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Cone visual pigments of monotremes: Filling the phylogenetic gap

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Abstract

We have determined the sequence and genomic organization of the genes encoding the cone visual pigment of the platypus (*Ornithorhynchus anatinus*) and the echidna (*Tachyglossus aculeatus*), and inferred their spectral properties and evolutionary pathways. We prepared platypus and echidna retinal RNA and used primers of the middle-wave-sensitive (MWS), long-wave-sensitive (LWS), and short-wave sensitive (SWS1) pigments corresponding to coding sequences that are highly conserved among mammals; to PCR amplify the corresponding pigment sequences. Amplification from the retinal RNA revealed the expression of LWS pigment mRNA that is homologous in sequence and spectral properties to the primate LWS visual pigments. However, we were unable to amplify the mammalian SWS1 pigment from these two species, indicating this gene was lost prior to the echidna-platypus divergence (~21 MYA). Subsequently, when the platypus genome sequence became available, we found an LWS pigment gene in a conserved genomic arrangement that resembles the primate pigment, but, surprisingly we found an adjacent (~20 kb) SWS2 pigment gene within this conserved genomic arrangement. We obtained the same result after sequencing the echidna genes. The encoded SWS2 pigment is predicted to have a wavelength of maximal absorption of about 440 nm, and is paralogous to SWS pigments typically found in reptiles, birds, and fish but not in mammals. This study suggests the locus control region (LCR) has played an important role in the conservation of photo receptor gene arrays and the control of their spatial and temporal expression in the retina in all mammals. In conclusion, a duplication event of an ancestral cone visual pigment gene, followed by sequence divergence and selection gave rise to the LWS and SWS2 visual pigments. So far, the echidna and platypus are the only mammals that share the gene structure of the LWS-SWS2 pigment gene complex with reptiles, birds and fishes.

Keywords: Monotremes, Cone pigments, Gene evolution, Locus control region, Marsupials

Introduction

Visual pigments involved in color vision exhibit a wide range of spectral sensitivity, ranging from ultraviolet to far red (Wang et al., 1999). Adaptations of vertebrates to different environments included selection for visual pigments with the appropriate spectral absorption (Yokoyama, 2000; Jacobs & Rowe, 2004). The molecular mechanisms of evolution and regulation of expression of the photo pigment genes involved point mutations that substituted opsin amino acids that contribute to spectral tuning, gene rearrangements (duplications and dispersions) as well as the formation of *cis*-regulatory elements that specify expression of the pigment genes in photo receptor classes (Ohno, 1970; Nathans, 1999; Bowmaker & Hunt, 2006). For example, the evolution of trichromatic color vision among primates involved the duplication of the ancestral pigment gene on the X-chromosome, followed by sequence divergence to form the LWS and MWS pigment genes.

Color vision relies on the differential expression of photo receptor pigments in different classes of cone cells. A locus control region (LCR) located upstream from the LWS pigment gene on the eutherian X chromosome controls this expression (Nathans et al., 1989; Wang et al., 1992; Deeb, 2004). Mutually exclusive expression of the LWS and MWS pigment genes in a single photo receptor cell is accomplished by stable interaction of the LCR with

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either the LWS proximal promoter to express the L pigment, or with the MWS gene proximal promoter to express the M pigment. Transgenic studies have shown that the LCR element plays a critical role in regulating the mutually exclusive expression of pigment genes in individual cones (Wang et al., 1999; Smallwood et al., 2002). The range of influence of the LCR appears to be limited, with only the first two genes in the array being expressed (Yamaguchi et al., 1997; Hayashi et al., 1999).

The common amniote ancestor is proposed to have had highly developed vision, with four cone pigments, one-rod pigment, and oil droplets for spectral tuning. This highly developed visual system is hypothesized to have been lost during early mammal evolution due to selection for more sensitive vision in the low light conditions of a nocturnal forager (Walls, 1942). Most eutherian mammals maintain the ancestral eutherian state of two cone opsins and a rod opsin providing dichromatic vision (Jacobs & Rowe, 2004; Bowmaker & Hunt, 2006).

Platypus and echidna are the only extant monotreme species and provide a unique insight into the common ancestor of all mammals. Monotremes diverged from therian mammals ~166 MYA, and provide insight into the common mammalian ancestor prior to the eutherian/marsupial divergence ~148 MYA, and the common amniote ancestor ~315 MYA. Australian marsupials, such as the tammar wallaby (*Macropus eugenii*), diverged from American marsupials such as the opossum (*Monodelphis domestica*) ~80 MYA and provide insight into the common marsupial ancestor (Wakefield & Graves, 2003, 2005). Primates are unique among eutherian mammals in having trichromatic color vision. This represents an independent evolution of spectral diversity in response to selection after returning to a diurnal lifestyle.

Two cone pigment genes of the tammar wallaby (*Macropus eugenii*) have previously been identified by cDNA cloning, consistent with observations indicating dichromatic color vision in behavioral and immunocytochemical studies (Hemmi, 1999; Hemmi & Grunert, 1999; Deeb et al., 2003). However, microspectrophometric measurements of photo pigments in the retina of four Australian marsupials: honey possum (*Tarsipes rostratus*), fat-tailed dunnart (*Sminthopsis crassicaudata*), quokka (*Setonix brachyurus*), and bandicoot (*Isoodon obesulus*), suggest potentially trichromatic color vision that has been proposed to span marsupials (Arrese et al., 2002, 2005). The cone pigment genes have been cloned by PCR from these four species and identified only a single MWS/LWS class opsin and a SWS1 pigment genes (Strachan et al., 2004; Arrese et al., 2006*a*). This lack of evidence for a third cone pigment does not support behavioral studies of the dunnart that suggest a capability for trichromatic vision (Arrese et al., 2006*b*).

The platypus genome sequence and gene annotation indicates the absence of a platypus functional SWS1 gene and the presence of an SWS2 gene (Warren et al., 2008). Recently published studies of the platypus visual pigments based on this preliminary genome data used PCR amplification from genomic DNA to confirm the ability of the encoded SWS2 protein to produce a functional product by *in vitro* expression of PCR products (Davies et al., 2007). In this study, we have analyzed platypus and echidna retinal RNAs to determine which photo receptor genes are expressed and determined the sequence of their transcripts.

Materials and methods

A 2.3-fold coverage bacterial artificial chromosome (BAC) library from a male tammar wallaby lymphocytes (Sankovic et al., 2006) was screened with the tammar wallaby opsin cDNA (Deeb et al., 2003). 100 ng of tammar wallaby MWS cDNA probe was labeled with P^{32}dCTP using the Megaprime labeling kit (Amersham) and hybridized at 65°C O/N in Church's Buffer (Sambrook & Russell, 2001). Filters were washed in 65°C 2×SSC and 65°C 0.1×SSC and exposed to film overnight. Positive clones were confirmed by PCR (Deeb et al., 2003). The BAC clones were sequenced at the University of Washington Genome Centre.

Tissue was obtained from animals captured at the Upper Barnard River, New South Wales, Australia, during breeding season (AEEC permit no. R.CG.07.03 to F.G., Environment ACT permit no. LI 2002 270 to J.A.M.G., NPWS permit no A193 to R.C.J., AEC permit no. S-49-2006 to F.G.).

Both genomic DNA and retinal RNA from a platypus and an echidna were isolated and used to PCR-amplify sequences encoding the LWS and SWS pigments. The primers used to amplify the LWS pigment were designed from consensus sequences that are highly conserved among several mammalian species. Genomic DNA was also used to determine the location of the SWS2 and LWS genes relative to each other. Consensus primers and the strategy used for amplification and sequencing of the entire mRNA were previously described (Deeb et al., 2003). Following the initial internal coding sequence determination, 5′ and 3′ RACE reactions were performed in order to amplify and sequence the entire mRNA using a Smart II RACE kit (Clontech). The PCR products were purified using a Qiagen PCR purification kit. Sequencing reactions were performed using Big Dye V3.1 (Applied Biosystems).

Repeats were identified using RepeatMasker (http://www.repeatmasker.org) and Repbase (Jurka et al., 2005). Genomescan (Yeh et al., 2001) and Blast (Altschul et al., 1997) results were manually integrated to provide the gene annotation for the tammar wallaby, and to supplement the automated annotation of platypus. Other Genome sequences (Table 1) and annotations were downloaded from the Ensembl genome browser (Hubbard et al., 2007). Sequence alignments were performed with clustalw 1.82 (Thompson et al., 1994) and visualized in Jalview (Clamp et al., 2004). Sequence logos were generated with Weblogo (Crooks et al., 2004). Phylogenetic footprinting was performed using multipipmaker (Schwartz et al., 2003). Presented data is for masked reference sequence in unchained mode.

Results

Two tammar wallaby BAC clones containing a complete *OPN1MW* gene were isolated. The smaller of the two clones, MeVIA22L1, is 92 kb in length and includes 58 kb of sequence upstream of the *OPN1MW* gene. The second clone, MeVIA35g12, which is 168 kb, overlaps the first by 55 kb to generate a 205 kb contig [genbank: AY737497.2]. These clones contain *OPN1MW*, a single *CXOrf2/TEX28* gene, and the *TKT2* gene.

We were able to amplify and sequence the entire platypus, and subsequently the echidna, LWS opsin mRNAs (Genbank: Platypus LWS GenBank accession # EU624413, Genbank: Echidna LWS GenBank accession # EU636011). The platypus and echidna opsin sequences are 93% identical and 96% similar to each other. Interestingly, there is one amino acid (asparagine) at position 21 of the platypus opsin that is missing from the echidna opsin. Asp^{21} has also been independently lost from the LWS opsin in some marsupials, including the wallaby, southern brown bandicoot, numba, and quokka, suggesting that the lack of this amino acid is not detrimental to the function of the opsin. The platypus and all known placental mammal L and M opsins do have Asp at posi-

Table 1. *Sequences used in this study*

Sequence	Genome Build	Chr accession	Start–End	Fig	Reference
Human LCR	NCBI36	*Chr X*	153059352–153059426	3	(Lander et al., 2001)
Mouse LCR	NCBI m36	Chr X	70376307–70376385	3	(Waterston et al., 2002)
Rat LCR	RGSC 3.4	Chr X	160090661–160090739	3	(Gibbs et al., 2004)
Cow LCR	Btau 2.0	Chr 7	6499657–6499735	3	Bovine Genome Consortium
Cow LCR		S44757S1	387–465	3	(Wang et al.)
Dog LCR	CanFam 1.0	Chr X	125028523–125028601	3	(Lindblad-Toh et al., 2005)
Monodelphis LCR	MonDom 4	Chr X	11815862–11815935	3	(Mikkelsen et al., 2007)
Tammar LCR	MeVIA22L1	AY737497.1	51575–51653	3	This study
Platypus LCR	OANA	UltraContig401	244828–248906	3	(Warren et al., 2008)
Human opsin region	NCBI36	Chr X	153022347–153222347	2	(Lander et al., 2001)
Mouse opsin region	NCBIM36	Chr X	70342064–70472064	2	(Waterston et al., 2002)
Rat opsin region	RGSC 3.4	Chr X	159914564–160109382	2	(Gibbs et al., 2004)
Dog opsin region	CanFam 1.0	Chr X	125028523–125028601	2	(Lindblad-Toh et al., 2005)
Opossum opsin region	MonDom 4	Chr X	11815862–11815935	2	(Mikkelsen et al., 2007)
Tammar opsin region	MeVIA22L1	AY737497.2	1–196721	2	This study
Platypus opsin region	OANA	UltraContig401	200001–350000	2	(Warren et al., 2008)

tion 21. The platypus has the same five amino acids at sites significant for spectral tuning as the human LWS opsin. These amino acids are A180, H197, Y277, T285, and A308. In humans, this opsin has been shown to have a λ_{max} of 555 nm. On the other hand, the corresponding echidna opsin has Ser at position 180 giving it an inferred λ_{max} of 560 nm. Overall, the platypus and echidna LWS opsins have an 85% identity and 95% similarity with the human L opsins.

The typical mammalian SWS opsins belong to the SWS1 class. Therefore, prior to availability of the genome sequence we attempted to amplify SWS1 coding sequences from both the platypus and echidna retinal mRNAs. All attempts, using several primers in different regions of the mRNAs failed (Fig. 1). The platypus genome sequence and gene prediction annotations by the sequencing consortium indicated that only exon 5 of the SWS1 gene was present (Davies et al., 2007; Warren et al., 2008). Subsequent attempts to amplify exon 5 of the SWS1 gene of the platypus and echidna genomes using primers designed against platypus exon 5 confirmed that this exon was not detected in retinal mRNAs (Fig. 1). Surprisingly, the platypus genome contained an SWS2

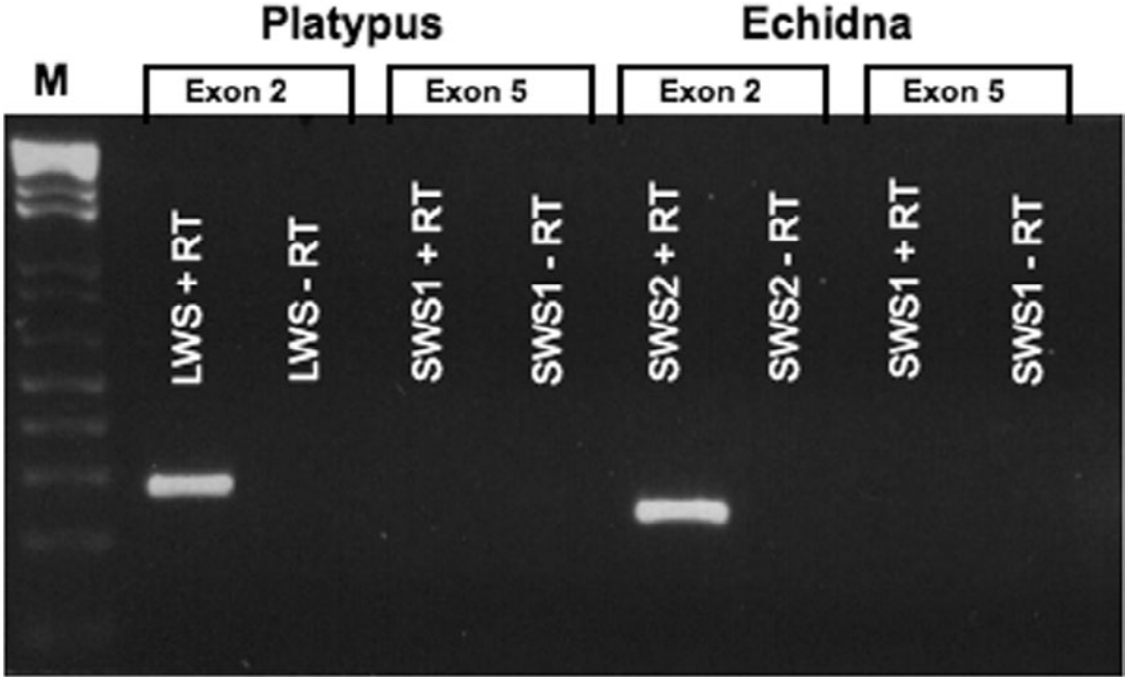

Fig. 1. Expression of platypus and echidna opsins. Expression of the SWS and LWS mRNAs in monotreme retinae. Retina RNA from the platypus and echidna retinae was used as template to RT-PCR amplify exons 2 and 5 of the SWS1, SWS2 and LWS coding sequences. M, 100 bp ladder marker. As shown, only the LWS and SWS transcripts are expressed in the retinae of the monotremes.

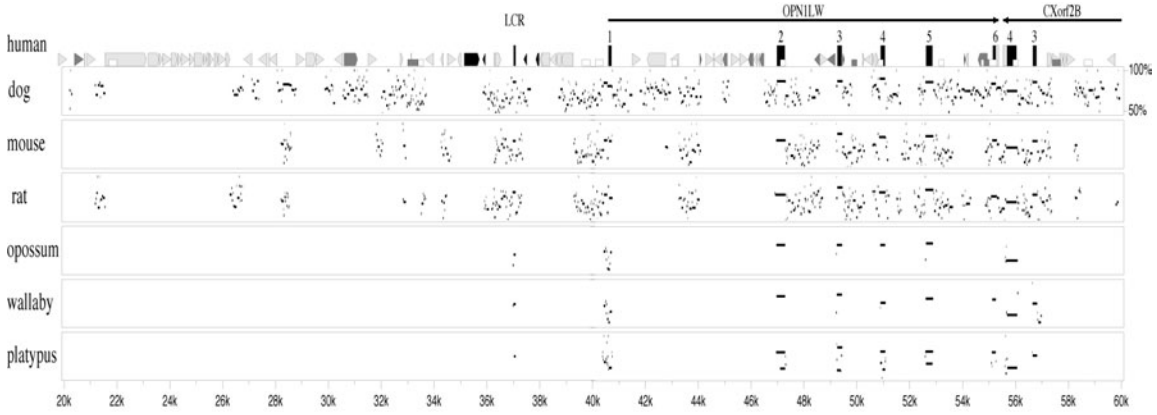

Fig. 2. Conservation of opsin locus. Pipmaker phylogenetic footprinting plot of the human OPN1LW gene and promoter compared to the orthologous region in mouse, rat, dog, opossum, wallaby and platypus. Conservation is indicated by horizontal lines representing a contiguous region of homology. Vertical height of the line indicates percentage homology, with 50% homology at the bottom of the panel and 100% the top. Human annotations are indicated above the plot. Repeats are represented by grey half height shapes. Exons are represented by black full height rectangles with an exon number above. The opossum, wallaby and platypus exhibit levels of conservation similar to the eutherian mammals within exons, however there is a significant reduction in non-exonic conservation. Only the proximal promoter and the locus control region exhibit significant conservation, clearly delineating these important control regions. Two conserved regions are present for each exon in platypus due to the presence of the SWS2 gene.

gene instead. We subsequently prepared primers complementary to the platypus SWS2 sequence and we were able to amplify the entire SWS2 mRNA sequences using both platypus and echidna retinal RNAs (Genbank: Platypus SWS2 GenBank accession # EU624412, Genbank: Echidna SWS2 GenBank accession # EU636021). The platypus and echidna SWS2 opsins are 96% identical and 99% similar. They are identical at all amino acid positions known to cause spectral shifts (L46, T49, F52, S91, T93, A94, M122, S127, A164, L207, C211, F261, A269, and S292). The echidna visual pigments are therefore expected to have the same spectral properties as platypus (Davies et al., 2007). A protein BLAST search, phylogenetic analysis (data not shown) and ortholog prediction from genome annotation (Warren et al., 2008) confirmed that the platypus and echidna SWS2 opsins are homologous to those of reptiles, birds and fish.

The proximity and orientation of the SWS2 and LWS opsin genes in the platypus was determined using the genome sequence. In the platypus, the SWS2 and LWS opsins are tandemly arranged with a ~20 kb gap. This is very similar to their orientation in reptiles, birds, and fish. To test if the echidna opsin genes are also similarly spaced, we performed a long range PCR reaction on echidna genomic DNA using a forward primer located in exon 4 of the SWS2 gene and a reverse primer located in intron 1 of the LWS gene. The amplified genomic fragment was ~15 kb in length.

Phylogentic footprinting identified significant regions of sequence conservation at the LCR and in 200 bases of the proximal *OPN1MW* promoter (Fig. 2). The marsupial and platypus sequences exhibited an extremely clean footprinting pattern, showing no conservation in intronic regions and clearly identifying coding regions and untranslated regions. In the platypus, a second, less conserved footprints corresponding to the SWS2 opsin was observed in *OPN1MW* coding regions. The low amount of noise in the conservation analysis allows clear delineation of the extent of the LCR. The 37 bp defined as the core LCR was not completely conserved in marsupials, and the 30 bp upstream of this sequence showed equivalent conservation (Fig. 3). Variation was observed in the CRX binding site within the 37 bp core LCR and a second CRX binding site in the 30 bp upstream showed stronger conservation. Significant flanking conservation was also observed outside the CTAATC core, indicating that sequences outside the core motif may contribute to the function of the LCR.

Discussion

We have shown that gene structure and expression of monotreme cone opsin genes bridge the phylogenetic gap between reptiles and other mammals. The L opsins of the monotremes are very homologous to the L and M opsins in other mammals. However, monotremes have SWS2, typical of reptiles, birds, and fishes, rather than the typical SWS1 gene in other mammals. The SWS1 gene in monotremes seems to have been lost, except for exon 5. The presence of a detectable exon 5 from the monotreme SWS1 gene in the platypus and echidna indicates that the deletion that removed the upstream exons is a relatively recent event. The absence of expression from platypus and echidna of this exon confirms that this deletion is not a genome assembly artifact, and dates the deletion prior to the echidna/platypus divergence ~21 MYA (Warren et al., 2008).

The mammalian ancestor therefore had an opsin complement that consisted of tandemly arranged SWS2 and M/LWS genes with a central LCR, and an SWS1 gene. As no RH2 class opsin has been detected in platypus this class of opsins was probably lost prior to the divergence of extant mammals. A proposed phylogeny pathway for the evolution of the opsin gene loci is shown in Fig. 4. Gene duplication, followed by sequence divergence resulted in the formation of photo pigment gene clusters each of which is regulated by a common *cis*-regulatory element like the LCR. We propose that LCRs in the genome have played a role in maintenance of such clusters, since translocation of a gene from a cluster may result in alteration in its spatial and temporal patterns of expression.

Identification and fine delineation of the regulatory element using phylogenetic footprinting is particularly valuable in the case of the LCR, as this element does not function as an enhancer in

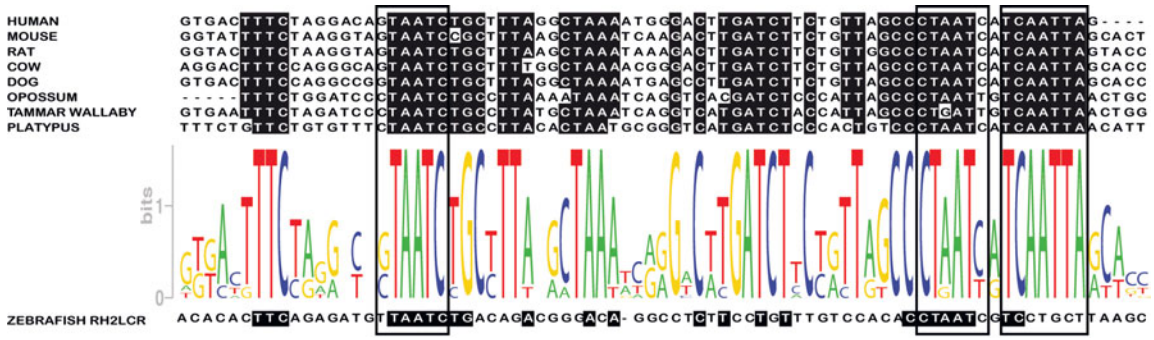

Fig. 3. The Opsin Locus Control Region.Multiple sequence alignment of the Locus Control Region. The previously defined extent of the LCR is underlined. The previously identified CRX and RX binding sites, and the proposed upstream CRX binding sites are boxed. The previously identified CRX binding site conforms to the consensus in eutherian species but is not completely conserved in marsupials. The upstream CRX binding site conforms to the consensus in marsupials and monotremes but is attenuated with a G in eutherians.

transient transfection studies. The requirement for stable integration into the genome for correct functioning severely limits the extent to which mutation and deletion strategies can be utilized to dissect the sequences responsible for its function.

Our analysis clearly indicates that the LCR is a larger element than the 37 bp previously identified by sequence conservation. The presence of two core TAAT-CRX binding motifs conserved in the LCR with a 39 bp spacing across all mammals strongly suggests that CRX binds to this region as a homodimer. The action of CRX as a dimer is supported by previous observations of double bands for CRX target genes *GUCY2D* and *ARR3* in gel mobility shift assays (Qian et al., 2005).

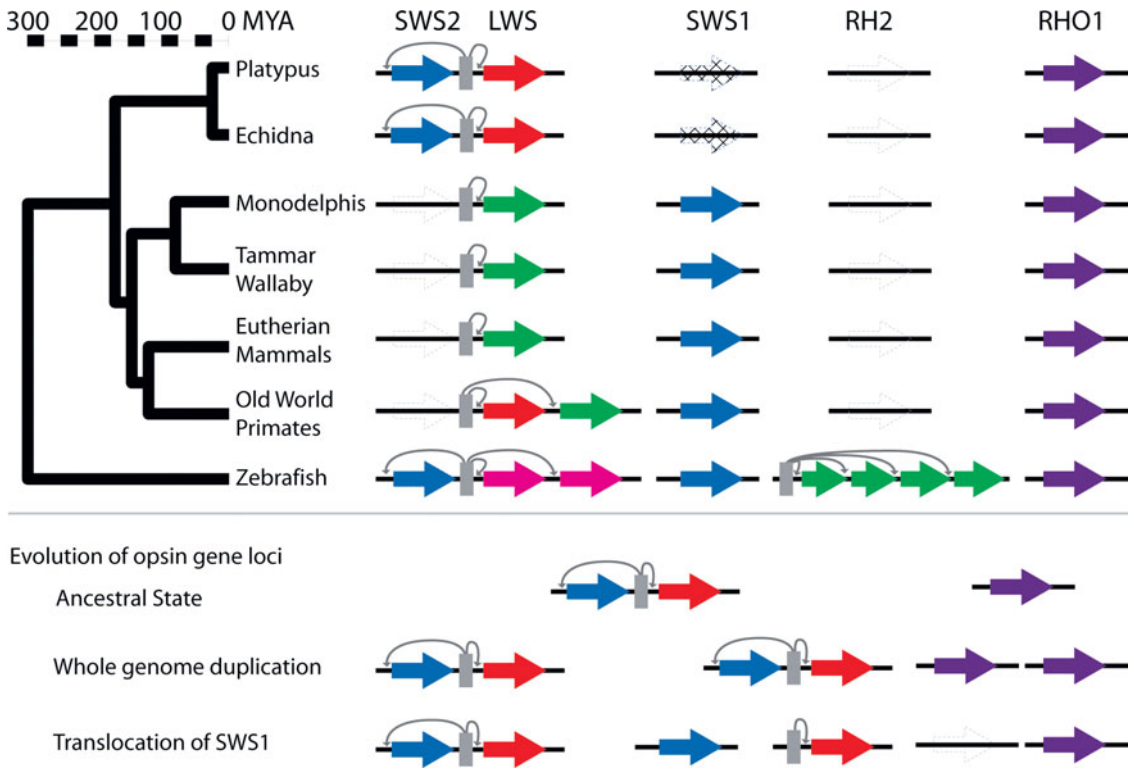

Fig. 4. Evolution of Visual Pigment Opsins.We propose that the tandem arrangement of SWS-LCR-LWS is an ancient ancestral arrangement that existed prior to the vertebrate whole genome duplication (Ohno, 1970; Dehal & Boore, 2005). After whole genome duplication a translocation seperated the SWS1 gene away from the LCR-RH2 locus and the duplicated RHO1 was lost. Loss of the RH2 opsin in mammals and SWS2 gene in therian mammals and lineage specific duplications of LWS and RH2 result in the observed opsin gene complements. The absence of an LCR results in duplications of SWS1 and RHO1 requiring mutations that provided regulation as well as spectral tuning before the duplication conveys any selective advantage, while duplication at the LWS and RH2 loci that maintain an LCR require only spectral tuning mutations to provide a selective advantage (Deeb et al., 2006).

The location of the LCR between the SWS2 and LWS genes in platypus suggests that the LCR in the ancestral mammal functioned as a bi-directional enhancer. The symmetrical conformation of the CRX binding sites to the consensus sequence may indicate selection for symmetry or strength in the monotreme ancestor that was relaxed after the deletion of the SWS2 gene.

Transgenic constructs that relocate the zebrafish RH2-LCR within the RH2 array show that this element is also capable of bi-directional function. Promoters located closer to the element exhibit higher rates of expression, and its influence extends over at least 35 kb (Tsujimura et al., 2007). Although the zebrafish RH2 enhancer exhibits functional and sequence similarities to the SWS2-LWS LCR it is unclear if this element represents a truly orthologous element that has been maintained since the initial opsin duplication occurred, or whether this element is an example of convergent evolution.

The absence of any detectable remnant of the SWS2 class opsin in both tammar wallaby and opossum indicates that the marsupial ancestor lacked a SWS2 gene. Combined with the absence of SWS2 in eutherian mammals, this suggests the SWS2 gene was lost in the therein ancestor prior to the marsupial/eutherian divergence. This excludes SWS2 opsin as a candidate photoreceptor for involvement in trichromatic colour vision reported for some Australian marsupials.

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