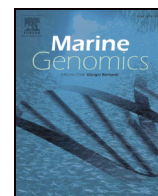




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Opsin evolution in the Ambulacraria

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ABSTRACT

Opsins — G-protein coupled receptors involved in photoreception — have been extensively studied in the animal kingdom. The present work provides new insights into opsin-based photoreception and photoreceptor cell evolution with a first analysis of opsin sequence data for a major deuterostome clade, the Ambulacraria. Systematic data analysis, including for the first time hemichordate opsin sequences and an expanded echinoderm dataset, led to a robust opsin phylogeny for this cornerstone superphylum. Multiple genomic and transcriptomic resources were surveyed to cover each class of Hemichordata and Echinodermata. In total, 119 ambulacrarian opsin sequences were found, 22 new sequences in hemichordates and 97 in echinoderms (including 67 new sequences). We framed the ambulacrarian opsin repertoire within eumetazoan diversity by including selected reference opsins from non-ambulacrarians. Our findings corroborate the presence of all major ancestral bilaterian opsin groups in Ambulacraria. Furthermore, we identified two opsin groups specific to echinoderms. In conclusion, a molecular phylogenetic framework for investigating light-perception and photobiological behaviors in marine deuterostomes has been obtained.

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1. Introduction

In animals, the prototypical molecules involved in photoreception and vision are opsin proteins (Terakita, 2005). Opsins are G-protein coupled receptors (GPCR) that consist of an apoprotein covalently bound to a chromophore (11-retinal) (Terakita, 2005). The nitrogen atom of the amino group of residue K296, situated in helix VII, binds to the retinal molecule through a Schiff-base linkage, forming a double bond with the carbon atom at the end of this molecule (Hargrave et al., 1983). Residue K296 is, therefore, crucial for light absorption, and its presence or absence can be used as a molecular fingerprint to judge whether or not a GPCR is a bona fide opsin.

Recent investigations on opsin phylogeny resolved six distinct groups present in metazoans: ciliary opsins, rhabdomeric opsins, Gopsins, neuropsins, peropsins, and RGR (RPE-retinal G protein-coupled receptor) opsins (Porter et al., 2012; Feuda et al. 2012; Terakita et al., 2012). A vast number of opsins are also expressed in non-ocular tissues (Porter et al., 2012; Koyanagi et al., 2005; Terakita et al., 2012).

With regard to opsin evolution in the deuterostomes, genomic and transcriptomic data of a number of chordates have been used to identify and characterize their opsins (e.g. Holland et al., 2008; Kusakabe et al., 2001). However, little attention has been paid to Ambulacraria, the sister group to all extant chordates, (i.e. cephalochordates, urochordates, and vertebrates, Edgcombe et al., 2011), a key clade to reconstruct the opsin set of the common ancestor of extant deuterostomes.

The present study integrates opsin sequences from two ambulacrarian sub-lineages: enteropneust Hemichordata (Harrimaniidae, Spengelidae,

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Ptychoderidae and Torquaratoridae), and the pentamer Echinodermata comprising five classes (Crinoidea, Ophiuroidea, Asteroidea, Holoturoidea and Echinoidea).

The phylogenetic relationship of echinoderms and hemichordates as sister groups within Ambulacraria, as shown in Fig. 1, was already suggested by Metschnikoff (1881), and supported by Nielsen (2012). The monophyly of Ambulacraria is also well supported by molecular phylogenetic analyses (Cannon et al., 2014; Telford et al., 2014). Moreover, Cannon and colleagues showed that the six hemichordate subgroups cluster into two monophyletic taxa, Enteropneusta and Pterobranchia (Rhabdopleuridae and Cephalodiscidae). Finally, Fig. 1 conforms to the Asterozoa hypothesis separating the Echinozoa (Echinoidea + Holothuroidea) and the Asterozoa (Asteroidea + Ophiuroidea), which is now well supported by recent molecular phylogenies (Cannon et al., 2014; Telford et al., 2014; O'Hara et al., 2014).

Other than a few structural investigations of eye-like structures in some asteroid species (e.g. the starfish optic cushion) and in enteropneust larvae (Brandenburger et al., 1973; Nezhlin and Yushin, 2004; Braun et al., 2015), the molecular mechanisms of echinoderm and hemichordate photoreception remained enigmatic until recently. Immunohistochemical studies indicated the presence of a putative rhodopsin in the asteroid *Asterias forbesi* and in the ophiuroid *Ophioderma brevispinum* (Johnsen, 1997). Subsequently, Raible et al. (2006) analyzed the 'rhodopsin-type' G-protein-coupled receptors family in an echinoid genome (*Strongylocentrotus purpuratus*). They predicted six bona fide opsin sequences, four of which were reported independently by Burke et al. (2006). Later, Ooka et al. (2010) cloned an "encephalopsin" orthologue in the sea urchin *Hemicentrotus pulcherrimus*. Recently, more opsin sequences have been found in sea urchins (*S. purpuratus*; *Paracentrotus lividus*), starfish (*Asterias rubens*), and brittle stars (*Ophiocoma nigra*, *Amphiura filiformis*) (Delroisse et al., 2013, 2014, 2015; Ullrich-Lüter et al., 2011, 2013). These studies highlighted the expression of ciliary and rhabdomeric opsins in various echinoderm tissues. Also, a large opsin gene repertoire was identified in the brittle star *A. filiformis*, pinpointing notable differences with findings from the previously published sea urchin genome (Delroisse et al., 2014). However, a comprehensive description of opsin diversity in echinoderms is

still lacking and almost nothing is known about hemichordate opsins.

Therefore, to characterize and describe the diversity of the opsin family in the Ambulacraria, we conducted a detailed analysis of 6 genomic and 24 transcriptomic sequence databases. This work represents the first attempt to describe and characterize the evolution of the opsin "toolkit" in the ambulacrarian lineage. We performed a phylogenetic study using the largest dataset of ambulacrarian opsin sequences to date, including representatives of a previously neglected group, Hemichordata.

2. Materials and methods

2.1. Data mining

Strongylocentrotus purpuratus opsins belonging to all the paralogous classes (Suppl. File 1) were used as starting query sequences for tBLASTx against transcriptomic and genomic databases including public databases (NCBI, JGI, Ensemble, Echinobase (www.echinobase.org/), BioInformatique CNRS-UPMC (<http://octopus.obs-vlfr.fr/>) and Genoscope (<http://www.genoscope.cns.fr/spip/Generation-de-ressources.html>). The parameters used across all our tBLASTx searches were the following: Matrix: Blosum62; gap penalties: existence: 11; extension: 1; neighboring words threshold: 13; window for multiple hits: 40. Additionally, our dataset was further enriched using various unpublished genomic and transcriptomic databases obtained from several independent research projects (Suppl. Files 1 and 2). This includes transcriptomes from adult specimens' tissues, such as cuverian tubules and integument from *Holothuria forskali*, muscle of *Parastichopus californicus*, radial nerve from *A. rubens*, arms from *Labidiaster annulatus*, *Ophiopsila aranea*, *Astrotonna agassizii* and *Antedon mediterranea*, proboscis from *Saccoglossus mereschkowskii* and *Torquaratorid* sp., whole adult body of *Leptosynapta clarki* and anterior part of the body from *Harrimaniidae* sp. and *Schizocardium braziliense*. Several other transcriptomes obtained from embryos or larvae from *P. lividus*, *Heliocidaris erythrogramma*, *Eucidaris tribuloides*, *Parastichopus parvimensis*, *Saccoglossus kowalevskii* and *Ptychodera flava* (Suppl. Files 1 and 2) were also screened. The absence of echinopsin-like sequences in other metazoans was checked using

Fig. 1. Ambulacrarian phylogenetic relationships and their adult forms. The Ambulacraria consist of two groups: Hemichordata, bilateral animals subdivided in six clades: Cephalodiscidae, Rhabdopleuridae, Harrimaniidae, Spengelidae, Ptychoderidae and Torquaratoridae, and the pentamer Echinodermata, comprising: Crinoidea, Ophiuroidea, Asteroidea, Holoturoidea and Echinoidea. For each class there is a representation of the adult body plan. The numbers represented on the figure correspond to the two hemichordate subgroups: 1. Pterobranchia and 2. Enteropneusta, and the two echinoderm subgroups 3. Eleutherozoa and 4. Crinozoa.

blast search analysis. The raw predicted opsin sequences used in this study are listed in the Suppl. File 3 in fasta format.

2.2. Alignment and phylogenetic analyses

Predicted protein alignments were performed with SeaView v4.2.12 (Galtier et al., 1996; Gouy et al., 2010) using the MUSCLE algorithm (Edgar, 2004). To improve phylogenetic reconstruction, N-terminal and C-terminal ends were trimmed and the alignment was manually corrected in order to minimize gaps and eliminate ambiguous and misaligned regions. Sequences that were shorter than 60 amino acids were removed to avoid bias. However, these could potentially correspond to true opsins and merit further study.

Maximum likelihood analyses (ML) of our dataset were conducted on Michigan State University's High Performance Computing Cluster using PhyML v3.0 (Guindon and Gascuel, 2003), and nodal support assessed with 1000 bootstrap replicates is indicated. The alignment is shown in Suppl. File 4 (phylib format) and Suppl. File 5 (image). A best-fit model analysis was performed using MEGA6 (following the AIC criteria) (Tamura et al., 2013; Kumar et al., 2008) and WAG+G+F amino acid substitution model was found to be the best suited (Whelan and Goldman, 2001). Three melatonin receptor sequences from *S. purpuratus* (Echinodermata) and three from *S. kowalevskii* (Hemichordata) were chosen as the best outgroup for the opsin phylogeny, as previously proposed by Plachetzki et al. (2010) and Feuda et al. (2014).

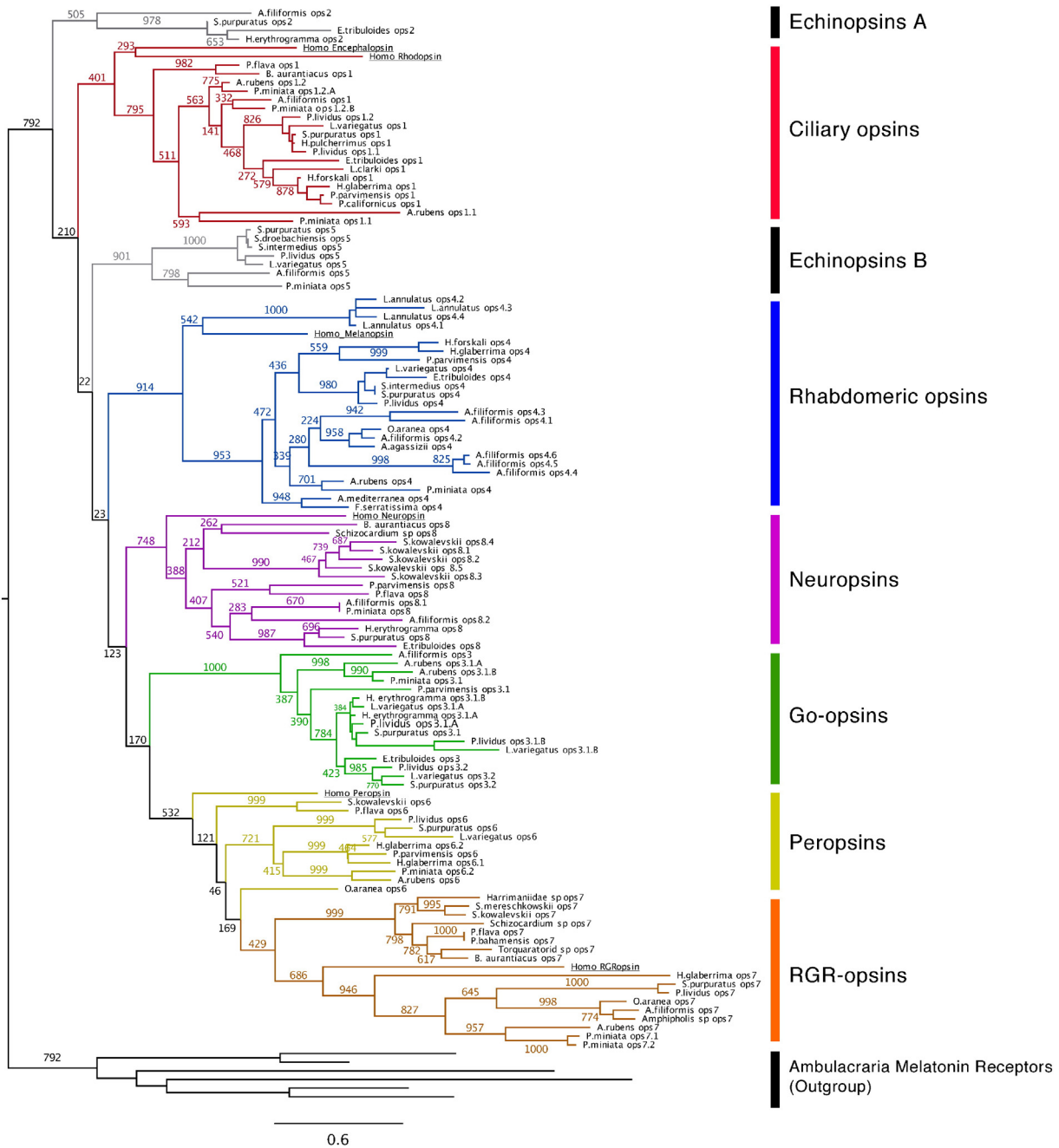


Fig. 2. Phylogenetic reconstruction of ambulacrarian opsins. 119 opsins from 31 different ambulacrarian species cluster in eight highly supported groups in this maximum likelihood (ML) based analysis. R-opsins in blue, c-opsins in red, Go-opsins in green, neuropsins in purple, peropsins in yellow and RGR-opsins in orange. Visualization was generated with fig tree. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.3. Consensus fingerprint of ambulacrarian opsin groups

Ambulacraria opsins were clustered according to their estimated position within opsin subfamilies and a multiple alignment of a 35 amino-acid long peptide region, including the 7th transmembrane domain with the opsin-specific lysine (K296), was performed with SeaView v4.2.12 for each opsin group supported in our phylogenetic tree. The selected region spanned residues 286 to 320 of the *Rattus norvegicus* rhodopsin sequence used as a reference (Palczewski et al., 2000). The consensus sequence was generated on the basis of the alignment for each class of ambulacrarian opsin using Geneious@8.1.5.

3. Results

3.1. Phylogeny and opsin distribution within ambulacrarian groups

Using a collection of both genomic and transcriptomic data (see Materials and methods and Suppl. File 2 for details), a final set of 119 protein sequences, representing 31 ambulacrarian species, was generated for our phylogenetic reconstruction, which included 6 outgroup sequences and 6 human reference opsin sequences (Suppl. Files 1 and 3 for raw predicted protein sequences). The trimmed opsin alignment is shown in the Suppl. File 5 (see Suppl. File 4 for the alignment phylip file). We employed maximum likelihood using the WAG+G+F model with melatonin receptors as an outgroup. Canonical opsin groups are well supported in our analysis (Fig. 2), demonstrating the presence of a complex opsin toolkit in Ambulacraria.

Interestingly, according to our data, two novel groups of opsins were found, which we have named echinopsin-A and echinopsin-B groups. Ad hoc BLAST searches against metazoan online database (NR, NCBI) clearly indicated the absence of these two opsin types outside the echinoderm lineage (Suppl. Fig. 6). The previously identified Sp-opsin2 and Sp-opsin5 belong to echinopsins-A and echinopsins-B, respectively (Raible et al., 2006).

A complete opsin profile including at least one representative of each prototypical opsin group (opsin 1–8) was detected in the sea urchin *S. purpuratus*, but not in *Lytechinus variegatus* or *P. lividus*. The genomes of the latter two species have not yet been comprehensively sequenced and annotated, and therefore some opsin genes may be missing due to incomplete sequence coverage. With the exception of echinopsin-B, a complete opsin profile was found in the genome sequence data of the starfish *Patiria miniata*. The starfish *A. rubens* radial nerve transcriptome also contained several opsins, including ciliary, Go-, RGR-opsins.

Surprisingly, rhabdomeric and Go-opsins do not seem to be present in hemichordates in our dataset. However, this requires confirmation through more extensive taxonomic sampling of hemichordate sequence data because, at present, only one hemichordate genome has been fully sequenced (*S. kowalevskii*). In several opsin groups we observed lineage-specific duplications: two c-opsins in *P. miniata* and *A. rubens*; five neuropsins in *S. kowalevskii*; four r-opsins in *L. annulatus* and six r-opsins in *A. filiformis*; two Go-opsins in the echinoids *L. variegatus*, *S. purpuratus* and *H. erythrogramma*. Nevertheless, some of these molecules present a short overlapping sequence, and therefore we cannot exclude that they could be part of unique genes. In this case, the number of genes would have been overestimated.

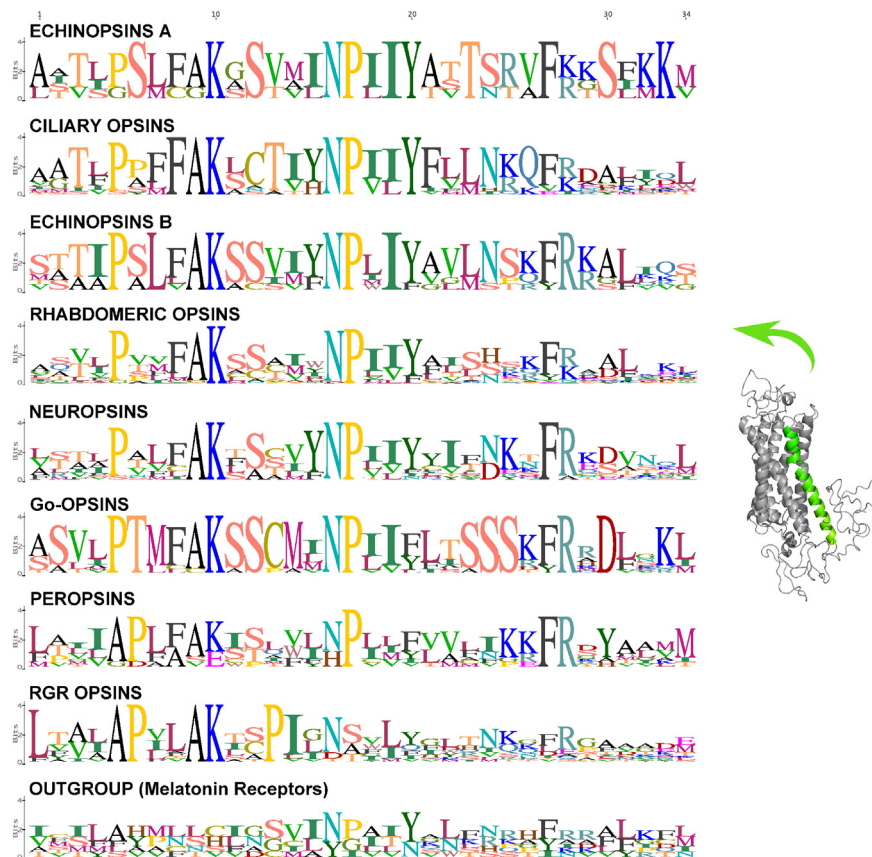


Fig. 3. Consensus sequences of different opsin groups. Graphical representations of opsin amino acid patterns within the multiple alignments of the 7th transmembrane domain and the protein G linkage site. The 7th transmembrane domain is highlighted in green in the tridimensional representation of a typical opsin receptor. Alignment is limited to the highly conserved regions including the opsin-specific lysine residue and the “NPxxY(x)₆F” pattern. The lysine residue involved in the Schiff base formation - equivalent to K296 of the *R. norvegicus* rhodopsin – is present in position 10. The pattern “NPxxY(x)₆F” (position 302–313 of the *R. norvegicus* rhodopsin sequence) is present in position 17–28. The size of each amino acid indicates the probability to find this specific amino acid for the considered position. Amino acid patterns of Melatonin receptors used as an outgroup in the phylogenetic analysis is also presented.

3.2. Alignment of the transmembrane domain and opsin fingerprint

In order to build a consensus fingerprint to distinguish the various ambulacrarian opsin groups, the 7th transmembrane domain and C-terminal tail region of our sequence dataset were aligned and a graphical representation was generated (Fig. 3). All sequences were characterized by the general structure of G protein-coupled receptors (GPCRs) comprising seven transmembrane (TM) domains. Numerous residues characteristic of opsins are present in the opsin sequences of Ambulacraria. However, as several sequences are partial, not all characteristic residues could be detected in all sequences. Most of the opsin sequences also contained the highly conserved lysine residue (equivalent to K296 of the *R. norvegicus* rhodopsin) critical for Schiff base linkage formed with retinal, except three sea-urchin peropsins (Sp-opsin 6, Pl-opsin 6, Lv-opsin 6) in which it is substituted by a glutamate (E). The dipeptide NP (position 302-303 of the *R. norvegicus* rhodopsin sequence) is also highly conserved among all the subfamilies except in peropsins (N/HP) and RGR-opsins, which show divergence in these residues (also rhabdomeric opsins to a lesser extent). Amino-acid conservation for each opsin group from our phylogenetic analysis is shown in Fig. 3. Ambulacrarian c-opsins, r-opsins and echinopsins-A displayed a highly conserved tyrosine (Y306). Conversely, the histidine (H310) appears distinctive of the ambulacrarian r-opsins (Fig. 3) and r-opsins in general

(human melanopsin, octopus rhodopsin and *Drosophila* Rh1-opsin). In our dataset the tripeptide SSS, positioned at residues 309–402 of the reference protein, is a distinctive feature of ambulacrarian Go-opsins.

These representations will be particularly useful in future studies in support of phylogenetic analysis to assign novel, unknown sequences to lineage-specific opsin groups.

4. Discussion

Our phylogenetic analyses showed ambulacrarian opsin sequences to be represented in all six prototypical bilaterian opsin groups: ciliary opsins, rhabdomeric opsins, neuropsins, Go-opsins, peropsins and RGR-opsins (Fig. 4). Even though ciliary opsins, peropsins and RGR-opsins are in general well supported in the literature, a relatively poor nodal support was obtained for these groups using our ambulacraria opsin data set. In addition we confirmed the presence of two novel echinoderm-specific opsin groups, which we have named echinopsins (echinopsin-A and echinopsin-B). These novel groups of opsins, which were found only in Echinozoa, Ophiurozoa and Asterozoa, respectively cluster as a sister group of all other opsins and as a sister group of all opsins except echinopsins-A and ciliary opsins (Fig. 4). A deeper analysis of these groups of proteins, including more hemichordate opsin sequences, is needed in order to determine if they represent an echinoderm or ambulacrarian novelty. Clear orthologs of echinopsin-A and

Phylum	Class	Species	TOTAL	R-opsins / opsins 4	C-opsins / opsins 1	Go opsins / opsins 3	Neuropsins / opsins 8	Peropsins / opsins 6	RGR-opsins / opsins 7	Echinopsins A / opsins 2	Echinopsins B / opsins 5		
Echinodermata	Echinozoa	<i>Strongylocentrotus purpuratus</i>	8	1	1	2	1	1	1	1	1	1	
		<i>Strongylocentrotus droebachiensis</i>	1										1
		<i>Strongylocentrotus intermedius</i>	2	1									1
		<i>Lytechinus variegatus</i>	6	1	1	2		1					1
		<i>Paracentrotus lividus</i>	7	1	1	2		1	1				1
		<i>Hemicentrotus pulcherrimus</i>	1		1								
		<i>Eucidaris tribuloides</i>	5	1	1	1	1					1	
		<i>Helicodaris erythrogramma</i>	3			1	1						1
	Holothurozoa	<i>Parastichopus californicus</i>	1		1								
		<i>Parastichopus parvamentis</i>	5	1	1	1	1	1					
		<i>Leptosynapta clarki</i>	1		1								
		<i>Holothuria forskali</i>	2	1	1								
		<i>Holothuria glaberrima</i>	4	1	1			1	1				
		Ophiurozoa	<i>Amphiura filiformis</i>	13	6	1	1	2			1	1	1
			<i>Ophiopsila aranea</i>	3	1					1	1		
			<i>Astrotomma agassizii</i>	1	1								
	<i>Amphipholis</i> sp		1							1			
	Asterozoa	<i>Asterias rubens</i>	6	1	2	1			1	1			
		<i>Patiria miniata</i>	10	1	2	1	1	1	2	2		1	
		<i>Labidiaster annulatus</i>	4	4									
Crinozoa	<i>Antedon mediterranea</i>	1	1										
	<i>Florometra serratissima</i>	1	1										
	Hemichordata	<i>Saccoglossus kowalevskii</i>	7				5	1	1				
<i>Saccoglossus mereschkowskii</i>		1							1				
<i>Harrimaniidae</i> sp. (Iceland)		1							1				
<i>Spengelidae</i> <i>Schizocardium</i> c.f. <i>braziliense</i>		2				1			1				
<i>Ptychodera flava</i>		4		1		1	1	1					
<i>Ptychodera bahamensis</i>		1							1				
<i>Balanoglossus</i> c.f. <i>aurantiacus</i>		3		1		1			1				
<i>Torquaratoridae</i> <i>Torquaratoid</i> sp. (Iceland)	1								1				

Fig. 4. Opsin distribution within the investigated ambulacraria species. For each species the number of opsin belonging to classical groups were reported. Those species for which no opsins were found are not reported in the table (for additional informations see Suppl. File 1). Species for which the genome data are available are in bold.

echinopsin-B where not encountered in any metazoan genome (except echinoderms), as shown in Suppl. Fig. 6. Nevertheless, we cannot state conclusively that these represent lineage-specific clades because new genomes could reveal that echinopsin-A and echinopsin-B are indeed not restricted to echinoderms or ambulacrarians.

Our analysis failed to reveal a rhabdomeric opsin (r-opsin) in hemichordates. The absence of such an opsin type is surprising because many enteropneust tornaria larvae possess eyespots that bear photoreceptors with clear microvillar surface enlargement (Brandenburger et al., 1973; Nezhlin and Yushin, 2004; Braun et al., 2015). So far, photoreception in microvillar photoreceptor cell types has been demonstrated to generally deploy opsins of the so-called rhabdomeric type (r-opsins), although co-expression of other opsin types in microvillar/rhabdomeric photoreceptors has been shown in recent studies (Randel et al., 2013). However, although our analysis reveals no such opsin in any of the examined enteropneust species, it should be noted that genomic information is only available from the direct developer *S. kowalevskii*, which does not have a larval (tornarian) stage in its life cycle. Moreover, most of hemichordate transcriptomes in our study were generated using adult tissues; it is therefore possible that the absence of r-opsin in this group of animals is due to a limitation of data availability from this understudied group of animals.

In contrast to the lack of r-opsins in enteropneusts, our analyses showed several cases of opsin gene duplication. Obviously in some instances the locus of duplication prompted a large expansion of the gene family, as is the case of the five neuropsins found in *S. kowalevskii*, and the six rhabdomeric opsins in *A. filiformis*, with the latter previously described by Delroisse et al. (2014). However, the fragmentary information about these duplicates makes it difficult to predict the exact number of functional opsin proteins in Ambulacraria. Whether or not these duplicated genes have sub-functionalized roles should be experimentally investigated by knock-out or silencing experiments.

Until recently, under-representation of many taxonomic groups in comparative studies of photoreceptor evolution has hidden the real extent of opsin diversity (Porter et al., 2012; Feuda et al., 2014). As more opsins have been characterized, these sequences have been classified into narrow pre-defined groups (e.g. Group 4 opsins), implying theoretical functional similarities that might not always be correct (Shichida and Matsuyama, 2009). At present, however, the rapidly increasing availability of entire genomes and transcriptomes provides a large number of sequences for investigating the evolution and functional diversity of the opsin family in greater detail. Likewise, our phylogenetic analysis of ambulacrarian opsins provides a better understanding of opsin evolution, nevertheless future metazoan genomes could certainly help to draw a more definitive evolutionary scenario.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.margen.2015.10.001>.

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