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Opsin detection in the sea urchin *Paracentrotus lividus* and the sea star *Asterias rubens*

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Abstract: Vision in metazoans is permitted by opsin expression in photoreceptor cells. These opsins can be mainly classified into two groups: the ciliary (c) and the rhabdomeric (r) opsins. Based on the r-opsin sequence of the sea urchin *Strongylocentrotus purpuratus*, degenerate primers were designed and used to amplify homologous opsin genes in the sea urchin *Paracentrotus lividus* and the sea star *Asterias rubens* by PCR. Partial r-opsin sequences were obtained for both species. These sequences are more similar between sea urchin species than between sea urchins and sea stars. In parallel, a commercial antibody, raised against the N-terminal domain of the rat rhodopsin, was used to detect c-opsins in Western blot experiments. Putative c-opsins were detected in the oral integument of *P. lividus*. We also detected c-opsins in the aboral integument of *A. rubens*, but not in the podia nor in the optic cushions. In all eventualities, it is therefore likely that two types of opsins co-occur in *A. rubens* and *P. lividus* as it is the case in *S. purpuratus*. In *A. rubens*, one opsin type would be located in the optic cushions (r-opsins) and another in the aboral integument (c-opsin). This could support the idea that sea stars possess two types of vision: the optic cushions, involved in visual perception of the environment, and the aboral integument, involved in non-visual photoreception.

Résumé : *Détection d'opsines chez l'oursin* Paracentrotus lividus *et l'étoile de mer* Asterias rubens. La vision chez les métazoaires est permise par l'expression d'opsines au sein des cellules photoréceptrices. Ces opsines peuvent être principalement classées en deux grands groupes: les opsines ciliaires (c) et les opsines rhabdomériques (r). Sur base de la séquence d'opsine-r découverte dans le génome de l'oursin *Strongylocentrotus purpuratus*, des amorces dégénérées ont été confectionnées pour amplifier les gènes homologues chez l'oursin *Paracentrotus lividus* et l'étoile de mer *Asterias rubens* par PCR. Des séquences partielles d'opsines-r ont été obtenues pour ces deux espèces. Ces séquences sont plus semblables entre espèces d'oursins qu'entre oursins et étoiles de mer. En parallèle, un anticorps commercial dirigé contre la partie N-terminale de la rhodopsin de rat (opsine-c) a été utilisé sur Western blots afin de détecter des opsines-c hypothétiques chez les deux espèces étudiées. Des opsines-c potentielles ont été détectées dans le tégument oral de *P. lividus*. Des opsines-c ont aussi été mises en évidence dans le tégument aboral d'*A. rubens* mais pas au niveau des podia ni des taches oculaires. Il est donc clair qu'au moins deux types d'opsines sont co-exprimées chez *A. rubens* et *P. lividus* comme c'est le cas chez *S. purpuratus*. Chez *A.rubens*, un type serait localisé dans les taches oculaires (opsine-r) et l'autre (opsine-c) dans le tégument aboral. Ces résultats supportent l'idée que les étoiles de mer possèdent deux types de photoréception: les taches oculaires, impliquées dans une perception plus visuelle de l'environnement et le tégument aboral impliqué dans une photoréception diffuse et non-visuelle.

Keywords: Echinoderms • Sea-urchins • Sea-stars • Photoreception • Opsin

Introduction

Since the publication of the Origin of species by Charles Darwin, the evolutionary story of metazoan photoreceptors has always been a subject of controversy. Although morphological comparisons supported the hypothesis of a convergent appearance of photoreceptors in evolutive lineages (Yoshida, 1966; Cobb, 1987), molecular studies do not support this hypothesis. On the contrary, sequence analyses suggest that the common ancestor of all the bilaterians, Urbilateria, possessed photoreceptors expressing opsins and other specific actors of the phototransduction cascade, and developing under the control of genes such as PAX6 (Arendt, 2003).

In metazoans, luminous information is detected through photosensitive pigments, the opsins, which are involved in both vision and non-visual photoreception. Classically animal photoreceptor cells are classified into two morphological types, the ciliary and the rhabdomeric photoreceptors, according to their membrane specializations (microvillar rhabdom and ciliary extensions, respectively) (Eakin, 1968). These specializations maximize the surface area where opsins can be expressed. In parallel, the "rhabdomeric/ciliary" distinction was also observed at the molecular level and the analysis of metazoan opsin and phototransduction protein sequences permitted to discover that specific phototransduction actors are associated to each type of photoreceptors cells (Yau & Hardi, 2009). The so-called c-opsins, for example, are present in ciliary photoreceptors whereas r-opsins occur in rhabdomeric photoreceptors. As a general rule, ciliary photoreceptors and c-opsins are involved in the visual processes of deutorostomes while rhabdomeric photoreceptors and r-opsins predominantly take part in protostome vision. However, ciliary photoreceptors can be found in protostomes and rhabdomeric one in deuterostomes but mainly involved in secondary photoreception processes (Nilsson, 2005).

Due to their sessile or slow-moving way of life, echinoderms have been considered as exhibiting only basic sensory capacities. However, many species are known to have complex responses to light such as colour change, covering reaction, spine or tube foot reaction, shade seeking, directed phototaxis, diurnal migration, regulation of reproductive cycles and even spatial vision (Yoshida, 1966; Hendler, 2004). The publication of the complete genome of the sea urchin *Strongylocentrotus purpuratus* boosted the investigations on the photosensory system of echinoderms. Raible et al. (2006) discovered a complex sensory receptor range in *S. purpuratus*, its genome coding for at least six opsin proteins homologous to metazoans opsins. Among them, one ciliary (Sp-opsin 1) and one rhabdomeric (Sp-opsin 4) opsins were detected. Excepted for the urchin *S. purpuratus*, little information is available about the presence and the diversity of opsin in other echinoderm species (Burke et al., 2006; Raible et al., 2006). Recently, Ullrich-Lüter et al. (2011) showed by immunodetections the expression of a r-opsin protein (homologous to Sp-opsin 4) in the tube feet of the sea urchin *Paracentrotus lividus* and in the optic cushions of the sea star *Asterias rubens*. To add new information on the photosensory capabilities of these two species, we first confirmed the presence of the opsin 4 gene in *P. lividus* and *A. rubens* by PCR amplification of genomic DNA, and we then investigated the expression of c-opsins by western blot using a commercial antibody against a mammalian rhodopsin (a ciliary opsin). These latter experiments allowed us to obtain important information about the areas where these opsins are expressed.

Material and Methods

Sampling

Individuals of *A. rubens* Linné, 1758 were collected intertidally in Audresselles (Pas-de-Calais, France). They were kept in a marine aquarium with closed circulation (13°C, 33 salinity) and fed mussels (*Mytilus edulis*, Linné, 1758.). Individuals of *P. lividus* were obtained from the marine station of Luc-sur-Mer (Normandie). They were kept in a similar aquarium and fed corn and algae.

Rhabdomeric opsin gene amplification

Degenerate primers, potentially amplifying r-opsin sequence fragments (+/- 400bp) in echinoderms, were designed based on a conserved region of the r-opsin alignment including *S. purpuratus* (opsin 4 - XM_003730498), *Homo sapiens* (NM_033282.3), *Platynereis sp.* (AJ316544.1), and *Drosophila melanogaster* (X65877.1). Their sequences are listed in Table 1.

Genomic DNA of *A. rubens* and *P. lividus* was extracted with the commercial Invitek Spin Tissue Mini kit (Invisorb). R-opsin fragments were amplified by semi-nested PCR using Ready-To-Go PCR Beads (Pharmacia). Semi-nested PCRs using two pairs of primers (4F2/4R2 for the first-round PCR and 4F3/4R2 for the second- and third-round PCR; see table 1) were performed to amplify opsin 4 fragments in *A. rubens* and *P. lividus*. First-round PCR

Table 1. degenerate primers used to amplify the opsin 4 gene fragment.

Primer ID	Bp number	Primer sequence
4F2	21	5' TCTGTTTGGMATIWISTCCAT 3'
4F3	20	5' TSYMTISCICCYTTCTTCGG 3'
4R2	20	5' GATGGCGGAASMCTTGGCIA 3'

conditions included an initial denaturation step of 5 min at 95°C followed by 40 cycles with a 30 sec denaturation step at 95°C, a 1 min annealing step at 43°C, and a 2 min elongation step at 72°C. These cycles were followed by a final elongation step of 2 min at 72°C. Products obtained with the first-round PCR were used as template for the second-round PCR. PCR conditions were identical except for the annealing step which was done at 45°C. Again, PCR products were used as template for the third round, a simple repetition of the second (using the same primer pair).

Amplification products were purified either with the commercial MSB® Spin PCRapace (Invisorb) or from a 2% agarose gel (Spin DNA extraction kit, Invisorb). Both strands of each PCR product were directly sequenced using the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems) and products were separated electrophoretically using an Applied Biosystems 3100 automated sequencer. Sequences were edited with Codon CodeAligner software (Codon Code Corporation, Dedham, MA), and Se-Al v2.0a11 (Rambaut, 1996). The alignment was first obtained using default parameter settings in Clustal X (Thompson et al., 1997), then corrected according to the amino acid alignment.

As reference sequences, trimmed bilaterian opsin sequences were added to the alignment of *A. rubens* and *P. lividus* opsin 4 sequences. Reference sequences were collected in Genbank databases and online accession numbers are the following : *Strongylocentrotus* opsin 4 XP_003730546.1, *Sepia* rhodopsin AAC26329.1, *Mizuhopecten* Gq O15973, *Platynereis* r-opsin CAC86665.1, *Branchiostoma* Mop Q4R1I4, *Xenopus* melanopsin AAC41235.1, *Drosophila* Rh6 NP_524368.3, *Branchiostoma* Ops3 BAC76023.1, Mus peropsin AAC53344.1, *Rattus* Opn5 NP_861437.1, *Branchiostoma* Opsin1 BAC76019.1, *Mizuhopecten* Go O15974, *Strongylocentrotus* opsin 3.2 GLEAN3_27633, *Strongylocentrotus* opsin 3.1 GLEAN3_27634, *Apis* pteropsin NP_001035057.1, *Anopheles* GPRop1 XP_312503.3, *Strongylocentrotus* opsin1 GLEAN3_05569, *Takifugu* TMT NP_001027778.1, *Branchiostoma* opsin 5 BAC76022.1, *Mus* encephalopsin NP_034228.1, *Platynereis* c-opsin AAV63834.1, *Danio* VAL opsin NP_571661.1, *Ciona* opsin1 NP_001027727.1, *Gallus* blue Opsin NP_990848.1, *Danio* MW4 opsin NP_571329.1, *Rattus* rhodopsin NP_254276.1, *Danio* SW opsin NP_571394.1, *Homo* MW Opsin NP_000504.1, *Danio* LW opsin NP_571250.1, *Gallus* Red NP_990771.1. In order to confirm the "opsin status" of the amplified opsin fragments, maximum-likelihood phylogenetic analyse was performed on the final alignment (146 amino acids). Tool used for calculating the PhyML tree is known as SeaView 4.2.12 (Galtier et al. 1996). We used the "blosum 62" substitution model and 8 gamma rate categories. Branch support values were estimated from 100 PhyML bootstrap replicates as bootstrap proportions (BP)."

Ciliary-opsin immunodetection on western blot

A commercial monoclonal antibody (Sigma-Aldrich O4886), raised against the N-terminal domain of rat rhodopsin, was used to detect putative c-opsins in *P. lividus* and *A. rubens* using western blot techniques. Sequence comparison between opsin 1 of *S. purpuratus* and mammal rhodopsin shows a high conservation of the N-terminal part, giving a strong argument for the using of the antibody.

In *P. lividus*, protein extractions were performed on freshly dissected pieces of oral and aboral integument. In *A. rubens*, extractions were done on podia, aboral integument and isolated optic cushions. The antibody was also tested on extracts from eyes of the rat, *Rattus norvegicus* (positive control), and of the crab, *Necora puber* (specificity control). All tissue samples were weighted, quickly frozen in liquid nitrogen and directly ground in extraction buffer (25mM Tris HCl, pH 7.2). Extracts were centrifuged (16000 g, 4°C, 15 minutes) and the supernatant containing soluble proteins was recovered as fraction A. The pellet was suspended in denaturing extraction buffer (25mM Tris HCl, SDS 2%, pH 7.2), the suspension was centrifuged and the second supernatant (fraction B) containing the majority of the transmembrane proteins was once again recovered. Finally, the pellet was re-extracted in the same denaturing extraction buffer in other to maximize the transmembrane protein extraction, allowing the collection of a third fraction (fraction C) after centrifugation. Protein concentrations in the different extracts and fractions were determined with the Bradford test using the Biorad Protein Assay Dye Reagent Concentrate (Bradford, 1976). For the calibration, a standard curve was constructed with different concentrations of Bovine Gamma globulins (BgG).

For gel electrophoresis, all protein extracts (fractions A, B, and C) were diluted to achieve a same protein concentration in each sample. They were then mixed with Laemmli buffer (4x – BioRad, Hercules, CA), incubated 2 minutes in a boiling water bath and centrifuged at 16000 g for 5 minutes. Protein separation was achieved using 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels (running conditions: 200V during approximately 45 min). Proteins separated by SDS-PAGE were blotted onto polyvinylidene fluoride (PVDF) membranes using 90 mM Tris–borate, 2.5 mM EDTA, 0.1% (w/v) SDS, and 25% (v/v) methanol as the transfer buffer. Running conditions were 200 mA constant current for 1 h (Transblot - BioRad, Hercules, CA). The PVDF membrane was blocked overnight in TBS-Tween 0.05%-BSA 1%, washed five times (TBS-Tween 0.05%-BSA 0.1%.), and incubated for 1 h in the primary antibody diluted 1:1,000 in TBS-Tween 0.05%-BSA 0.1%. After five more washes, the membrane

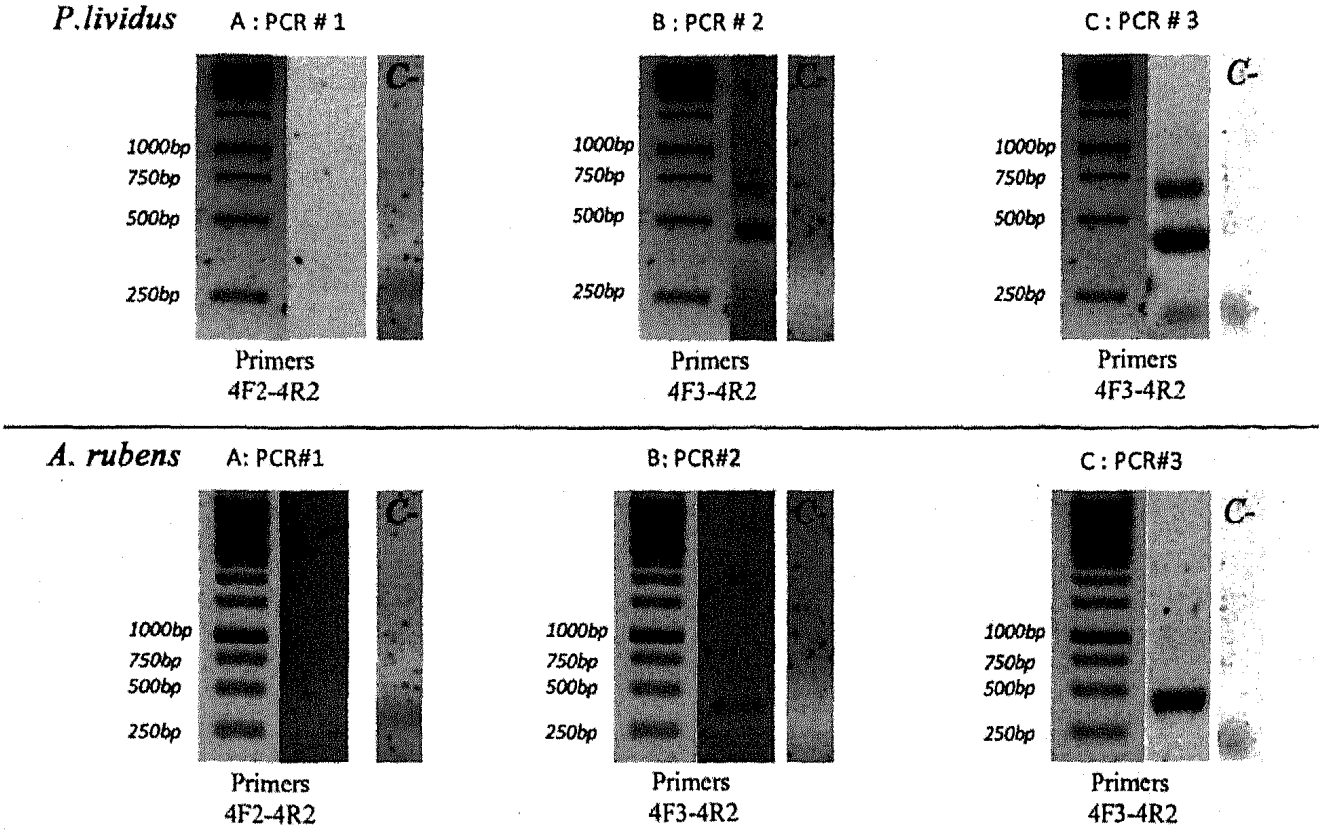

Figure 1. Semi-nested PCR performed on genomic DNA extracted from *P. lividus* and *A. rubens*. Negative controls are represented as followed: "C-". Negative controls were reamplified as the PCR samples.

was incubated for 1 h in the secondary antibody (sheep anti-mouse antibody conjugated to ECL peroxidase; GE Healthcare) diluted 1:10,000 in TBS-Tween 0.05%-BSA 0.1%. After five final washes, the membrane was incubated with ECL detection reagents (Amersham Biosciences, Piscataway, NJ) for 1 min and exposed to X-ray film.

Results

Rhabdomeric opsin gene amplification

Amplifications of the r-opsin gene were performed in *A. rubens* and *P. lividus*. A fragment of 400 base pairs was obtained by nested-PCR for both species (Fig. 1).

Based on *S. pupuratus* genome, the first primer pair used in the semi-nested PCR (4F2-4R2) theoretically amplifies a fragment of around 2000 bp. This long fragment is the target of the second primer pair (4F3-4R2) amplifying a fragment of 447 bp. After the first PCR, no band was visible in both species. The second PCR, performed on the first PCR product, permitted to obtain two bands at around 450 and 700 bp for *P. lividus* and one band at around 400 bp for *A. rubens*. After the third PCR, the bands corresponding to the expected size (around 400 bp) were excised and the DNA was retrieved and sequenced. A DNA sequence of 421 bp was obtained for *P. lividus* and one of 428 bp for *A. rubens*.

After translation into protein sequences, the obtained sequences were aligned and compared with the amino acid sequence of opsin 4 from *S. purpuratus* (Fig. 2). Considering the 129-amino-acid alignment, there is a 96% similarity between the sequences of *P. lividus* and *S. purpuratus* and a similarity ranging from 60 to 70% between the sequence of *A. rubens* and those of the two sea-urchins (Table 2). One amino acid (methionine in position 221) seems to be present only in the sea star and could be interpreted as a genetic mutation (insertion – deletion) (Fig 2).

To look for proteins homologues to the sequences obtained for *A. rubens* and *P. lividus*, BLAST searches were carried out against NCBI online data-bases. The results confirmed that these partial predicted protein sequences belong to the 7-transmembrane receptor family (rhodopsin family). The 10 first hits for each sequence indeed all

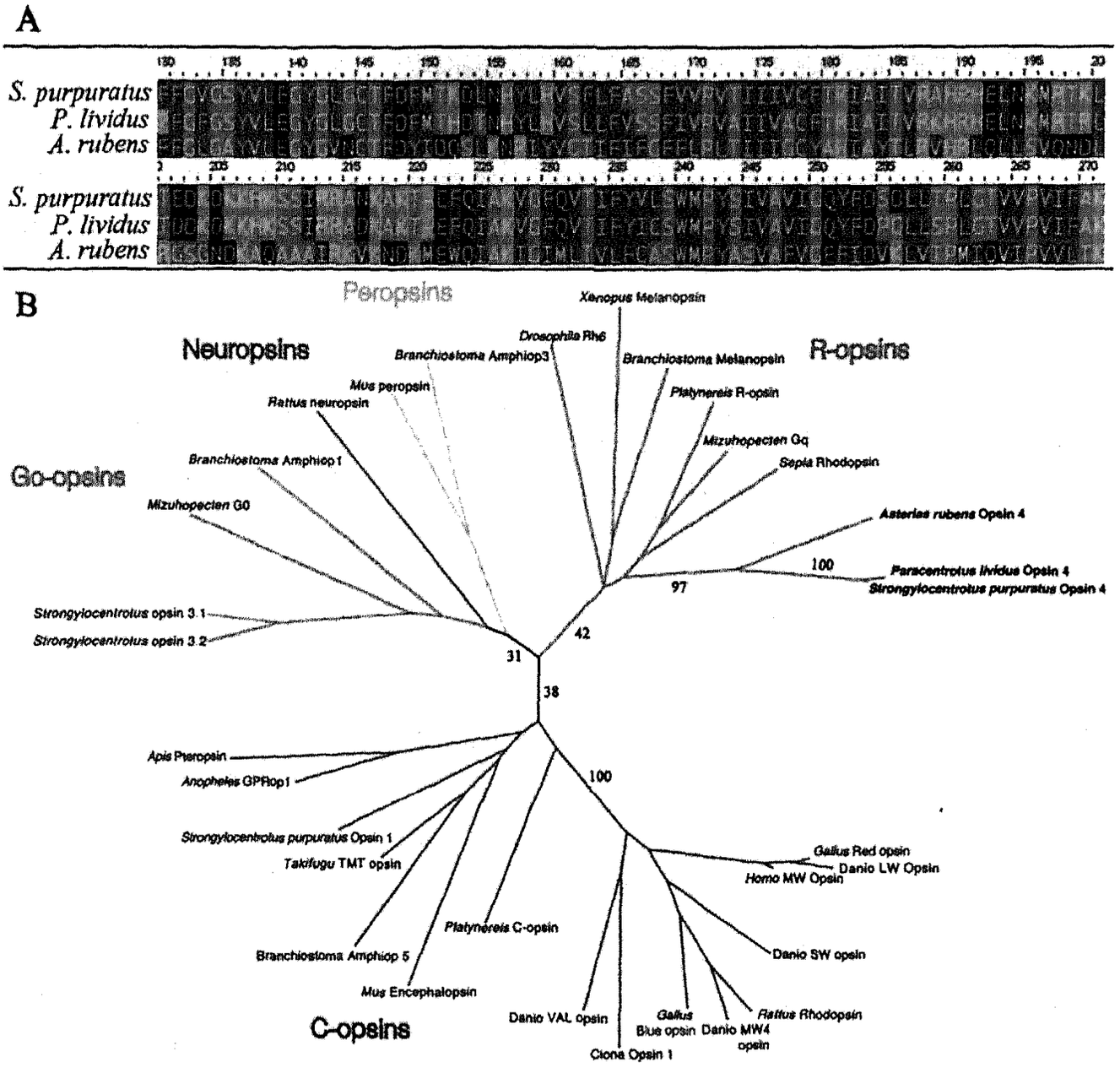

Figure 2. A. Predicted amino acid alignment of the opsin 4 sequences of *S. purpuratus*, *P. lividus* and *A. rubens*. The Se-Al software color code (v2.0a11) was used to highlight amino acids with similar physicochemical properties in the same color. B. Maximum-likelihood phylogenetic tree of bilaterian opsins including opsin 4 fragments of *Asterias rubens* and *Paracentrotus lividus*. See text for details. Main bootstrap values are presented in the tree.

correspond to r-opsin genes from echinoderms, molluscs, plathyhelminthes or arthropods.

Ciliary opsin immunodetection

By western blot experiments, two characteristic immunoreactive bands were detected in rat eye extracts at apparent molecular weights of 39 and 78 kDa (Fig. 3), corresponding to rhodopsin monomers and dimers, respectively (Johnsen, 1997). On the other hand, no labelling was observed for the crab eye extracts (Fig. 3), indicating that the antibody is specific of c-opsins. Putative c-opsin-like proteins were detected in the oral integument of *P. lividus* (Fig. 3), but not in the aboral integument. The portions of integument used included the body wall of the animal and its external appendages (spines, pedicellariae and podia). We also detected c-opsin-like proteins in the aboral integument of

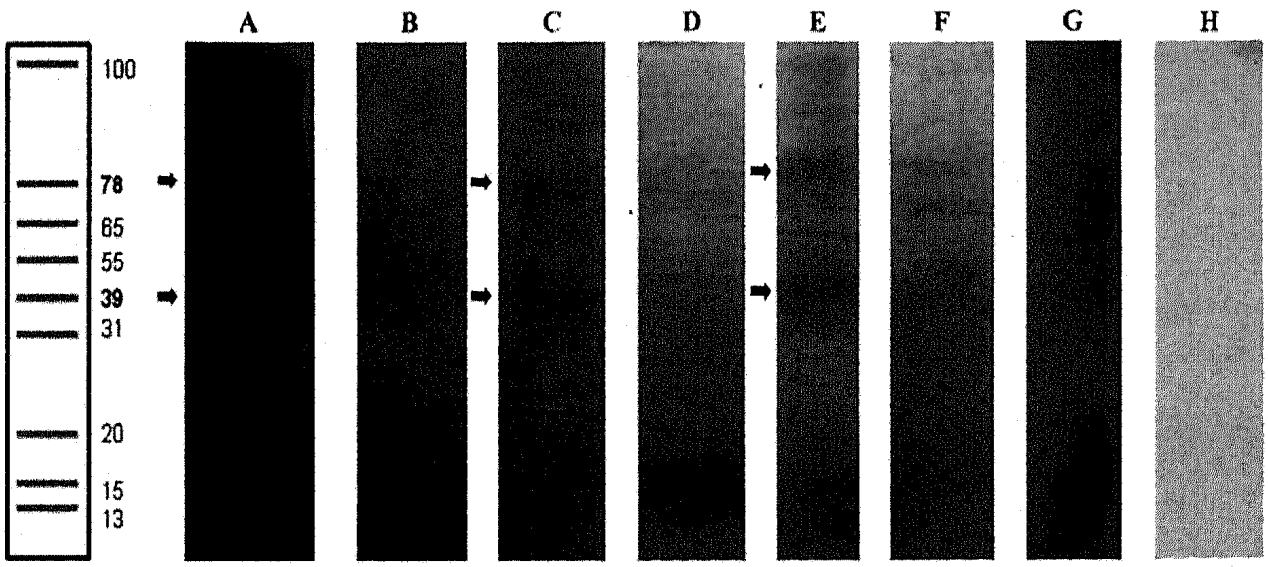

Figure 3. Western blots performed on proteins extracted from different tissues of *Rattus norvegicus*, *A. rubens et P. lividus* and immunostained with anti-bovine rhodopsin antibodies. A: *R. norvegicus* eye (positive control), B: podia of *A. rubens*, C: aboral integument of *A. rubens*, D: Optic cushions of *A. rubens*, E: oral integument of *P. lividus*, F: aboral integument of *P. lividus*, G: same as F but without primary antibody (negative control), H: *N. puber* eye (specificity control).

Table 2. Comparison between the partial sequence (129 amino acids) of Sp-opsin 4 and those of the two predicted r-opsins of *A. rubens* and *P. lividus* (mismatches: different amino acids, conservative mismatches: different amino acids with similar physicochemical properties, non-conservative mismatches: different amino acid with different physicochemical properties).

			Pair of species		
			S. purpuratus - *P. lividus*	*S. purpuratus* - *A. rubens*	*P. Lividus* - *A. rubens*
Mismatches	Total	Number	17	82	85
		Percentage	≈ 12%	≈ 58%	≈ 60%
	Conservative	Number	9	34	38
		Percentage	≈ 6%	≈ 24%	≈ 27%
	Non-conservative	Number	8	48	47
		Percentage	**≈ 6%**	**≈ 34%**	**≈ 33%**
General similarity (%)			**≈ 94%**	**≈ 66%**	**≈ 67%**

A. rubens, but not in the oral integument nor in the podia (Fig. 3). Furthermore, no antibody labelling was observed in extracts from the optic cushions.

Discussion

This study brings new information on light perception in echinoderms. Our results, although still preliminary, confirm that sea urchin and sea star express at least two types of opsins, both homologous to visual opsin of metazoans. Genomic DNA amplification and sequencing shows that r-opsins are present in the genomes of *P. lividus* and *A. rubens* (R-opsin status confirmed by phylogenetic analysis). These r-opsin sequences have been compared after translation into amino acid sequences and show a moderate similarity (between 60 and 70%) between sea stars and sea urchins. Among sea urchins (*P. lividus* and *S. purpuratus*), however, the similarity is much higher (96%). By immunodetection experiments on western blots, putative c-opsins were also detected in the tissues of both *P. lividus* and *A. rubens*. This method has the advantage that it also provides information on the site of expression of the targeted molecules. C-opsins were detected in the oral integument of the sea urchin *P. lividus*. If we compare literature information (e.g., Johnsen, 1997; Burke et al., 2006; Raible et al., 2006) with our results, it is likely that opsins in *P. lividus* are expressed in podia (mainly present in oral integument) and pedicellariae, as it is the case in *S. purpuratus* (Raible et al., 2006). In *A. rubens*, c-opsins are expressed in the aboral integument but not in the podia nor in the optic cushions. The latter are however known to contain rhabdomeric photoreceptor and r-opsins (Eakin & Brandenburger, 1979; Ullrich-Lüter et al., 2011). In view of our results, it seems that the distribution of opsins, revealed by the antibody, would be different in sea urchins and in sea stars. However, more detailed studies are needed to confirm these findings.

This study emphasizes the apparent complexity of the

light perception processes in echinoderms for which different opsins appear to be expressed in different areas. In *A. rubens*, one opsin type would be located in the optic cushions and another in the aboral integument. This could support the idea that sea stars possess two types of vision mediated by different opsins: the optic cushions containing r-opsins and involved in visual perception of the environment, and the aboral integument containing c-opsins and involved in a more diffuse photoreception. In asteroids, the evolution and specialization of the optic cushions could have led to a reduction of the photoreception by the podia. Surprisingly this hypothesis seems to indicate that, in echinoderms, rhabdomeric photoreceptor and r-opsins play here a key role in "visual" process, contrary to what occurs in the majority of Deuterostomes for which the main visual processes is most of the time mediated by ciliary photoreceptors. This hypothesis, already proposed by Ullrich-Lüter et al. (2011) for sea urchins, seems to be applicable to the sea-stars.

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