovine rhodopsin [5,6]. This antisera is monospecific for rod-opsin [7,8] and was used at a dilution of 1:2000 in PBS-T.

Following fixation, several isolated pineals and retinas were embedded in Araldite resin (Durcupan, Fluka). Blocks were sectioned (1.5 μm) with glass knives on a Reichert-Jung Ultracut Ultramicrotome. After removal of the resin with sodium ethoxide, immunocytochemical protocols were identical to those used for paraffin sections.

2.2. Fugu cDNA library screen

Gridded cDNA libraries constructed from eye and brain of the puffer fish *F. rubripes* were obtained from the UK HGMP Resource Centre, Cambridge, UK, and probed with a goldfish (*Carassius auratus*) rod-opsin cDNA probe. The probe was labelled with α-^{32}P-dCTP (Amersham) using an Oligonucleotide Labelling kit (Pharmacia) according to the manufacturer's instructions. The labelled probe was 'cleaned up' with an MicroSpin S-200 HR Column (Pharmacia) prior to hybridisation against the filters in 0.5 M sodium phosphate buffer, pH 7.2, 7% SDS at 62°C. The filters were then washed on 0.2×SSC at 62°C and exposed against X-ray film at −80°C for 14 h with two intensifying screens. Positive clones identified were obtained from the UK HGMP Resource Centre.

2.3. mRNA extraction and RT-PCR

Retinas and pineal complexes were dissected from pre-smolt Atlantic salmon (*S. salar*) and mRNA extracted using a QuickPrep Micro mRNA Purification kit (Pharmacia). Single-stranded cDNA was synthesised using a 3′RACE System (Gibco BRL) for use in polymerase chain reactions (PCRs) using the supplied antisense primer (AUAP) and a sense degenerate oligonucleotide primer (TELROD5′; 5′-caaccATGAAYGGNACNGARGG-3′) designed to consensus sequence of the 5′UTR (lowercase above) and coding region (uppercase above) junction of previously sequenced rod-opsin genes: goldfish, *C. auratus* (L11863); common carp, *Cyprinus carpio* (U02475); zebrafish, *Danio rerio* (AF109368); Japanese medaka, *Oryzias latipes* (AB001606); sandgoby, *Pomatoschistus minutus* (X62405); *Neoniphon sammara* (U57536); and puffer fish, *F. rubripes* (AF201471 and AF201472). PCRs were performed in a total volume of 25 μl with 12.5 pmol of each primer, 0.5 nmol each of dATP, dCTP, dGTP and dTTP, and 0.25 units of Bio*Taq* polymerase (Bioline) in the manufacturers NH_4 buffer at an annealing temperature of 58°C and at 2.0 mM $MgCl_2$. The amplified products from three separate PCRs were cloned into pGEM-T-Easy (Promega) prior to sequencing to eliminate the possibility of PCR generated sequence errors.

2.4. Sequencing

All nucleotide sequence determination was carried out on an ABI PRISM 377 DNA Sequencer (Perkin Elmer) using the ABI PRISM BigDye Terminator Cycle Sequencing kit (Perkin Elmer). Both strands of all clones were sequenced.

2.5. Tissue expression

Gene specific oligonucleotide primer pairs were designed to unique regions of the 3′UTR of the salmon retinal rod-opsin (RETUTRF, 5′-CCACAAAGAAGACTTCTGCTC-3′, corresponding to bases 1105–1125; RETUTRR, 5′-GCGATTTATTACGTTGCCTTG-3′, complementary to bases 1537–1517 of AF201470), and salmon pineal rod-like opsin (PINUTRF, 5′-AACCCTGACTCCTTACCT-3′, corresponding to bases 1081–1101; PINUTRR, 5′-ACATTATTAACACTAGTCCTG-3′, complementary to bases 1904–1884 of AF201469) sequences. PCRs using the above primer pairs (at an annealing temperature of 58°C and at 1.5 mM $MgCl_2$) were carried out using salmon retinal and pineal cDNA. Primer pair RETUTRF/RETUTRR yields a fragment of 433 bp in length and primer pair PINUTRF/PINUTRR yields a fragment of 824 bp in length. PCR products were separated by agarose gel electrophoresis.

2.6. Sequence and phylogenetic analysis

Database searches were conducted using the BLAST [9] server at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Nucleotide and amino acid alignments were carried out using the ClustalX 1.8 suite of programs using default values ([10]; ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). For phylogenetic purposes, the nucleotide alignments were adjusted using the SeaView program to maintain codon integrity prior to analysis in the Phylo_win package (both programs - [11]; http://pbil.univ-lyon1.fr/). Maximum parsimony and neighbour-joining trees were constructed with bootstrap confidence values based on 1000 replicates.

3. Results and discussion

3.1. Opsin immunocytochemistry of the Atlantic salmon central nervous system (CNS)

In this study we have employed two antibodies, CERN 858 (anti-rod opsin) and CERN 874 (anti-cone opsin), which have been used previously to characterise both retinal and extra-retinal photoreceptors [4,12–15]. On sections of *S. salar* retina

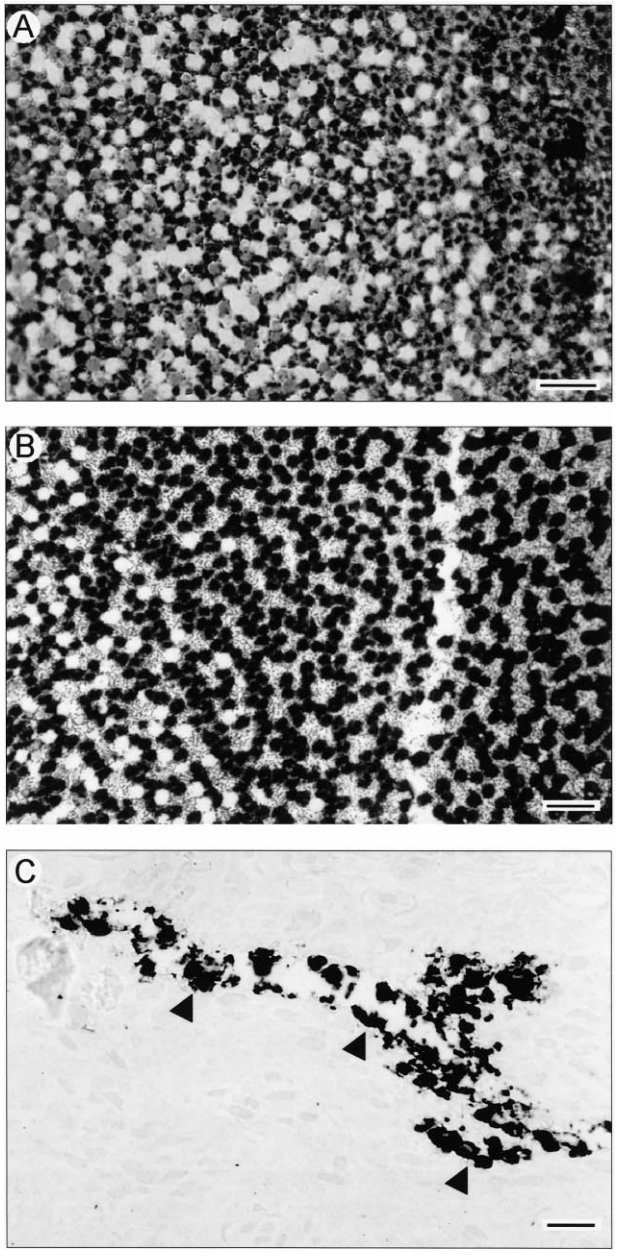

Fig. 1. Sections through the *S. salar* retina showing the retinal 'mosaic' of photoreceptors (A and B). A: Anti-cone opsin antisera (CERN 874). B: Anti-rod opsin antisera (CERN 858). C: Section through the pineal showing pinealocyte outer segments (arrows) labeled with anti-rod opsin antisera (CERN 858). Scale bar: 25 μm.

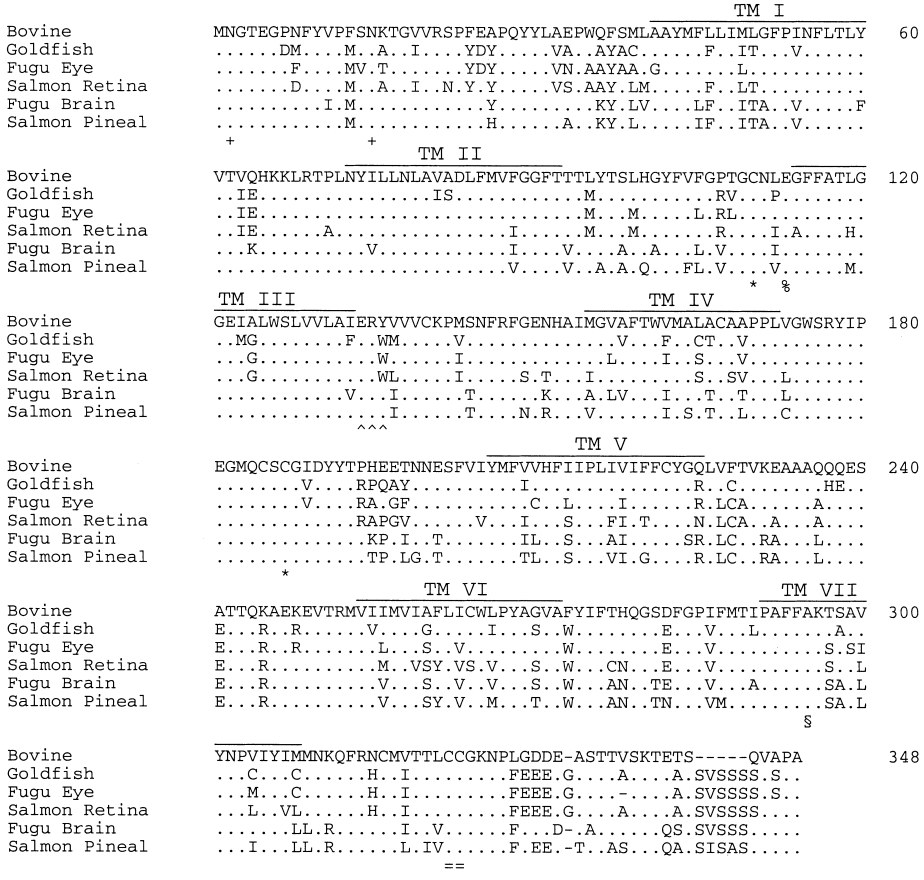

Fig. 2. Amino acid alignment of the retinal rod- and pineal/brain rod-like opsins with bovine (K00506) and goldfish (L11863) rod-opsin. Membrane embedded residues of the α-helices are indicated according to the model by Baldwin [48]. Conserved functional opsin features include: glycosylation sites Asn-2 and Asn-15 (+); disulphide bridge Cys-110 and Cys-187 (*); chromophore attachment site Lys-292 (§), and Schiff base counterion Glu-110 (%); palmitoylation site Cys-322 and Cys-323 (=); transducin binding Glu136Arg137Tyr/Trp138 (–).

(Fig. 1a,b) these antibodies labelled either rod or cone outer-segments in a manner which duplicated the rod and cone photoreceptor mosaic previously described in this species [16]. Thus these antibodies were shown to be sufficiently specific to differentiate between rod and cone opsins in *S. salar* without cross-reactivity. In the pineal of the same species, numerous pinealocytes were labelled with both of these antibodies (Fig. 1c; anti-cone data not shown). Comparison of consecutive (1.5 μm) semi-thin sections of the pineal suggested that rod and cone opsins were not co-expressed in the same cells (data not shown). We failed to identify rod-like opsin immunolabelling in other areas of the salmon CNS.

Table 1
Amino acid identity shared by the *F. rubripes* brain rod-like opsin and the *S. salar* pineal rod-like opsin with the retinal rod-opsins of other vertebrate groups and the cone opsins of another teleost (goldfish)

Organism	Species	GenBank	Identity (%)	
			Fugu	*Salmo*
Alligator rod	*Alligator mississippiensis*	U23802	80.5	76.8
Coelacanth Rh1	*Latimeria chalumnae*	AF131257	79.4	75.5
Chicken rod	*Gallus gallus*	S29152	78.5	75.1
Salamander rod	*Ambystoma tigrinum*	U36574	77.7	75.7
Bovine rod	*Bos taurus*	K00506	76.8	75.4
Cat shark rod	*Galeus melastomus*	Y17586	77.4	76.3
Goldfish rod	*C. auratus*	L11863	74.3	72.0
Eel fresh-water rod	*A. anguilla*	L78007	77.7	75.7
Eel deep-sea rod	*A. anguilla*	L78008	77.7	74.6
Puffer fish (eye)	*F. rubripes*	AF201471	74.5	72.8
Salmon (retina)	*S. salar*	AF201470	76.5	75.1
Puffer fish (brain)	*F. rubripes*	AF201472	–	85.8
Salmon (pineal)	*S. salar*	AF201469	85.8	–
Goldfish blue cone	*C. auratus*	L11864	46.7	44.5
Goldfish green cone	*C. auratus*	L11865	67.1	66.4
Goldfish red cone	*C. auratus*	L11867	41.1	39.8
Goldfish UV cone	*C. auratus*	D85863	41.9	41.9

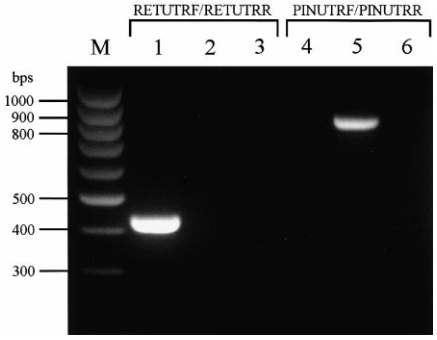

Fig. 3. Tissue specific expression of retinal rod- and pineal rod-like opsin in *S. salar* assayed by RT-PCR using gene specific primer pairs RETUTRF/RETUTRR (retinal rod, 433 bp) and PINUTRF/PINUTRR (pineal rod-like, 824 bp). PCR products were resolved by 3% agarose gel electrophoresis and visualised with ethidium bromide. Lanes: M, 100 bp marker (MBI Fermentas); 1 and 4, retinal cDNA; 2 and 5, pineal cDNA; 3 and 6, no DNA.

3.2. Isolation and sequence of retinal rod- and pineal/brain rod-like opsins

To determine whether the rod-opsins in the pineal are identical to the rod-opsins of the retina we used a combination of RT-PCR and cDNA library screening to isolate the retinal rod-opsins and pineal/brain rod-opsins of two species of teleost fish *S. salar* (Atlantic salmon) and *F. rubripes* (puffer fish). Using primer pairs TELROD5′/AUAP we were able to generate two PCR fragments that encompassed the coding region and 3′UTR of the rod-opsins from the retina and pineal of *S. salar*. The retinal rod-opsin has a 1065 bp ORF (predicting a 354 amino acid protein) and a 481 bp 3′UTR (GenBank: AF201470), whilst the pineal rod-opsin has a 1062 bp ORF (predicting a 353 amino acid protein) and a 910 bp 3′UTR (GenBank: AF201469). Screening the *F. rubripes* eye cDNA library with a goldfish rod-opsin probe identified multiple clones that potentially contained a rod-opsin sequence, whereas only a single clone was identified in the *F. rubripes* brain cDNA library, clone 16h22. Three clones from the eye library were selected and obtained from the UK HGMP Resource

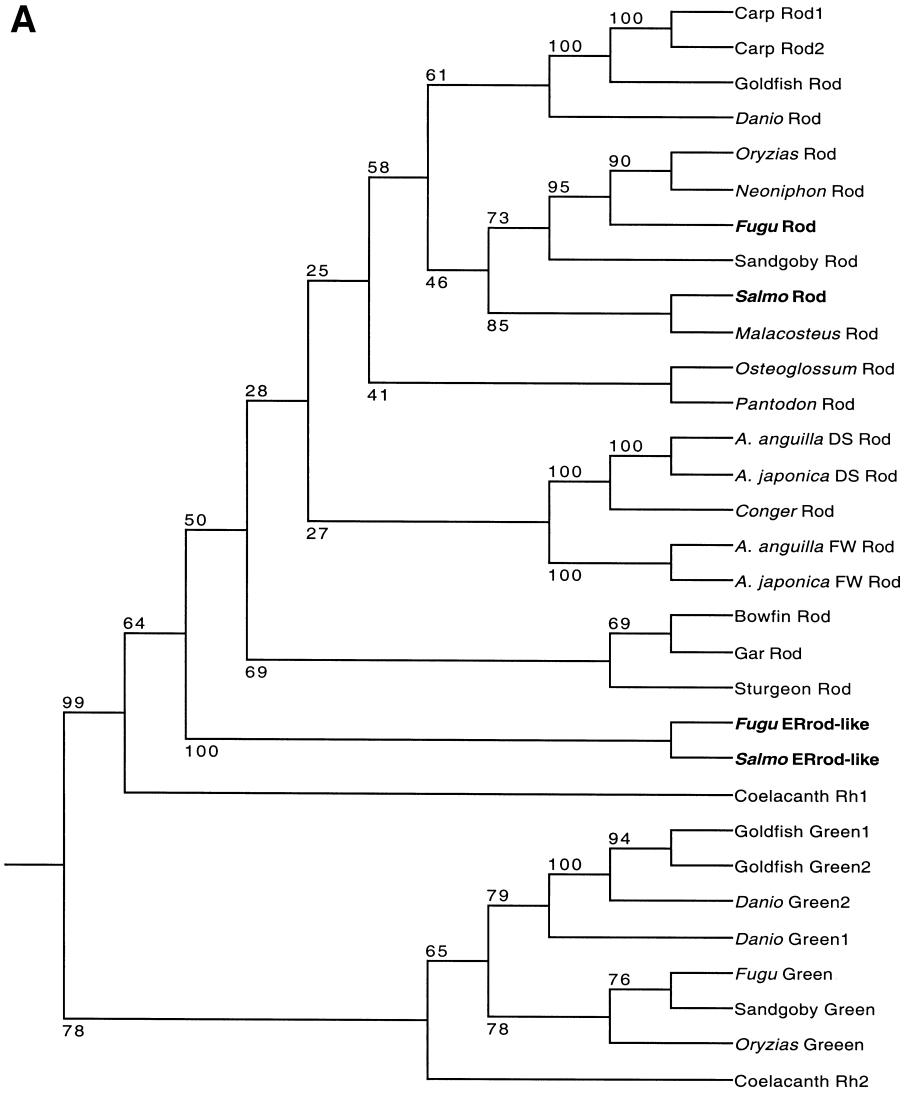

Fig. 4 (*to be continued*).

Centre. Plasmid DNA was prepared and the clone insert excised by digestion with *Eco*RI and *Xho*I and then subjected to agarose gel electrophoresis. The longest of the three inserts was chosen for characterisation - clone 27l6. Clone 27l6 contains a 1513 bp insert (excluding the poly-A tail) consisting of 49 bp of 5′UTR, a 1062 bp open reading frame (ORF) predicting a 353 amino acid protein, and a 402 bp 3′UTR (GenBank: AF201471). Similarly, clone 16h22 contains a 1906 bp insert (excluding the poly-A tail) consisting of 122 bp 5′UTR, a 1062 bp ORF predicting a 353 amino acid protein, and a 722 bp 3′UTR (GenBank: AF201472).

Intra-species comparison of the *S. salar* and *F. rubripes* eye/retina and pineal/brain rod-opsins indicates that they share only 72.9% and 74.7% identity at the coding nucleotide level, respectively (74.5% and 72.8% identity at the amino acid level). Inter-species comparison of the amino acid sequences indicates that the pineal/brain rod-opsins share 85.8% identity and the eye/retina rod-opsins share 84.2% identity. However, BLAST analysis of the *S. salar* and *F. rubripes* pineal/brain rod-opsin amino acid sequences indicate that they generally share greater identity with other classes of vertebrate rod-opsin than with intra-species or inter-species teleost retinal rod-opsins (data not shown). Pairwise comparison of the *S. salar* and *F. rubripes* pineal/brain rod-opsins with the retinal rod-

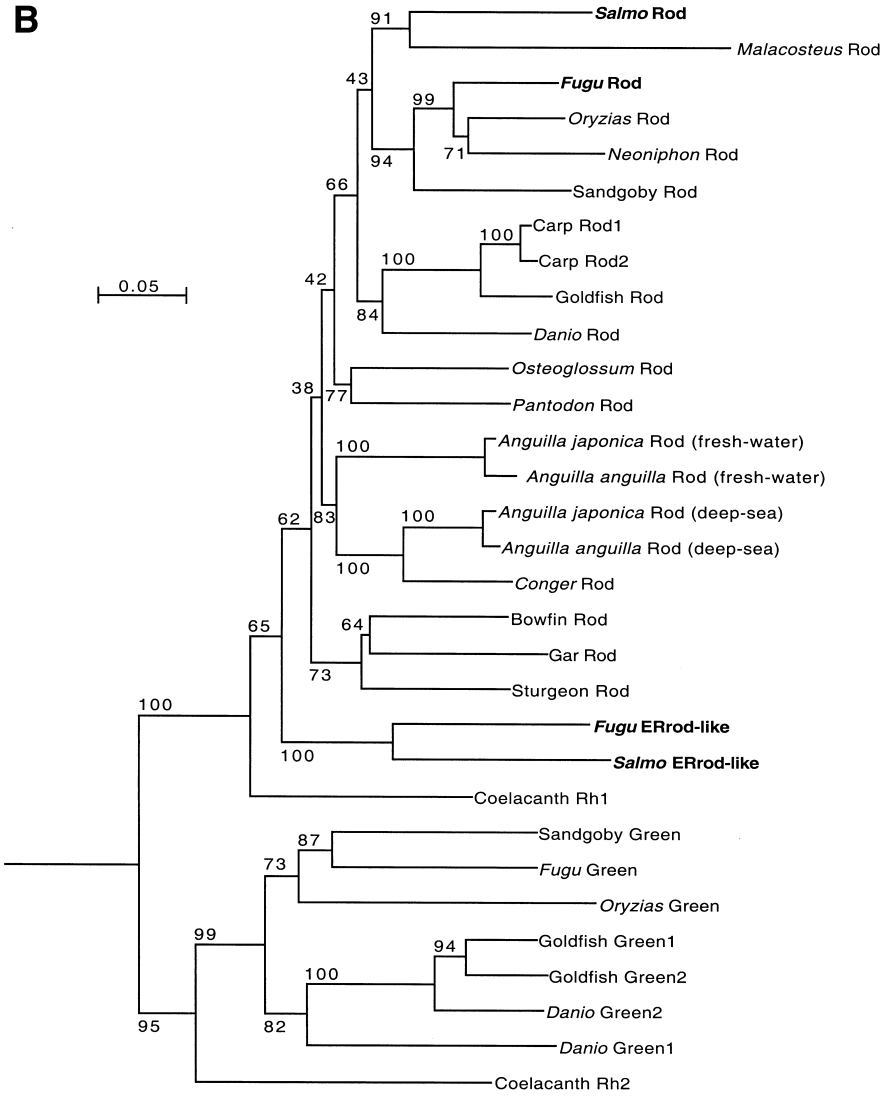

Fig. 4. Maximum parsimony (A) and neighbour-joining (B) trees of the rod-, ERrod-like and green cone opsins of members of the Actinopterygii (ray-finned fishes) and a lobe-finned fish, the coelacanth (Sarcopterygii), constructed from nucleotide data. The blue cone-opsins of goldfish (L11864), zebrafish (AF109372), cavefish (AF134762-AF134766), and Japanese medaka (AB001602), as an outgroup. Bootstrap confidence values are based on 1000 replicates. The neighbour-joining tree is corrected by the Kimura method [42] and the scale bar is calibrated in substitutions per site. Nucleotide accession numbers: *S. salar* rod, AF201470; *S. salar* ERrod-like, AF201469; *F. rubripes* rod, AF201471; *F. rubripes* ERrod-like, AF201472; goldfish rod, L11863; carp rod1, U02475; carp rod2, Z71999; zebrafish rod, AF109368; *O. latipes* rod, AB001606; *N. sammara* rod, U57536; sandgoby rod, X62405; *Malacosteus niger* rod, AJ224691; *Osteoglossum* sp. rod, AF137209; *Pantodon buchholzi* rod, AF137210; *A. anguilla* deep-sea (DS) rod, L78008; *A. anguilla* fresh-water (FW) rod, L78007; *A. japonica* deep-sea (DS) rod, AJ249203; *A. japonica* fresh-water (FW) rod, AJ249202; *Conger conger* rod, S82619; bowfin rod, AF137208; gar rod, AF137207; sturgeon rod, AF137206; coelacanth Rh1, AF131257; coelacanth Rh2, AF131258; goldfish green1, L11865; goldfish green2, L11866; zebrafish green1, AF109369; zebrafish green2, AF109370; *F. rubripes* green, AF226989; sandgoby green, Y18679; *O. latipes* green, AB001603.

opsins of representatives of non-teleost of vertebrate are shown in Table 1. The absence of classical a rod photoreceptor cell in the teleost pineal/brain therefore enables us to define these novel opsins as being rod-like in character.

From Fig. 2, it can be seen that both pineal/brain rod-like opsins exhibit the characteristic motifs of opsins (numeration with respect to bovine rod-opsin). Asn2 and Asn15 at the amino terminus on the extracellular side are two glycosylation sites [17]; Cys-110 in the first extracellular loop and Cys-187 in the second extracellular loop which are required for correct conformational folding of the opsin molecule [18]. A further two cysteine residues, Cys-322 and Cys-323, are palmitoylated and anchor the carboxyl tail so forming a fourth cytoplasmic loop [19]. The chromophore, 11-*cis*-retinal, is covalently bound via a protonated Schiff base to Lys-292 in the seventh α-helix (TM VII), and the Glu-113 residue acts as the counterion to the Schiff base [20]. An ER(Y/W) motif at positions 134–136 [21], and Ser-240 [22], are necessary for binding of the α-subunit of transducin and are conserved, as are three sites that are considered essential for transducin activation Leu-226, Thr-243 [23] and Tyr-306 [24]. However, between the salmon pineal rod-like opsin and retinal rod-opsin there are subtle changes in the residues that form the transducin binding pocket; 136–139 and 247–251 [25]. Two substitutions are observed between the rod- and rod-like opsins, Trp136Tyr and Val137Iso. Although these changes are not major, both tryptophan and tyrosine have aromatic side-chains while valine and isoleucine have large aliphatic side chains. These differences raise the possibility that different α-subunits of the G-protein transducin may be utilised. Collectively, these results suggest that the pineal/brain rod-like opsins will form a stable photopigment that will activate a phototransduction cascade that is the same or very similar to that present in the retina.

3.3. Spectral tuning of the rod and rod-like opsins

The spectral tuning of opsins has recently been extensively reviewed by Bowmaker and Hunt [26], and we have used their model to define potential intra-specific and inter-specific amino acid substitutions that may be involved in spectrally tuning the rod- and rod-like opsins of *F. rubripes* and *S. salar*. However, since the λ-max of the retinal rod- and pineal/brain rod-like opsins of either species have yet to be determined, any conclusions drawn will be based on probability rather than observation.

We have identified three intra-specific and one inter-specific site that may be involved in the spectral tuning of these two opsins, with the substitutions at all four sites (which face into the chromophore binding pocket) involving the replacement of non-polar residues by hydroxyl-bearing polar residues. Position 164 in TM IV is occupied by an alanine in *S. salar* retinal rod-opsin and by a serine in *S. salar* pineal rod-like opsin. Substitution of alanine by serine at the equivalent position in longwave cone opsins (180) causes a 2–7 nm red shift [26]. Two further putative intra-specific site substitutions are Ser168Ala and Ser264Cys between the retinal rod-opsin and pineal rod-like opsin of *S. salar*, though amino acid substitution at these sites have yet to be shown to have spectral tuning properties. The single inter-specific spectral tuning site is position 261 which is occupied by phenylalanine in both opsin types from *F. rubripes* and by tyrosine in both *S. salar* opsin types. Yokoyama et al. [27] have shown that a Tyr261Phe substitution in the rod-opsin of the cavefish, *Astyanax fasciatus*, causes an 8 nm blue shift in λ-max of the pigment, and similar substitutions have been shown to cause 7–9 nm shifts between primate longwave-red and longwave-green cone pigments [26].

At an intra-specific level, few conclusions may be drawn as to whether a spectral shift exists between the rod- and rod-like opsins of *F. rubripes*, but the Ala164Ser substitution observed in *S. salar* suggests that the pineal rod-like opsin will be slightly red-shifted when compared to the retinal rod-opsin. A red-shift in photosensitivity that might be expected in the haemoglobin filtered light environment of encephalic photoreceptors [28]. Furthermore, the Tyr261Phe substitutions observed between both opsin types of *S. salar* and *F. rubripes* suggest that the opsins of *S. salar* will be red shifted when compared to those of *F. rubripes*. These observations may be explained by considering that pre-smolt *S. salar* live in fresh water, where the level of dissolved organic material is higher than in the sea water environment inhabited by *F. rubripes*. These dissolved solids tend to favour red-shifted visual pigments [29,30].

3.4. Tissue specific expression of the rod- and rod-like opsins

Tissue expression patterns of the salmon retinal rod and pineal rod-like opsin genes were assessed by gene specific PCR on retinal and pineal cDNA using primer pairs RETUTRF/RETUTRR and PINUTRF/PINUTRR. From Fig. 3 it can be seen that the retinal rod-opsin is uniquely expressed in the retina whilst the pineal rod-like opsin is exclusively expressed in the pineal. This is in contrast to the expression of rod-opsin in pigeon where the same transcript appears to be expressed in both the retina and the deep brain [31]. On the basis of the expression pattern of the two opsin forms, we have termed the pineal rod-like opsins 'extra-retinal rod-like opsin' (ERrod-like opsin). The nature of the cDNA library (brain) from which the *F. rubripes* ERrod-like opsin was isolated precludes us from determining specific sites of expression within the CNS, but we predict a similar distribution to that observed in *S. salar*.

Whilst this manuscript was being considered for publication, a paper was published by Mano et al. [32] describing a novel opsin (Exo-rhodopsin) in zebrafish. Exo-rhodopsin is clearly a member of the rod-like gene family we describe here. We would like to emphasise that the term 'rhodopsin' refers to any vitamin A_1 (11-*cis*-retinal) based photopigment, whilst the term 'porphyropsin' refers to any vitamin A_2 (3-dehydroretinal) based photopigment [33]. Since teleosts may have both $A_1$and A_2 chromophores in both the retinal and pineal photoreceptors [34], the term 'exo-rhodopsin' may lead to confusion as to the nature of the chromophore utilised by this novel family of photopigments. Thus, for clarity, we suggest the term ERrod-like opsin.

3.5. Evolution of the retinal rod- and ERrod-like opsins

We have constructed both maximum parsimony and neighbour-joining trees (see [11] for details of algorithms) for the rod-opsins and green cone opsins from fish (which have been termed as being rod-like also [26]), with the addition of the ERrod-like opsins (Fig. 4). From both the maximum parsimony tree (Fig. 4a) and the neighbour-joining tree (Fig. 4b), it can be seen that the lineage of the ERrod-like opsins is well supported and forms a second group extant from the rod-

opsins of other Actinopterygii (ray-finned fishes) after their divergence from the Sarcopterygii (lobe-finned fishes) such as the coelacanth, *Latimeria chalumnae*.

This suggests that a gene duplication event has occurred at some point early in the lineage of the ray-finned fishes prior to their radiation - a gene duplication that is separate from, for example, the duplication that has occurred in the Elopomorpha giving rise to deep-sea and fresh-water retinal rod-opsins in the European eel, *Anguilla anguilla* [30]. Similarly, the allelic variation present in the rod-opsins of the carp, *C. carpio*, [35,36] probably results from the tetraploid genome of this species [37]. It is unlikely that tetraploidy is the cause of the differences we see between the retinal and extra-retinal opsins since *F. rubripes* is diploid [38]. *S. salar* is also diploid [39], although, salmonid fish have been described as existing in a quasi-tetraploid state since they have originated from a tetraploid ancestor and the diploidisation process is yet to be fully completed [40].

In order to estimate when the gene duplication occurred, we have used a method similar to that employed by [41] to determine the divergence of the rod-opsin and ERrod-like opsin genes. Pairwise comparison of the nucleotide sequences (substitutions per synonymous site between species corrected for multiple substitutions by the Kimura method [42]; data not shown) of the retinal rod-opsins of *F. rubripes* and *S. salar* with those of three Cyprinids (goldfish, L11863; zebrafish, AF109368; carp, U02475, Z71999) yield an average value of 0.229 substitutions per synonymous site (K_1). Pairwise comparison of the nucleotide sequences of the ERrod-like opsins of *F. rubripes* and *S. salar* with their respective retinal rod-opsins yields an average value of 0.336 substitutions per synonymous site (K_2). The emergence of the Ostariophysi (of which the Cyprinids are members) about 140 million years ago (Mya) provides a value for T_1. Applying the formula $K_1/(2T_1)$ gives a rate of divergence (r) of 8.1786×10^{-10} substitutions/site/year [43]. Using this value of r to set the molecular clock of teleost rod-opsins, we can estimate the time of divergence of the rod-opsin and ERrod-like opsin genes (T_2) with the following formula $T_2 = K_2/(2r)$ [43] at approximately 205.4 Mya. Thus, the divergence of the rod- and ERrod-like opsins appears to have occurred prior to the divergence of the Chrondrostei (sturgeons) from the Neopterygii (teleosts, gars and bowfin) which is estimated as occurring some 200 Mya [44]. Interestingly, both trees show the co-segregation of the rod-opsins of the more primitive members of the Actinopterygii (sturgeons, bowfin and gar) as being distinct from those of the Teleostei, and this probably reflects the indefenite classification of these primitive groups [45,46]. Analysis of the opsins present in the pineal of sturgeon and the primitive members of the Neopterygii such as bowfin and gar will confirm or contradict our proposed evolutionary lineage of the retinal rod- and ERrod-like opsins of the Actinopterygii.

4. Conclusions

Because of the differing photosensory tasks, light environment, development, and evolutionary history of retinal and extra-retinal photoreceptors, we decided to examine the assumption that the rod-opsin photopigments in these photoreceptor organs are the same. In two species of teleost fish, cDNA ERrod-like opsins were isolated which share only 74% nucleotide and amino acid identity with their corresponding rod-opsins from the retina. The basis for the sequence differences between the ERrod-like opsins and retinal rod-opsins remains unclear, and may be related to the differing photosensory roles of the retinal and extra-retinal photoreceptors, and/or as a result of genetic drift of these opsins after the gene duplication event. Functional analysis of the ERrod-like photopigments, and the isolation of additional extra-retinal opsins should help resolve these alternatives. An additional example of pineal specialisation is the recent discovery of retinal and pineal specific arylalkylamine *N*-acetyltransferase (AANAT) genes isolated from two teleost fish - trout and pike. The evolution of two AANAT genes may represent a strategy for tissue optimisation of the photic regulation of melatonin synthesis [47]. It is therefore possible that a number of the photosensory elements of the fish pineal might be specialised for encephalic light detection.

Acknowledgements: This publication is the result of research sponsored by the Biotechnology and Biological Sciences Research Council (BBSRC), UK; EU BioMed2 and DGE PV97 1285 (Spain). The authors are grateful to the help and assistance provided by Robert Turner, Tania Joyce and Ian Morris (Imperial College). We thank Dr W.J. de Grip for the generous gift of anti-opsin antibodies. Our gratitude also extends to the UK HGMP Resource Centre, Hinxton, Cambridge, CB10 1SB for supplying the *F. rubripes* gridded cDNA libraries and clones used in this study.

References

[1] Shand, J. and Foster, R.G. (1999) in: Adaptive Mechanisms in the Ecology of Vision (Archer, S.N., Djamgoz, M.B.A., Loew, E.R., Partridge, J.C. and Vallerga, S., Eds.), pp. 197–222, Kluwer Academic Publishers, Dordrecht.
[2] Wald, G. (1968) Science 162, 230–239.
[3] Foster, R.G. and Soni, B.G. (1998) Rev. Reprod. 3, 145–150.
[4] Foster, R.G., Garcia-Fernandez, J.M., Provencio, I. and DeGrip, W.J. (1993) J. Comp. Physiol. A 172, 33–45.
[5] Margry, R.J.C.F., Jacobs, C.W.M., DeGrip, W.J. and Daemen, F.J.M. (1983) Biochim. Biophys. Acta 742, 463–470.
[6] Schalken, J.J. and DeGrip, W.J. (1986) Exp. Eye Res. 43, 431–439.
[7] Schalken, J.J. (1987) Ph.D. Thesis, University of Nijmegen.
[8] Janssen, J.J.M. (1991) Ph.D. Thesis, University of Nijmegen.
[9] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) J. Mol. Biol. 215, 403–410.
[10] Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) Nucleic Acids Res. 25, 4876–4882.
[11] Galtier, N., Gouy, M. and Gautier, C. (1996) Comput. Appl. Biosci. 12, 543–548.
[12] Foster, R.G., Grace, M.S., Provencio, I., Degrip, W.J. and Garcia-Fernandez, J.M. (1994) Neurosci. Biobehav. Rev. 18, 541–546.
[13] Garcia-Fernandez, J.M. and Foster, R.G. (1994) Cell Tissue Res. 275, 319–326.
[14] Grace, M.S., Alones, V., Menaker, M. and Foster, R.G. (1996) J. Comp. Neurol. 367, 575–594.
[15] Garcia-Fernandez, J.M., Jimenez, A.J., Gonzalez, B., Pombal, M.A. and Foster, R.G. (1997) Cell Tissue Res. 288, 267–278.
[16] Wagner, H.J. (1990) in: The Visual System of Fish (Douglas, R.H. and Djamgoz, M.B.A., Eds.), pp. 109–157, Chapman and Hall, London.
[17] Hargrave, P.A. (1977) Biochim. Biophys. Acta 492, 83–94.
[18] Karnik, S.S., Sakmar, T.P., Chen, H.B. and Khorana, H.G. (1988) Proc. Natl. Acad. Sci. USA 85, 8459–8463.
[19] Ovchinnikov Yu, A., Abdulaev, N.G. and Bogachuk, A.S. (1988) FEBS Lett. 230, 1–5.
[20] Sakmar, T.P., Franke, R.R. and Khorana, H.G. (1989) Proc. Natl. Acad. Sci. USA 86, 8309–8313.
[21] Franke, R.R., Konig, B., Sakmar, T.P., Khorana, H.G. and Hofmann, K.P. (1990) Science 250, 123–125.
[22] Pepe, I.M. (1999) J. Photochem. Photobiol. B 48, 1–10.

[23] Yang, K., Farrens, D.L., Hubbell, W.L. and Khorana, H.G. (1996) Biochemistry 35, 12464–12469.
[24] Cai, K., Klein-Seetharaman, J., Farrens, D., Zhang, C., Altenbach, C., Hubbell, W.L. and Khorana, H.G. (1999) Biochemistry 38, 7925–7930.
[25] Acharya, S., Saad, Y. and Karnik, S.S. (1997) J. Biol. Chem. 272, 6519–6524.
[26] Bowmaker, J. and Hunt, D.M. (1999) in: Adaptive Mechanisms in the Ecology of Vision (Archer, S.N., Djamgoz, M.B.A., Loew, E.R., Partridge, J.C. and Vallerga, S., Eds.), pp. 439–462, Kluwer Academic Publishers, Dordrecht.
[27] Yokoyama, R., Knox, B.E. and Yokoyama, S. (1995) Invest. Ophthalmol. Vis. Sci. 36, 939–945.
[28] Foster, R.G. and Follett, B.K. (1985) J. Comp. Physiol. A 157, 519–528.
[29] Lythgoe, J.N. (1979) The Ecology of Vision, Clarendon Press, Oxford.
[30] Archer, S., Hope, A. and Partridge, J.C. (1995) Proc. R. Soc. Lond. B Biol. Sci. 262, 289–295.
[31] Wada, Y., Okano, T., Adachi, A., Ebihara, S. and Fukada, Y. (1998) FEBS Lett. 424, 53–56.
[32] Mano, H., Kojima, D. and Fukada, Y. (1999) Mol. Brain Res. 73, 110–118.
[33] Archer, S.N. (1999) in: Adaptive Mechanisms in the Ecology of Vision (Archer, S.N., Djamgoz, M.B.A., Loew, E.R., Partridge, J.C. and Vallerga, S., Eds.), pp. 25–41, Kluwer Academic Publishers, Dordrecht.
[34] Tabata, M., Suzuki, T. and Niwa, H. (1985) Brain Res. 338, 173–176.
[35] Tsai, H.J., Shih, S.R., Kuo, C.M. and Li, L.K. (1994) Comp. Biochem. Physiol. B Biochem. Mol. Biol. 109, 81–88.
[36] Lim, J., Chang, J.L. and Tsai, H.J. (1997) Biochim. Biophys. Acta 1352, 8–12.
[37] Zhang, H., Okamoto, N. and Ikeda, Y. (1995) Gene 153, 231–236.
[38] Bradford, C.S., Miller, A.E., Toumadje, A., Nishiyama, K., Shirahata, S. and Barnes, D.W. (1997) Mol. Mar. Biol. Biotechnol. 6, 279–288.
[39] Johnston, I.A., Strugnell, G., McCracken, M.L. and Johnstone, R. (1999) J. Exp. Biol. 202, 1991–2016.
[40] Hordvik, I. (1998) Immunol. Rev. 166, 153–157.
[41] Hunt, D.M., Fitzgibbon, J., Slobodyanyuk, S.J., Bowmaker, J.K. and Dulai, K.S. (1997) Mol. Phylogenet. Evol. 8, 415–422.
[42] Kimura, M. (1980) J. Mol. Evol. 16, 111–120.
[43] Li, W.-H. and Graur, D. (1991) Fundamentals of Molecular Evolution, Sinauer Associates, Sunderland, MA.
[44] Cantatore, P., Roberti, M., Pesole, G., Ludovico, A., Milella, F., Gadaleta, M.N. and Saccone, C. (1994) J. Mol. Evol. 39, 589–597.
[45] Alexander, R.M. (1981) The Chordates, Cambridge University Press, Cambridge.
[46] Bond, C.E. (1996) Biology of Fishes, Saunders College Publishing, Orlando, FL.
[47] Coon, S.L., Begay, V., Deurloo, D., Falcon, J. and Klein, D.C. (1999) J. Biol. Chem. 274, 9076–9082.
[48] Baldwin, J.M., Schertler, G.F. and Unger, V.M. (1997) J. Mol. Biol. 272, 144–164.