Evolutionary ecology of the visual opsin gene sequence and its expression in turbot (Scophthalmus maximus)

CURRENT STATUS: UNDER REVIEW

BMC Evolutionary Biology

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DOI: 10.21203/rs.3.rs-19116/v1

SUBJECT AREAS
Evolutionary Developmental Biology
Evolutionary Biology

KEYWORDS
turbot, benthic life, adaption, opsin, heterochronic shift
Abstract
Background As flatfish, turbot undergo metamorphosis as part of their life cycle. In the larval stage, turbot live at the ocean surface, but after metamorphosis they move to deeper water and turn to benthic life. Thus, the light environment differs greatly between life stages. The vision system plays a great role in organic evolution, but reports of the relationship between the visual system and benthic life are rare. In this study, we reported the molecular and evolutionary analysis of opsin genes in turbot, and the heterochronic shifts in opsin expression during development.

Results Our gene synteny analysis showed that subtype RH2C was not on the same gene cluster as the other four green-sensitive opsin genes (RH2) in turbot. It was translocated to chromosome 8 from chromosome 6. Based on branch-site test and spectral tuning sites analyses, E122Q and M207L substitutions in RH2C, which were found to be under positive selection, are closely related to the blue shift of optimum light sensitivities. And real-time PCR results indicated the dominant opsin gene shifted from red-sensitive (LWS) to RH2B1 during turbot development, which may lead to spectral sensitivity transition from red to green.

Conclusions We demonstrated that RH2C may be an important subtype of green opsin gene that was retained by turbot and possibly other flatfish species during evolution. Moreover, E122Q and M207L substitutions in RH2C may contribute to the survival of turbot in the bluish colored ocean. And heterochronic shifts in opsin expression may be an important strategy for turbot to adapt to benthic life.

Background
To survive, all organisms must react to changes in the physical environment, such as temperature, circadian rhythm of light, and humidity [1]. Thus, to frame the evolutionary perspective about the molecular basis of organismal adaption and biology, sensory systems are generally selected as ideal models [2]. Vision is closely related to behaviors such as foraging, mating, parental care, and avoiding predation. As it allows for almost instantaneous transmission of information, vision likely plays a great role in organic evolution [3]. Vision formation involves retinal reception, integration, and higher-order brain processing [4, 5]. Retinal reception is mediated by visual pigments, which consist
of one opsin protein, a group of G protein-coupled receptors, and one chromophore (11-cis-retinal, A1, or 11-cis-3, 4-dehydroretinal, A2) [3].

Due to water absorption and scattering, the photic environment of the aquatic system in which fish live changes rapidly with depth, especially in the ocean. In order to adapt to different spectral conditions, teleosts possess five classes of visual opsin genes: 1) RH1 (rhodopsin) for dim light; 2) RH2 (rhodopsin-like opsin) for green; 3) SWS1 (short wavelength-sensitive type 1) for ultraviolet; 4) SWS2 (SWS1-like opsin) for blue; and 5) LWS (long wavelength-sensitive) for red. With the exception of RH1, which is expressed in rod photoreceptors, the other four classes are expressed in cone photoreceptors [3]. The cone opsin genes derived from two rounds of whole genome duplication [6]. It is believed that after several additional duplication events, diverse opsin repertoires were maintained among different species [7-12]. RH2 and LWS duplication events were most prevalent in ray-finned fish, and tandem duplication seems to have produced more duplicates [13]. Due to substitution in key sites, the multiple opsin subtypes from duplication generally have different spectral peak absorbance ($\lambda_{\text{max}}$), which helps enrich the visual system [2, 3, 7, 10].

In addition to the adaptive evolution of gene sequences, heterochronic shifts in visual opsin expression are an important mechanism of spectral tuning [14, 15]. For example, single cones in rainbow trout (Oncorhynchus mykiss) switch opsin expression from SWS1 to SWS2 during the juvenile period [16]. In winter flounder (Pleuronectes americanus), only RH2 was expressed in the premetamorphic retina, whereas RH1, SWS2, and LWS were also expressed in the postmetamorphic retina [17]. Besides ontogenetic changes, plasticity of visual opsin expression in response to different photic environments is an important strategy that allows rapid adaptation to environmental changes. Plastic opsin expression was reported to have a profound effect on Nile tilapia (Oreochromis niloticus) and guppy (Poecilia reticulate) during development [18, 19]. It was also reported for the adult stage of fish such as red shiner (Cyprinella lutrensis) and bluefin killifish (Lucania goodei) [20, 21]. Additionally, a recent study showed that opsin coexpression might be a novel mechanism for modulating color vision [22]. However, it is unclear whether the direction and extent of opsin expression plasticity are limited by ontogeny [23].
The turbot (Scophthalmus maximus) is an important aquaculture species with great commercial value. As a flatfish, metamorphosis is a critical part of its life history. In the early stage, it lives at the ocean surface. After metamorphosis, turbot move to deep water and enter a benthic phase. This change in habitat results in a great shift in environmental conditions, and its visual system may change accordingly. However, visual characteristics and the opsin expression pattern of turbot and their relationship to benthic life remain unknown. In the present study, we investigated the selection pressure acting on turbot and eight other teleost species and conducted spectral tuning sites and synteny analyses to evaluate the adaptive evolution of turbot visual opsin genes. We also investigated the heterochronic shifts during development of turbot. Results of this study enrich the understanding of sensory adaptation in demersal fish.

Results

**Phylogeny and syntenic analysis of turbot visual opsin**

Figure 1 shows an unrooted visual opsin phylogenetic tree of turbot and eight other species constructed using the neighbor-joining method. The tree confirmed the identities of turbot opsin genes: RH1, SWS1, SWS2, RH2A1, RH2A2, RH2B1, RH2B2, RH2C, and LWS. Unlike other single-copy opsin genes, turbot have five RH2 paralogs. Figure 2 shows the results of comparative synteny analyses among six selected teleost species. In general, opsin gene positions were conserved among teleosts. For example, the RH1 gene was typically positioned between the gene loci of prickle2a and ren. In addition to RH2C, four other RH2 genes of turbot were located in tandem on chromosome 6. By comparing the synteny region containing RH2C, we found that although the genomic regions both downstream and upstream were otherwise maintained, RH2C was missing in the other five species (Fig. 2B). When we expanded the analysis to all fish genomes that could be analyzed, the RH2C loci was still not found. Similarly, LWS-1, LWS-2, and LWS-3 of guppy formed a tandem gene cluster, while LWS-4 was located on chromosome LG21 (Fig. 2C). Additionally, SWS1 was not present in the tongue sole genome (Fig. 2A).

**Natural selection and spectral tuning sites analysis**

To evaluate the selective constraints acting at the branch level in teleosts, branch-specific selection
analysis was performed. In all cases of opsin genes, the free-ratio model provided a better fit, indicating the heterogeneity of $\omega$ values (nonsynonymous/synonymous rate ratio, $\omega = dN/dS$) among branches (Table 1). For the branch-site models, the test was conducted on a particular branch of the tree. The LRT comparisons between model A and the null model revealed several sites under positive selection on nine branches of the tree (red labeled branches in Fig. 1). These sites under episodes of positive selection are listed in Table 2. Among the lineages selected in the phylogenetic tree, only LWS lacked a positive selection site. Furthermore, positive results were found for all branches of zebrafish except LWS. Among all selected branches, the SWS2 branch of zebrafish had the most sites. Based on amino acid multiple alignments, we surveyed the main tuning sites involved in spectral sensitivity. Tables 3 and 4 show the results for RH2 and RH1, respectively, and the others are shown in Tables S2–4. Asparagine (N) was present at site 83 in five flatfish species, while the other teleost genomes had aspartic acid (D) in RH1; there were no differences in the other three sites. With the exception of Atlantic halibut, the other four flounders all had changes of glutamic acid (E) to glutamine (Q) at site 122 and methionine (M) to leucine (L) at site 207 in RH2C. For SWS1, sites 46 and 114 of turbot contained phenylalanine (F) and alanine (A), respectively, but serine (S) was present in those locations for the other four flatfish species. Moreover, in the b branch of SWS2 in the tree (Fig. 1), all four species had the same amino acid substitution of valine (V) for threonine (T) at site 99. We also found a turbot-specific amino acid site (122S) in SWS2.

**Divergence time of turbot RH2 genes**

The time that the turbot RH2 genes diverged was estimated by MCMCTree using the soft fossil constraints method, and then we obtained an evolutionary pathway (Fig. 3). We speculated that five RH2 genes were the product of several duplication events. The proposed first divergence time was 166 (139–193) million years ago (Mya) in the Jurassic, which formed RH2A and RH2B/RH2C. The third divergence (about 71 Mya) created RH2B and RH2C. The divergence times of RH2A1/RH2A2 and RH2B1/RH2B2 were estimated to be 87 (51–128) and 34 (15–55) Mya, respectively. The divergence of RH2B1/RH2B2 was the latest one, and it occurred in the Tertiary.
Heterochronic shifts in opsin gene expression

In these experiments, expression levels of visual opsin genes in turbot at different developmental stages were determined by qPCR. Eight of the visual opsin genes were expressed at a low level at 0.5 and 18 months of age, but RH1 was highly expressed at 18 months of age. From 1 to 9 months of age, RH1, SWS2, and SWS1 expression significantly increased and LWS, RH2A1, RH2B2, and RH2C expression significantly decreased as turbot grew. No significant change was detected in RH2A2 and RH2B1 throughout ontogeny (Fig. 4). Among the RH2 genes, RH2A1/RH2B2 and RH2A2/RH2B1 exhibited the same expression pattern throughout ontogeny, and the pattern for RH2C was closer to that of RH2A2 and RH2B1. When we analyzed the proportional opsin gene expression of each cone opsin gene, we found that LWS, RH2B1, and RH2C were the three genes present in the highest proportions. The maximum LWS expression level was 53.85% at 1 month of age, while that of RH2B1 was 63.27% at 9 months of age. Thus, the dominant opsin shifted from LWS to RH2B1 during turbot development (Table. 5).

Discussion

Molecular evolution of RH2 and RH1

Through phylogenetic analysis, we characterized the visual opsin genetic component of turbot. Five green opsin genes are present in the turbot genome, and this number is the largest among the sequenced fish species to date, along with Pacific bluefin tuna (Thunnus orientalis) [10, 25]. Our gene synteny analysis showed that RH2C was not on the same gene cluster as the other four RH2 genes. In addition, the RH2C locus was absent in other teleost species analyzed in this study. Unfortunately, our gene synteny analysis failed to cover all species of fish, including some flatfish such as barfin flounder, spotted halibut, and Japanese flounder. We do not know yet whether their RH2Cs are arranged in tandem with other RH2 genes on the chromosome. Additionally, despite the close genetic relationship of tongue sole, it lacks the RH2C locus. We predicted that RH2C might have originated from flatfish-specific duplication, after which it was translocated to chromosome 8 from chromosome 6 in turbot. It is generally accepted that gene duplication may generate redundant genes, which is usually followed by degenerative mutations on one member of the pair and even gene loss [13, 26,
Subsequently, some flatfish lineages lost RH2C, whereas other species such as turbot retained it. Although a previous study found no signals of positive selection on visual opsin genes of turbot [25], we detected 10 amino acid sites of RH2C, and sites 122 and 207 are related to spectral sensitivity. In barfin flounder, the $\lambda_{\text{max}}$ of RH2C is blue shifted by approximately 16 nm compared to RH2B [28]. Additionally, mutagenesis experiments in coelacanth indicated that both E122Q and M207L cause blue shift of optimum light sensitivities [3, 29, 30]. Thus, we speculated that the $\lambda_{\text{max}}$ of RH2A1, RH2A2, and RH2C are blue shifted in turbot. Moreover, in turbot multiple copies of RH2 form green opsins with different $\lambda_{\text{max}}$, as is found in zebrafish and medaka [7, 8]. Different spectral peak absorbance is beneficial because it allows the fish to discriminate a wider spectrum of light. It may enhance color vision and contribute to prey detection in the bluish ocean [10]. Based on the loss of RH2A function in the genus Verasper [11] and our results showing low expression levels of RH2B2 and pairs of RH2A, we deduce that RH2B1 and RH2C are the major RH2 genes in turbot. Furthermore, the retention of RH2C is an adaptation of turbot to the spectral environment in the deep sea due to its short-shift of $\lambda_{\text{max}}$. Additionally, some studies have shown that opsin genes are tied to nuptial and body coloration [31, 32], but further work is required to confirm this function. Regarding the RH1 genes, all flatfish species studied herein have the substitution of D83N, which has been demonstrated to cause blue shift in cattle and chameleons [33]. However, this site was not positively selected. Therefore, whether this replacement is beneficial for benthic adaptation needs further study. For the other three sites, no differences were found in nine fish species [34-36].

**Heterochronic changes in opsin expression**

Heterochronic changes in a variety of traits have been reported, including opsin expression [15, 37-40]. A study of cichlids revealed that subfunctionalization of heterochronic changes in expression is critical for preservation of opsin genes [41]. Furthermore, altering opsin expression patterns during ontogeny is an important mechanism for modulating color vision [14]. In order to adapt to different life cycle stages, organisms retain three patterns of heterochronic changes in opsin gene expression. For instance, in cichlid fishes, Nile tilapia showed a normal pattern of opsin gene expression that
changes dynamically from a larval gene set to a final adult set. In contrast, cichlids from Lake Victoria had only an adult gene set with little change through time (direct developing pattern), and rock dwellers from Lake Malawi had a reduced rate of change (neotenic pattern) [15]. In the current study, we found a normal pattern of visual opsin expression in turbot. Turbot undergo metamorphosis during growth and development, which is accompanied by changes in the spectral environment: the bright daylight and long wavelength spectrum environment during the pelagic phase and the dim light and short to medium wavelength spectrum environment in the benthic phase. Transformation of light environments in turn might lead to shifts in opsin gene expression and spectral sensitivity [42]. Our results showed that turbot undergo heterochronic shifts in opsin gene expression, which may alter spectral sensitivity and contribute to scotopic vision. Specifically, the increased expression level of RH1 is beneficial for observation in diminishing luminance when the fish enter deep water. As for cone opsin genes, the highest proportional expression level of LWS at the early developmental stages is gradually replaced by RH2B1 at the later developmental stages during ontogeny. This variation means that the visual sensitivity shifts from red to green during adaptation to the deep sea environment. Downregulation of LWS and upregulation of RH1 expression level were also found during development in barfin flounder (Kasagi et al. 2015). Similarly, no rod photoreceptors were found in retinas of larval winter flounder (Pseudopleuronectes americanus), indicating no expression of RH1, whereas three types of photoreceptors with different $\lambda_{\text{max}}$, including rods, were present in adult retinas. These results indicate increased RH1 expression and a shift in spectral sensitivity [42]. In our study of turbot, we found low expression of cone opsins and a slight increase in proportional level of LWS at 18 months of age. Thus, color vision seems not to be important for turbot in the deep sea, and higher LWS expression may occur in preparation for reproduction.

**Genetically based versus environmentally triggered variation**

The developmental progression in opsin expression is strongly linked to shifts in spectral sensitivity, which is generally considered to be an adaptation strategy to ambient light changes that occur over the life cycle. It is not yet clear whether heterochronic shifts in opsin expression are genetically determined or environmentally triggered. Generally speaking, opsin expression should not need to
change if there are no differences in the environmental spectrum. However, in the present study, we found that the turbot, which has been artificially domesticated for a long time, still showed variation of opsin expression in the same spectral environment during different developmental stages. Thus, it retains the developmental progression of the wild state. Hence, we speculate that the normal pattern of turbot opsin expression is a pre-programmed developmental event, and not environmentally triggered. Cichlids from Lake Malawi show bounded plasticity, which occurs within the genetically controlled expression profile is consistent with our hypothesis [43].

Conclusions
Our results indicate that evolutionary changes in gene sequences and heterochronic shifts in opsin expression are the main ways that turbot adapt to environmental photic variations from the pelagic to the benthic period. Specifically, the positive selection of E122Q and M207L of RH2C is closely related to a blue shift of the spectral peak absorbance, and RH2C may be an important green opsin gene retained by some flatfish species, including turbot, after gene duplication. In addition, spectral sensitivities tuned by heterochronic shifts in opsin expression is another strategy to adapt to different ambient light spectra during the life cycle. And we propose that the heterochrony is pre-programmed.

Methods
Phylogenetic and synteny analysis
To identify the visual opsin gene repertoire of turbot, zebrafish opsin sequences were used as BLASTp query sequences with e-value < $10^{-10}$, and they were downloaded from the chromosome of the reference genome. Opsi sequences of four flatfish (spotted halibut, *Verasper variegatus*; Japanese flounder, *Paralichthys olivaceus*; barfin flounder, *Verasper moseri*, and Atlantic halibut, *Hippoglossus hippoglossus*) and four freshwater species living in shallow water (zebrafish, *Danio rerio*; medaka, *Oryzias latipes*; guppy; and zebra mbuna, *Maylandia zebra*) were obtained from GenBank. For accession numbers, see previous research [11, 28, 44, 45]. Phylogenetic relationships among the visual opsin nucleotide sequences were inferred using MEGA 7 software [46] by applying the neighbor-joining method [47] and maximum composite likelihood model algorithm [48]. The reliability of tree topology was evaluated by bootstrap analysis with 1000 replications and uniform rates among
sites. Genomicus synteny [49] and Ensembl genome browsers were used to assess the syntenic regions between turbot and five other teleost genomes (tongue sole, Cynoglossus semilaevis; Japanese medaka HNI; zebra mbuna; zebrafish, and guppy).

**Branch and branch-site test of selection**

To estimate the differences in selection pressure between five flatfish and four freshwater fish, branch-specific models and branch-site models of maximum likelihood were implemented in the CODEML program of PAML4.9i [50]. Tree topologies of each gene were the same as those obtained in section 2.1. First, we compared the one-ratio model and free-ratios model in the branch test of selection [24]. We then compared null Model A against the alternative Model A in the branch-site test to determine whether positive selection was present; different sets of foreground lineages were marked with letters in each gene (Fig. 1) [51]. The likelihood ratio test (LRT) was used to compare all alternative models and their corresponding null models. The Bayes Empirical Bayes method was used to obtain the posterior probability of sites under positive selection [52].

**Analysis of spectral tuning sites**

Representative spectral tuning sites of amino acids were compared among the opsins of five flatfish and four freshwater species. The sites were referenced from a previous study [10]. The amino acid sequence alignments were accomplished using ClustalX [53]. The number of amino acid sites was standardized to bovine rhodopsin, except for blue opsin, which was standardized to barfin flounder SWS2A.

**Estimation of turbot RH2 divergence times**

The divergence time of turbot RH2 genes was estimated by MCMCTREE within PAML4.9i [50]. The neighbor-joining tree of six species (turbot; Atlantic salmon, Salmo salar; fugu, Takifugu rubripes; common carp, Cyprinus carpio; lungfish, Neoceratodus forsteri, and rainbow trout) was acquired by MEGA 7 using the same method as described above. The fossil calibrations were adopted from TimeTree [54]. The tree topology and fossil calibrations were set as (((((((S.maximus_RH2B1, S.maximus_RH2B2), S.maximus_RH2C), T.rubripes_RH2), (S.maximus_RH2A1, S.maximus_RH2A2)), (S.salar_RH2, O.mykiss_RH2)) ‘>1.86<2.27’, (C.carpio_RH2-1, C.carpio_RH2-2)) ‘>2.05<2.55’,
N. forsteri RH2). The RootAge was set as '<6.0'.

**Quantification of turbot opsin RNA expression**

Laval and juvenile turbot were bred at Shenghang Aquatic Science and Technology Company (Weihai, Shandong Province, China). The ages of the individuals used for analysis of heterochronic shifts in opsin gene expression were: 0.5 month (15 days post hatch), 1 month, 4 months, 9 months, and 18 months. All individuals for mRNA expression analysis were euthanized with 300 mg/L of MS-222 (Sigma, Shanghai, China) before being decapitated. The eyes were removed and immediately stored in liquid nitrogen until analyzed.

Total RNA was extracted using the RNA Isolation Kit (Vazyme Biotech Co, Nanjing, China). RNA purity and concentration were examined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Shanghai, China), and RNA integrity was verified by gel electrophoresis. The PrimeScript RT reagent kit with gDNA Eraser was used to synthesize first-strand cDNA from 0.5 μg of total RNA (TaKaRa, Dalian, China). Using the Bio-Rad CFX Connect™ Real-Time PCR System (Bio-Rad, Hercules, CA, USA), quantitative real-time PCR (qPCR) was conducted with TB Green Premix Ex Taq (TaKaRa) following the manufacturer's protocol. Melting curves were plotted to confirm amplification specificity. Using the $2^{-\Delta\Delta Ct}$ method, the relative expression level of each opsin gene was normalized to β-actin, which was selected as the internal reference gene after evaluating the expression pattern of eight commonly used housekeeping genes [55]. Table S1 shows the specific primers for qPCR. The PCR mixture (20 μl) contained 10 μl of TB Green Premix Ex Taq, 7.6 μl of PCR-grade water, 1.6 μl of cDNA, and 0.4 μl of each of the primers. The qPCR reaction was performed in triplicate with the program of 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s, 57/58 °C for 30 s, and 72 °C for 30 s. Proportional opsin expression was determined as a fraction by calculating the proportion of each cone opsin (Ti) relative to the total cone opsin expression (Tall) as follows:

$$\frac{T_i}{T_{all}} = \frac{1}{(1 + E_i)^{C_x}} \sum \frac{1}{(1 + E_i)^{C_x}}$$
where \( E_i \) represents the PCR efficiency for each pair of primers and \( C_{ti} \) is the critical cycle number for each gene [56, 57].

To assess significance of the change in opsin expression between different stages, the least significant difference (LSD) post hoc test with 95% confidence level was used. Data were analyzed using SPSS 23.0 software. Relative expression data are shown as the mean ± standard deviation, and proportional expression data are shown as averages.

Declarations

**Ethics approval and consent to participate**

All experiments were performed in accordance with the relevant national and international guidelines and approved by the Institutional Animal Care and Use Committee, Institute of Oceanology, Chinese Academy of Sciences.

**Funding**

This work was supported by the National Key R&D Program of China (Grant no. 2017YFB0404000), National Natural Science Foundation (Grant no. 41976122), Key R&D Program of Shandong Province (Grant no. 2018GHY115031), Key R&D Program of Guangdong Province (Grant no. 2019B020215001) and China Agriculture Research System (CARS-47).

**Acknowledgements**

We thank International Science Editing (http://www.internationalscienceediting.com) for editing this manuscript. Thanks to Dr. Hao Song for his comments on the article.

**Availability of data and materials**

All NCBI accession numbers used in this study are listed in Additional file 2. The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Not applicable.

**Author Contributions**
Wang, Y., Li, X. and Li, J. designed the study; Zhou, L., Wu, L., Ma, X., Xu, S. and Du, T. participated in sampling. Wang, Y conducted all analysis; Wang, Y., Li, X. and Li, J. contributed to writing the paper.

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## Tables

**Table 1. Statistics of branch-specific model analysis of turbot and other teleost species.**

| gene | lnL(1\(\omega\) model) | lnL(free \(\omega\) model) | Likelihood ratio test | \(P\) Values |
|------|--------------------------|----------------------------|-----------------------|----------------|
| LWS  | -4964.165032             | -4922.267355              | 83.795354             | <0.001        |
| SWS1 | -4253.724995             | -4240.774669              | 25.900652             | 0.0391        |
| SWS2 | -8001.473925             | -7970.454002              | 62.039846             | <0.001        |
| RH2  | -10370.70927             | -10296.73427              | 147.94999             | <0.001        |
| RH1  | -3734.012013             | -3705.674753              | 56.67452              | <0.001        |

The likelihood ratio test is to determine whether the free \(\omega\) model fits the data significantly better than the one \(\omega\) model. The \(\omega\) for each branch are not shown. lnL, ln likelihood.
Table 2. Parameter estimates of branch-site models and predicted positively selected sites.

| opsin | clade | lnL | LRT | P  | Positively selected sites |
|-------|-------|-----|-----|----|---------------------------|
| RH2   | a     | -10169.10 & -10169.10 | 0   | 1  | -                         |
|       | b     | -10166.54 & -10164.23 | 4.62 | 0.0315 & 19(0.881) | 31(0.628) | 95(0.945) | 109(0.882) | 122(0.8) | 511(0.501) | 207(0.966) | 266(0.604) | 273(0.949) | 320(0.578) |
|       | c     | -10169.10 & -10169.10 | 0   | 1  | -                         |
|       | d     | -10167.62 & -10165.03 | 5.16 | 0.0230 & 105(0.612) | 217(0.993) |
|       | e     | -10168.60 & -10168.58 | 0.03 | 0.8636 & -                    |
|       | f     | -10166.92 & -10162.26 | 9.32 | 0.0022 & 49(0.944) | 95(0.733) | 270(0.982) | 307(0.907) |
|       | g     | -10148.85 & -10136.84 | 24.01 | <0.001 & 17(0.945) | 239(0.528) | 254(0.952) | 286(0.502) | 290(0.594) | 297(0.581) | 321(0.757) | 322(0.966) | 323(0.9) | 325(0.979) | 328(0.649) | 329(0.770) | 330(0.772) |
| RH1   | a     | -3629.453 & -3625.696 | 7.51 | 0.0061 & 35(0.769) | 126(0.674) | 196(0.858) | 210(0.879) | 235(0.911) | 236(0.959) | 281(0.773) |
|       | b     | -3632.72 & -3632.72 | 0   | 1  | -                         |
|       | c     | -3632.72 & -3632.72 | 0   | 1  | -                         |
|       | d     | -3630.82 & -3630.82 | 4E-06 | 1   | -                         |
| SWS2  | a     | -7840.16 & -7831.84 | 16.63 | <0.001 & 2(0.572) | 5(0.605) | 10(0.815) | 14(0.801) |
|       | b     | -7851.02 & -7848.07 | 5.88 | 0.0152 & 38(0.560) | 99(0.904) | 276(0.687) | 299(0.983) |
|       | c     | -7852.17 & -7850.19 | 3.97 | 0.0463 & 18(0.894) |
|       | d     | -7852.21 & -7852.21 | 0   | 1  | -                         |
|       | e     | -7852.13 & -7852.10 | 0.06 | 0.8135 & -                    |
|       | f     | -7852.21 & -7851.96 | 0.50 | 0.4798 & -                    |
| SWS1  | a     | -4194.06 & -4194.06 | 0   | 1  | -                         |
|       | b     | -4194.59 & -4193.82 | 1.54 | 0.2146 & -                    |
|       | c     | -4189.36 & -4185.92 | 6.87 | 0.0087 & 8(0.895) | 9(0.860) | 10(0.788) | 11(0.765) | 16(0.704) |
|       | d     | -4828.06 & -4828.06 | 0   | 1  | -                         |
| LWS   | a     | -4828.06 & -4828.06 | 0   | 1  | -                         |
|       | b     | -4828.06 & -4828.06 | 0   | 1  | -                         |
|       | c     | -4828.06 & -4828.06 | 0   | 1  | -                         |
|       | d     | -4828.06 & -4828.06 | 0   | 1  | -                         |

InL, ln likelihood; LRT, likelihood ratio test; - , no positively selected sites.

Table 3. Comparison of representative spectral tuning sites among teleost RH2 opsins.
Table 4. Comparison of representative spectral tuning sites among teleost RH1 opsins.

| Tuning site         | 83  | 122 | 261 | 292 |
|---------------------|-----|-----|-----|-----|
| spotted halibut     | N   | E   | F   | A   |
| Barfin flounder     | N   | E   | F   | A   |
| Atlantic Halibut    | N   | E   | F   | A   |
| Japanese flounder   | N   | E   | F   | A   |
| turbot              | N   | E   | F   | A   |
| cichlids            | D   | E   | F   | A   |
| medaka              | D   | E   | F   | A   |
| guppy               | D   | E   | F   | A   |
| zebrafish           | D   | E   | F   | A   |

Marked in red are two positively selected sites of RH2C.

Table 5. Proportional expression of cone opsin genes of turbot at different stages.

| Age(month) | LWS  | RH2B1 | RH2C | Other |
|------------|------|-------|------|-------|
| 0.5        | 47.98a | 28.13a | 16.12a | 7.77a |
| 1          | 53.85a | 26.14a | 13.29a | 6.71ab |
| 2.5        | 47.43a | 34.13a | 14.51a | 3.93b |
| 4          | 24.71b | 53.39b | 17.35a | 4.55b |
| 9          | 14.64c | 63.27b | 18.74a | 3.35ab |
| 18         | 29.22b | 57.19b | 11.22b | 2.37b |

Different letters represent statistically significant differences between stages ($P<0.05$).

Additional Files
Additional file 1: Table S1-4.

Additional file 2: NCBI accession numbers used in this study.

Figures
Phylogenetic relationships of the turbot opsin genes and other teleost opsin genes based on
the neighbor-joining method. The bootstrap test (1000 replicates) scores are shown on the nodes. Branches marked in red or green were selected for branch-site models analysis: red indicates that several sites under positive selection were detected, and green indicates the opposite. Different letters marked near the branches represent the settings of the different foreground branches introduced in section 2.2.

Figure 2

Synteny analyses of visual opsin genes between turbot and five other teleost species (tongue sole, medaka, zebra mbuna, zebrafish, and guppy). (A) SWS1, (B) RH2, (C) LWS and SWS2, (D) RH1. Different gene families are represented by colored pentagons, and the direction of the pentagon indicates gene orientation. The dashed lines indicate that two adjacent genes in that species are not directly linked.
Figure 3

An inferred evolutionary pathway of turbot RH2 genes. The number on each node of the dendrogram (left) represents a duplication event, whereas the right side shows the changes in RH2 gene orientations during evolution.
Figure 4

Visual opsin expression of turbot at different stages. Gene expression was measured by quantitative Real-Time PCR (qPCR) with the TB Green Premix Ex Taq assay, and mRNA expression levels of each gene were averaged over several individuals: 0.5 month (15 days post hatching (dph), n > 30), 1 month (30 dph, n > 30), 2.5, 4, 9, and 18 months (n = 3). Different letters represent statistically significant differences between stages (P < 0.05).

Supplementary Files
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