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RESEARCH ARTICLE

Partial Opsin Sequences Suggest UV-Sensitive Vision is Widespread in Caudata

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Abstract Ultraviolet (UV) vision exists in several animal groups. Intuitively, one would expect this trait to be favoured in species living in bright environments, where UV light is the most present. However, UV sensitivity, as deduced from sequences of UV photoreceptors and/or ocular media transmittance, is also present in nocturnal species, raising questions about the selective pressure maintaining this perceptual ability. Amphibians are among the most nocturnal vertebrates but their visual ecology remains poorly understood relative to other groups. Perhaps because many of these species breed in environments that filter out a large part of UV radiation, physiological and behavioural studies of UV sensitivity in this group are scarce. We investigated the extent of UV vision in Caudata, the order of amphibians with the most nocturnal habits. We could recover sequences of the UV sensitive SWS1 opsin in 40 out of 58 species, belonging to 6 families. In all of these species, the evidence suggests the presence of functional SWS1 opsins under purifying selection, potentially allowing UV vision. Interestingly, most species whose opsin genes failed to amplify exhibited particular ecological features that could drive the loss of UV vision. This likely wide distribution of functional UV photoreceptors in Caudata sheds a new light on the visual ecology of amphibians and questions the function of UV vision in nocturnal animal species.

Keywords SWS1 opsin gene · Ultraviolet vision · Caudata · Paralog gene · Tuning site · Nocturnal species · Amphibian · Sliding window · Ka/Ks

Introduction

Many invertebrates (Briscoe and Chittka 2001; Porter et al. 2007) and vertebrates (Yokoyama 2002; Bowmaker 2008) see in the ultraviolet (UV) range (300–400 nm). UV sensitivity is the ancestral state for vertebrates (Collin et al. 2003; Tresize and Collin 2005). It is used in communication (Bennet et al. 1996; Smith et al. 2002; Whiting et al. 2006), foraging (Siitari et al. 1999; Li and Lim 2005; Novales Flamarique 2013), and orientation (Eugene and Buchmann 1974; Edrich 1979; Kawamura et al. 2009). At the molecular level, UV sensitivity is enabled by the presence in the retina of a UV-sensitive (UVS) short-wavelength sensitive opsin (SWS1). When struck by a photon, the chromophore, covalently linked to the opsin, isomerizes, which leads to a conformal change of the opsin protein that initiates the transduction of the neural signal. In some other species, the SWS1 opsin is present, but a different sequence of amino acids at key tuning sites changes the structural configuration of the protein, shifting the light absorbance properties of the photoreceptor to a non-UV range shifted to longer wavelengths (Yokoyama 2000).

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Because the contribution of UV radiation to total irradiance from sunlight is relatively modest (Johnsen 2012), one would primarily expect UV vision in species living in bright environments, where transmission in the UV range is the highest. For instance, there is very little UV radiation available under forest canopies (Endler 1993), which likely makes the use of UV wavelengths less useful for animal species living in this environment. Similarly, absorption by water and dissolved organic compound may restrain the use of UV wavelengths to shallow aquatic environments and clear waters (Morris et al. 1995; Crump et al. 1999; Johnsen 2012). Moonlight is also several orders of magnitude lower than sunlight, which renders nocturnal colour vision more challenging because of the trade-offs involved between sensitivity and spatial and temporal resolutions (Warrant et al. 2004). Nevertheless, some nocturnal species are capable of colour vision at night (Warrant et al. 2004; Carvalho et al. 2006; Johnsen et al. 2006; Kelber and Roth 2006). In addition, the red-shifted spectrum of moonlight relative to sunlight (Johnsen 2012) would make UV vision in nocturnal species less likely but nocturnal species within particular lineages are UV-sensitive, which has been well documented in mammals such as rodents, marsupials and bats (Peichl 2005; Zhao et al. 2009). In the case of violet-sensitive (VS) primates (Peichl 2005; Veilleux et al. 2013) and UVS bats (Zhao et al. 2009), the loss of SWS1 functionality seems associated with species ecology and light environment (cave, closed canopy). The long history of nocturnality, in bats at least, seems to rule out evolutionary inertia to explain the retention of SWS1 (Perry et al. 2007; Zhao et al. 2009; Moritz et al. 2013; Veilleux et al. 2013).

Amphibians have been considered the second most nocturnal group of vertebrates after bats (Hölker et al. 2010). Indeed, many species have fully nocturnal habits on land and/or live in environments with low light intensity (forest litter for instance) (Rafaëlli 2007). They often mate, breed and spend their larval stage in freshwater habitats where UV is strongly scattered and excess attenuation by dissolved organic carbon occurs (Morris et al. 1995; Crump et al. 1999). Caudata tend to exhibit a more aquatic lifestyle than anurans and some groups, like the large Plethodon family, are forest dwellers (Rafaëlli 2007). Hence, UV vision could be unfavoured in amphibians, particularly in Caudata, but evidence to the contrary from physiological measurements, behavioural tests, or field observations already exists for a few species of Anura (Dietz 1972; Govardovskii and Zueva 1974; Ries et al. 2008) and Caudata (La Touche and Kimeldorf 1979; Perry and McNaughton 1991; Deutschlander and Phillips 1995; Przyrembel et al. 1995; Secondi et al. 2012; Korenyak and Govardovskii 2013).

Even if species active at dusk may benefit from a short-wave shifted spectrum and a higher relative contribution of UV radiation (Rickel and Genin 2005; Johnsen 2012), widespread UV vision in mostly nocturnal and aquatic breeding animals would be puzzling. To our knowledge, the SWS1 opsin has only been sequenced in two species of Caudata, *Ambystoma tigrinum* (Xu et al. 1998) and *Cynops pyrrhogaster* (Sakakibara et al. 2002). Results seemed to indicate a peak of maximum absorption in the UV range. However, it seems no physiological or behavioural evidence of UV sensitivity has been provided in these species yet. We investigated here the potential for UV sensitivity in Caudata. We sequenced the SWS1 opsin gene from species belonging to nine out of the ten living families of Caudata. This approach allows to discriminate functional from non-functional genes and to predict whether the SWS1 gene has the ability to confer UV sensitivity (Ödeen and Håstad 2003; Porter et al. 2007; Zhao et al. 2009; Hoffman et al. 2012; Veilleux et al. 2013). This work is a first step to investigate the distribution and function of UV vision in Caudata and more generally in amphibians.

Methods

Sample Collection and Amplification of Opsins

Lissotriton and *Triturus* samples were obtained from natural populations. The remaining samples were acquired from private collections of hobbyists from France and Germany. We took tissue samples or buccal swabs when the former method was unpractical or too invasive. DNA was extracted with the DNeasy extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. We successfully recovered and amplified partial SWS1 sequences from 40 out of 58 available species, representing 6 out of the 9 tested Caudata families (Table S1). The sequences were deposited in GenBank (accession numbers KP744926-66).

Based on the known SWS1 sequences of *A. tigrinum* and *C. pyrrhogaster*, we designed PCR primers to amplify SWS1 for other Caudata (Table S2). In vertebrates, this opsin has five exons and four introns (Yokoyama 2008). Depending on the group and species, SWS1 sequences with introns span between 1 kb and more than 19 kb so that long range PCR is necessary to recover full sequences. Based on preliminary work on several Caudata species, we estimated that the length of intron 1 alone is >7 kb. Furthermore, long range PCR seemed very sensitive to primer mismatch, generating only a few successful amplifications out of the 58 tested species. The same limitations were met when attempting to reconstruct the near-full coding sequence by concatenating PCR products generated by internal primers (pers. obs.). Additionally, concatenation of exons cannot yield the recovery of the entire sequence

because internal primers forbid the recovery of exon ends. Therefore, recovering a near-full coding sequence from genomic DNA was not practical. Instead, we targeted, amplified and sequenced a partial gene sequence, based on the locations of 9 known tuning sites (TS) at positions 46, 49, 52, 86, 90, 91, 93, 109 and 113, within the first 3 out of the 7 transmembrane regions (TM) of the protein (Yokoyama 2008). This fragment was located in exon 1 and ranged from 199 to 299 bp depending on the primers used for each targeted species (see primers used in Table S2). All amino acid positions in this study refer to those of the bovine rhodopsin (Nathans and Hogness 1983). Within exon 1, we could not retrieve data for many sequences at sites 114, 116, 118, which are close to the exon end. However, these sites are not known to induce major sensitivity shifts if not associated with other changes among the recovered tuning sites (Yokoyama 2008). Substitutions within TM IV to VII have been shown to have negligible effects on absorbance profiles (Takahashi and Yokoyama 2005).

The structural stability of the protein was assessed by characterizing amino acids at key positions using existing Genebank sequences for Caudata, the partial exon 1 fragment data generated in this study, but also the few products of long range PCRs we could obtain (primers and species in Table S2).

Typical PCR mix was 1× Green GoTaq Flexi buffer, 0.03 U μl^{-1} GoTaq DNA polymerase (Promega, Madison, WI, USA), 0.33 $\mu g\ \mu l^{-1}$ of Bovine Serum Albumine, 2 mM MgCl2, 200 µM dNTPs, 300 nM of each primer, 2 ng μl^{-1} of template DNA. Typical PCR conditions had a 3 min initial denaturation step at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C and a 10 min final extension at 72 °C. Typical long range PCR conditions were: 1× GoTaq Long PCR Master Mix (Promega), 300 nM of each primer, 2 ng μl^{-1} of template DNA. Products with a clear, single band were purified through a standard Exo/SAP protocol and sequenced on an ABI 3730 (Applied Biosystems, Carlsbad, CA, USA). Sequences are available on GenBank (accession numbers: KP744926-66).

Genetic Analyses

Partial sequences were checked visually and edited in Geneious 6.1.5 (Biomatters, New Zealand), aligned in Bioedit 7.2 (Hall 1999) using the ClustalW alignment tool, and trimmed manually to exclude primer and intron sequences from subsequent analyses. SWS1 sequences from *Anolis carolinensis* (class Reptilia, UVS) and *Silurana tropicalis* (order Anura, VS), as well as reference Caudata sequences for *A. tigrinum* and *C. pyrrhogaster* were retrieved from GenBank. The nine tuning sites for each species were then identified on the alignment. Ancestral state reconstruction of tuning sites was performed in Mesquite 3.03 (Maddison and Maddison 2015) by retracing the character matrix of amino acids with the maximum parsimony method at the nine recovered tuning sites on the phylogenetic tree reconstructed from Pyron and Wiens (2011). Phylogenetic analyses of the partial SWS1 DNA fragment within exon 1 and corresponding amino acid sequences were also performed following the protocol presented in Text S1.

K_a/K_s (also called dN/dS or ω), the ratio of non-homologous substitutions (K_a) by homologous substitutions (K_s), is often used as a proxy to identify variation in selective pressure within a gene (Schmid and Yang 2008), including for opsins (Hoffmann et al. 2007; Zhao et al. 2009). K_a/K_s Ratios <1 suggest purifying selection, whereas ratios around 1 point at relaxed selection, and ratios >1 at positive selection (Yang and Bielawski 2000). We performed separate analyses for Caudata ($n = 41$, i.e. 40 species recovered, one with 2 paralog sequences), Salamandridae ($n = 25$) and Plethodontidae ($n = 7$), the latter being the families within the order Caudata with the largest sample sizes. We used the integrative analysis implemented in the HyPhy-based Datamonkey webserver (Kosakovsky Pond et al. 2005; Delport et al. 2010) to detect positive and negative codon selection on the Caudata, Salamandridae and Plethodontidae datasets. The analysis detect codons under selection using three methods: single-likelihood ancestor counting (SLAC), fixed effects likelihood (FEL) and random effects likelihood (REL). The approach allows for a conservative decision rule (i.e. it retains the codons for which all methods give significant tests) and a liberal one (it retains the codons for which at least one method give a significant test). The model of nucleotide substitution was set to K80 (112211), in agreement with the closest model chosen by jModelTest 2.1.5 (Text S1). For other settings, the default parameters were used, including significance levels (p values ≤ 0.1 for SLAC and FEL, REL Bayes factor ≥ 50).

Sequence-wise K_a/K_s ratio were estimated through the Datamonkey webserver with SLAC and REL (Kosakovsky Pond et al. 2005; Delport et al. 2010), but also in DnaSP v5 (Librado and Rozas 2009). A sliding window analysis of the K_a/K_s ratio was performed to highlight conserved regions. Our a priori hypothesis was that purifying selection should be stronger in regions containing key tuning sites for the protein structure (ratio close to zero). Sliding windows of 10–50 nucleotides with incremental steps of 2–20 nucleotides were tested. Respective window and step sizes of 25 and 5 nucleotides were finally chosen empirically to compromise between a sufficient number of values to plot along the sequence and stable, interpretable patterns of variation of the K_a/K_s ratio.

Results

The reconstructed SWS1 tree (Fig. S1) had a very similar configuration to that of the phylogenetic tree based on several genes (Pyron and Wiens 2011) (Fig. S2). While the sequence reflects the general evolutionary pattern of the genome, we also found strong evidence that the opsin gene is conserved and functional in most of the Caudata species we analyzed. No stop codons were found in the recovered sequences. Based on all available sequences, from this study and from those recovered in Genebank, the structural stability of the proteins was assessed by checking the presence of key amino acids at particular positions that assure a functional spatial configuration (Palczewski et al. 2000). The conserved K^{296}, to which the chromophore is covalently attached, was found for all species for which we have the near complete SWS1 sequence (*A. tigrinum, C. pyrrhogaster, Lissotriton vulgaris, Lissotriton helveticus*). C^{110} and C^{187} which allow the formation of a conserved disulfide bridge between them (Karnik et al. 1988; Palczewski et al. 2000) were systematically found in all recovered species. The tripeptide (D/E)R(Y/W) motif (Kim et al. 1997; Palczewski et al. 2000), which is involved in the propagation of phototransduction, was also examined and the $E^{134}/R^{135}/Y^{136}$ motif was systematically found in our sequences. The presence of conserved residues N^{55} and W^{161} was also verified (Palczewski et al. 2000).

Regarding known tuning sites, as expected, the S90C mutation that shifts SWS1 opsin from a violet sensitive (VS) to a ultraviolet sensitive (UVS) form, and has been only found in birds (Wilkie et al. 2000; Yokoyama et al. 2000), was not observed in Caudata species (all S^{90}). The Schiff base linking the opsin to the chromophore can be in a protonated or unprotonated state depending on mutations affecting its counterion, i.e. amino acid at position 113 and other tuning sites within the binding pocket (Hunt et al. 2007). In this regard, F^{86}, S^{90} and the counterion E^{113}, which are found in the vertebrate ancestor, were also systematically encountered in our sequences (except for *Notophtalamus viridescens a* sequence, NVa), suggesting an unprotonated Schiff base link with the chromophore implying UV sensitivity through the presence of F^{86} (Hunt et al. 2007). Substitutions at tuning sites were few. In fact, with the exception of the NVa sequence, mutations were only observed at sites 93 and 109 (Fig. 1). The ancestral V^{109} (Yokoyama 2008) was substituted in at least five several separate events by another non-polar amino acid, I^{109}. Three other independent mutations, in *Rhyacotriton cascadae*, *C. pyrrhogaster*, and *N. viridescens a*, resulted in M^{109} instead, another non-polar amino acid. Similarly, most sequences had T^{93} (polar), while *Hydromantes strinatii* and *C. pyrrhogaster* had S^{93} (polar) and non-polar A^{93} was found in *Ranodon sibericus*.

We found two sequences for *N. viridescens*, both recovered from each of two individuals by using small variations in PCR conditions. Their phylogenetic positions indicate that they are paralogs (Fig. S1). One sequence (NVb) has a tuning sites haplotype similar to the majority of the species in its family (*A. tigrinum* haplotype). The other sequence (NVa) has mutations at four different tuning sites (F49L/F86V/T93P/I109M). Interestingly, while I109M was also found in *C. pyrrhogaster* and *Rhyacotriton cascadae*, no other sequences retrieved from the other Caudata species had similar L^{49}, V^{86} and P^{93} mutations. L^{49} and P^{93} were also found in the VS SWS1 sequence from *S. tropicalis* (along with a different H^{86} mutation). Furthermore, F86V probably involves protonation in the Schiff base, suggesting a shift to a VS opsin (Hunt et al. 2007). In fact, the opposite V86F mutation in UV SWS1 opsin of the guinea pig was shown to produce a ~54 nm shift towards shorter wavelengths, achieving UV sensitivity (Parry et al. 2004).

For Caudata, Salamandridae, and Plethodontidae K_a/K_s ratios were consistent across all methods and much lower than 1 (Table 1). The REL $K_a/K_s = 0.368$ for Plethodontidae ($n = 7$) exhibited the highest ratio, but although REL is the recommended method for datasets of 5–15 sequences (Delport et al. 2010), it is also more sensitive to false positive than other methods and could overestimate the ratio. The sliding window analysis for all recovered species (Fig. 2a) revealed that the ratio varied slightly but generally stayed below 0.16 along the sequence. The ratios were low (<0.1), especially around regions densely populated by known tuning sites (amino acids 46–52, 86–93, 109–113, respectively). One possible exception occurs around amino acid position 86 in Salamandridae, where the ratio was >0.6 (Fig. 2b), but close inspection of the alignment reveals this is mainly the result of amino-acid substitutions that have not been identified as tuning sites within the range of the 25 nucleotide window (data not shown). In the sliding window analysis of Salamandridae (Fig. 2b), the ratio slightly exceeded 1in two regions centered around nucleotide positions 245 and 315, maybe suggesting relaxed purifying selection around these sites. These regions seemed to flank the region comprising TS 86–93, which is well conserved aside from very few mutations (Fig. 1). Conversely, when testing for individual codon selection, no codon was found to be under positive selection in the Salamandridae dataset regardless of the method used (Table 1).

Regarding the analysis of codons under selection performed on the entire Caudata dataset (Table S3), no positively selected codon was found across all three methods (SLAC/FEL/REL). Positive selection was detected for one codon (position 97) in one method, with marginal significance (REL Bayes factor = 50.7). On the contrary, significant purifying

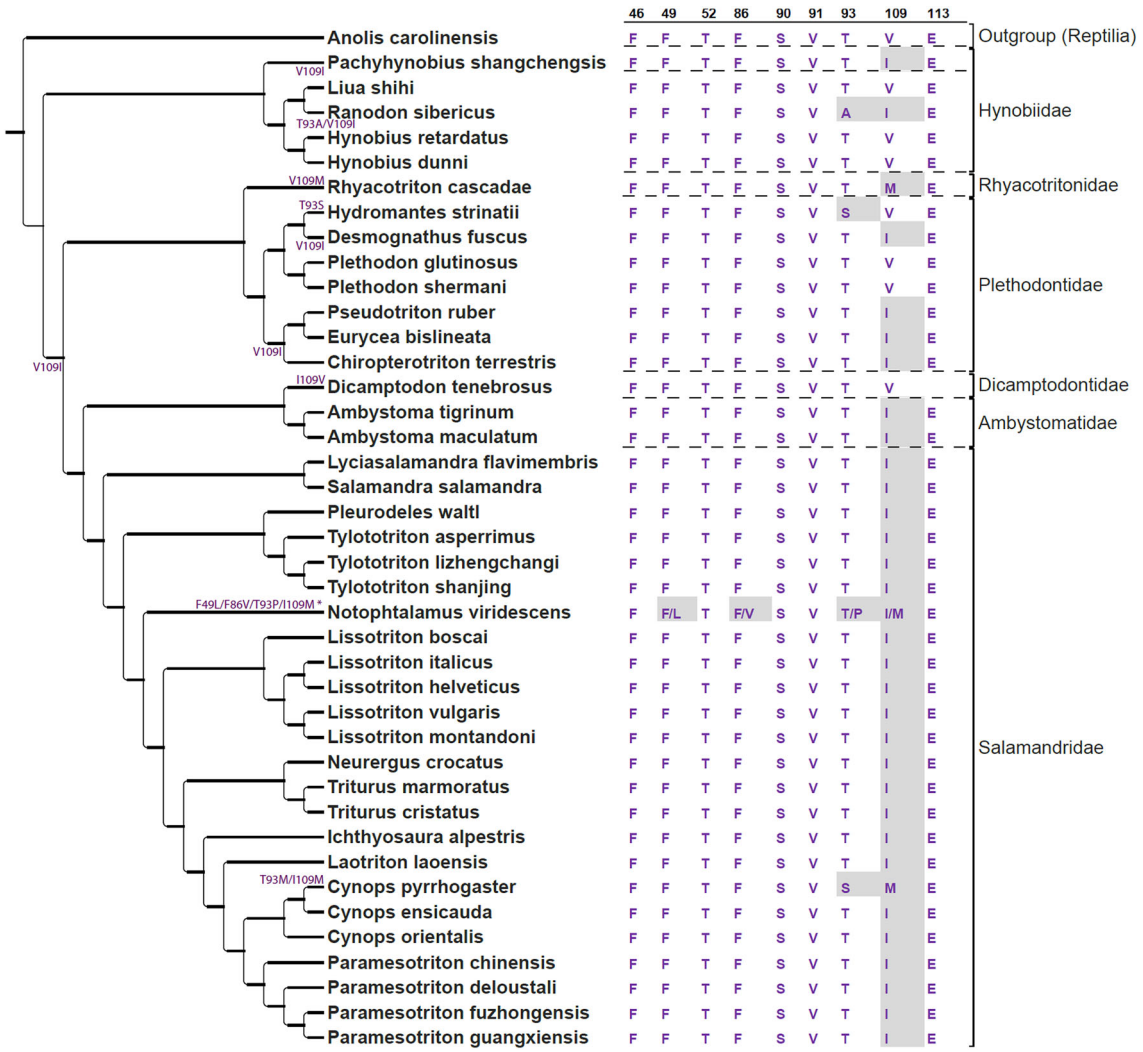

Fig. 1 Inferred mutations of the SWS1 opsin at key tuning sites in Caudata. *Left*: phylogenetic tree adapted from Pyron and Wiens (2011). *Right*: amino acids at nine known tuning site positions. The most parsimonious mutation events inferred by ancestral state reconstruction in Mesquite (*purple*) are placed within the tree. *Grayed boxes* show elements that differed from the *C. carolinensis* outgroup, but also from the amphibian ancestor (Yokoyama 2008), and the common Caudata ancestor as reconstructed from our sequence dataset (which ancestor shares the same tuning sites as *C. carolinensis*). For *N. viridescens* paralogs, *slash bars* indicate alternative amino acid at tuning sites (NVb/NVa). *Asterisk*: combination of mutations for NVa (Color figure online)

selection in the three methods was detected for 32 out of 99 codons (Table 1) (61 codons in at least one of three methods), including TS 46,52,90,93,113. Significant negative selection was also detected for TS 49, but for 2 out of 3 methods (excluding REL) while the value for TS 91 was significant for FEL only. TS 86 and 109, showed no significant signs of selection for any of the three methods.

Discussion

Evidence of Functional SWS1 Opsin Genes

We amplified the SWS1 gene in most Caudata families. Three lines of evidence support the hypothesis that the gene is structurally stable and functional in these families. First, we observed no stop codons and found the expected residues that ensure protein stability at several key positions. We found L^{296}, that forms a Schiff base linkage with the chromophore, and the disulfide bridge between C^{110} and C^{187} that is found in most G protein–coupled receptors and functioning retinal-binding opsins (Palczewski et al. 2000). We could also observe N^{55}, that bonds to two other structural residues, and W^{161} that is likely involved in signal transfer (Lin and Sakmar 1996; Palczewski et al. 2000). Nonetheless, a word of caution is necessary, since we only characterized a partial sequence of the SWS1 gene. In theory, the remaining fragments of the sequences might still contain stop codons, lack essential residues or have frame-shifting deletions or insertions resulting in the

Table 1 Summary of the SWS1 partial sequence analyses for Caudata order, Salamandridae and Plethodontidae families

	K_a/K_s method	Caudata (n = 41)	Salamandridae (n = 25)	Plethodontidae (n = 7)
Total number of codons		99	99	99
Negatively selected codons (min–max)	SLAC, FEL, REL	32–61	9–30[a]	2–26
Positively selected codons (min–max)	SLAC, FEL, REL	0–0	0–0	0–1
Ka/Ks methods	K_a/K_s DnaSP	0.056	0.127	0.051
	SLAC (HyPhy)	0.086	0.148	0.084
	FEL (HyPhy)	N/A[b]	N/A[b]	N/A[b]
	REL (HyPhy)	0.133	0.195	0.368

K_a and K_s in DnaSP v5 were computed with the Jukes and Cantor correction. SLAC/FEL/REL are three methods developed to detect codons under selection available in the HyPhy-based Datamonkey webserver. Note that "min" is the number of codons under selection detected by all methods and "max" is the number codons under selection as detected by at least one of the methods. For codon selection, only significant results are reported

[a] For Salamandridae, the REL analysis indicated that the Bayes factor could not be calculated given that K_s was equal to 0 for all codons but that this pointed at purifying selection across the sequence

[b] Mean K_a/K_s along the sequence cannot be calculated by the program for the FEL method

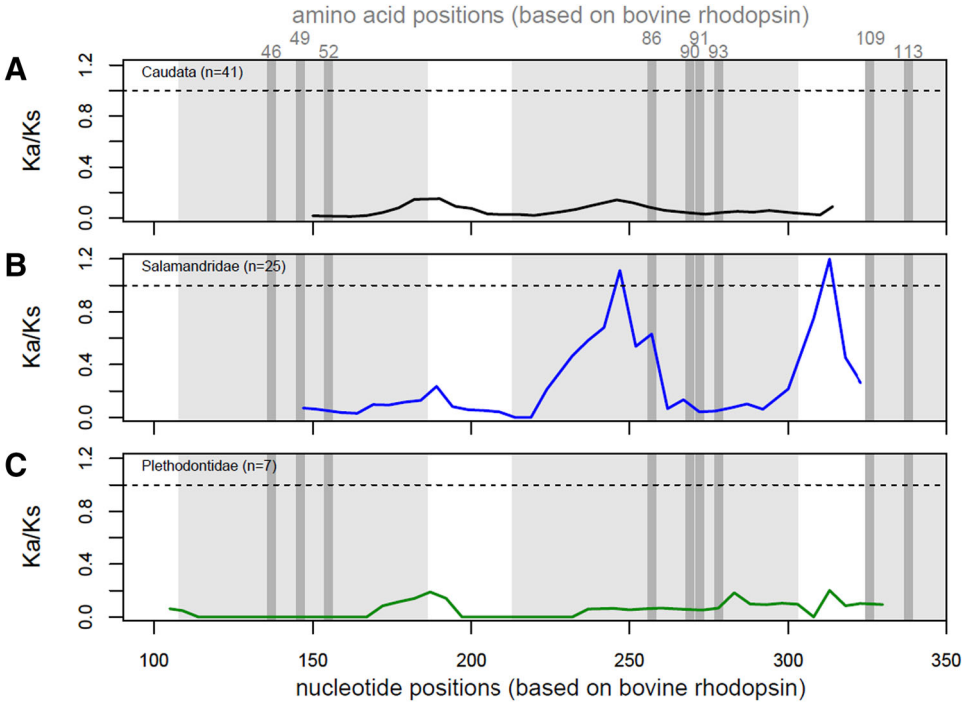

Fig. 2 K_a/K_s sliding window analyses of the recovered SWS1 opsin fragment. A. K_a/K_s sliding window analysis for our 41 recovered fragments (Caudata). B. K_a/K_s sliding window analysis for Salamandridae only (n = 25). C. K_a/K_s sliding window analysis for Pletodontidae only (n = 7). *Grayed areas* indicate transmembrane helices I, II and III (*left* to *right*). *Vertical gray lines* are positioned at key tuning sites. Nucleotide and codon positions are respectively given on the *lower* and *upper* x-axis

loss of protein function or formation (Carvalho et al. 2006). While events of this kind in only a few of the studied species could go unnoticed, a widespread loss of function in Caudata seems very unlikely since the lack of selection on the lost protein would result in the accumulation of random mutations, including deletions and insertions, eventually affecting key residues in at least some of the sequenced fragments.

Second, tuning sites known to confer UV sensitivity were largely conserved. Most species shared the haplotype of *A. tigrinum* for which UV sensitivity has been ascertained (Perry and McNaughton 1991). We found only a few substitutions at four tuning sites. Substitutions on two sites concerned one single sequence (NVa). The main substitution was V109I. UV sensitivity has in fact been demonstrated in at least four other Salamandridae species, by microspectrophotometry in *Pleurodeles waltl, Cynops orientalis* and *Lissotriton vulgaris* (Korenyak and Govardovskii 2013), behavioral experiments in *L. vulgaris* (Secondi et al. 2012) and electroretinographic measurements

in *Taricha granulosa* (La Touche and Kimeldorf 1979). *T. granulosa* does not have SWS1 sequence data because it was not sampled in our study, but the remaining of this group of species, where UV-sensitivity was previously demonstrated, all carry the I^{109} substitution. Furthermore, evidence of UV-sensitivity from species with this haplotype is also brought by larvae of *L. vulgaris* and *L. helveticus* that show varying levels of SWS1 expression in response to changes in UV exposure (J. Secondi, unpublished data). In total, 89.7 % of species with recovered SWS1 sequences possessed either V^{109} or I^{109}. The rarer M^{109} aminoacid residue involved an amino acid with the same properties as the two others (non-polar), suggesting limited impact on the protein structure and absorbance. In vertebrates, key substitution at site 86, 90, and 93 cause sensitivity shifts UVS to VS pigments and vice versa even if shifts are not consistent between species (Hauser et al. 2014). We observed substitutions at site 86 and 93 in four species from four genera. Only the co-occurrence of a Valine at site 86 and a Proline at site 93 in one copy of *N. viridescens* may induce a shift to a VS pigment (Carvalho et al. 2012). Other identified absorbance shifts, including missing 114, 116 and 118 sites, may require co-occurring sets of substitutions which were not observed in our surveyed tuning sites (Yokoyama 2008). For this reason, we do not expect the lack of data for these sites to affect our general conclusions about UV sensitivity. Additionally, the unprotonating effect on the Schiff base linkage with the chromophore of E^{113}, which was found in all recovered sequences, is necessary to produce UVS pigments whereas protonation likely produces VS pigments (Hunt et al. 2007).

Third, in the absence of selection, non-functional genes are expected to accumulate random mutations occurring at a neutral rate (Page and Holmes 2009), even on small evolutionary scales (Lynch and Conery 2000). Instead, our data suggests strong purifying selection along the partial sequence in Caudata, which characterizes a conserved, likely functional region. Indeed, we observed significant purifying selection of individual codons and very low K_a/K_s ratios for Caudata, Salamandridae, and Plethodontidae overall and along the sequence, especially in regions of key tuning sites but also in another region comprising no known tuning sites (approximately amino acid positions 64-78). Like for tuning sites, this region could be important to maintain opsin structure but caution should be applied to the interpretation of patterns which are not confirming an a priori hypothesis (Schmid and Yang 2008). Nonetheless, overall values were consistent with those found in bats possessing functional SWS1 (Zhao et al. 2009) and strongly suggested purifying selection of SWS1 sequences in Caudata.

Opsin duplication has been reported in all opsin gene classes, with many examples for the RH2 and LWS classes (Davies et al. 2012). Confirmed SWS1 paralogs have only been reported in the smelt *Plecaglossus altivelis* though (Minamoto and Shimizu 2005). We identified a second case of duplication in SWS1 for *N. viridescens*, with the NVb sequence corresponding to the UVS SWS1 ortholog forms in Caudata, and the NVa sequence to a paralog VS SWS1 form. Since no other species in the order has revealed the presence of SWS1 paralogs so far, it is unclear if the duplication is specific to this species or has appeared earlier and is present in other related species. The possible selective advantage of possessing UVS and VS opsins simultaneously should also be further investigated.

Implications of the Widespread Distribution of UV Sensitive Opsin Sequences in Caudata

In Caudata there is no report of oil droplets (Bowmaker 2008).We do not know about light transmission for cornea, lens or the rest of ocular media but the likely presence of functional UV-sensitive SWS1 opsins in so many species seems inconsistent with the widespread occurrence of UV filters in this group. Indeed, in other taxa, ocular media transmittance data of fish (Siebeck and Marshall 2007), jumping spiders (Hu et al. 2012), mammals (Douglas and Jeffery 2014), lacertid lizards (Pérez i de Lanuza and Font 2014) and birds (Lind et al. 2014) concur that species with photoreceptors having absorbance peaks in the UV range do not filter UV light (Hofmann et al. 2010). Conversely, these studies show that the opposite is not always true. Some species that do not express photoreceptors absorbing maximally in the UV still have transparent ocular media letting UV through. This is understandable, as some species still perceive UV light through the margin of their SWS1 α absorbance peak or through the secondary (β) absorbance peak of other photoreceptors (Hofmann et al. 2012; Pérez i de Lanuza and Font 2014; Douglas and Jeffery 2014).

Therefore, according to our analyses, UV sensitivity seems widespread across Caudata. Sequences were not retrieved from all species though, which may be due to changes on primer hybridization sites of functional genes, or reflect the presence of non-functional genes. It is interesting to note that many non-amplified species exhibit particular ecological features. Some entirely or partially live in caves (*Proteus anguinus*, *Hydromantes italicus, Salamandra algira*). Others are described as living in potentially dim environments such as the forest litter (*Bolitoglossa dofleini*, *Eurycea lineola, Pseudoerycea cephalica*), or as having a strong aquatic (*Siren intermedia, Amphiuma tridactylum*, *Necturus lodingi, N. maculosus, Desmognathus quadramaculatus*) or terrestrial lifestyle (*S. Algira, H. italicus, Thorius troglodytes, E. lineola, P. Cephalica, Bolitoglossa platydactyla, B. rufescens, B. dofleini*). For some of these species, we successfully

amplified functional LWS, suggesting that DNA quality was adequate (*Onychodactylus koreanus*, *S. intermedia*, *Tylototriton verrucosus, N. lodingi, A. tridactylum, P. cephalica, B. platydactyla, B. rufescens*). The sensory ecology of species can drive relaxed selection on SWS1 opsin gene or entire cone loss (Kawamura and Kubotera 2004; Tan et al. 2005; Perry et al. 2007; Zhao et al. 2009; Jacobs 2013; Veilleux et al. 2013). It is therefore possible that some of the failed amplifications reflect the loss of functional SWS1 gene in species experiencing low or no UV radiation. Furthermore, although this could only be achieved partially on a limited number of species, such as *L. vulgaris* and *L. helveticus* (Secondi et al. 2012; Korenyak and Govardovskii 2013), J. Secondi unpublished data), we recognize that an integrative approach combining complete gene sequences, gene expression, microspectrophotometry and behavioural experiments on a few key representative species would be very helpful to confirm the findings of this study. We expect this approach to support the evidence that the UVS opsin sequences from our data are, in fact, representative of a widespread use of UV vision in Caudata. Given the expected extent of UV sensitivity in Caudata, one could wonder about Anurans. The common ancestor to all amphibians was UVS (Tresize and Collin 2005). Demonstrations of UV sensitivity (Dietz 1972; Govardovskii and Zueva 1974; La Touche and Kimeldorf 1979; Perry and McNaughton 1991; Deutschlander and Phillips 1995; Przyrembel et al. 1995; Secondi et al. 2012) and indirect evidence like the development of mating coloration (Ries et al. 2008) exist for a few species. Finally, many species exhibit partial diurnal habits. Despite evidence that some Pipidae have a violet-sensitive SWS1 opsin (Starace and Knox 1998), ecological and biological facts suggest that UV vision may have been largely overlooked in amphibians.

The apparent wide distribution of UV sensitivity in Caudata contrasts with the usual view considering UV as deleterious to amphibians (Blaustein et al. 1997). Actually, many temperate amphibians increase their activity at dusk and early night at a time when they could benefit from a short-wave shifted irradiance spectrum (Johnsen 2012). Melin et al. (2012) have also speculated that the presence of an intact, blue-shifted SWS1 in the aye–aye could be an adaptation to its crepuscular activities. Large variations in the colour pattern of Caudata species, and more largely in amphibians, exist (Rafaëlli 2007) but quantitative analyses of colouration have been carried out in a few species only and the mate selection process is poorly known for many families relative to other groups like birds or fishes. Salamandridae are interesting in this regard. Adults breed in water and some species exhibit colourful skin patches which reflect UV, in the genera *Lissotriton* (Secondi et al. 2012), *Triturus, Salamandra, Ichtyosaura*, or *Ommatotriton* (JS pers. obs.). Thus, visual sexual communication and partly diurnal habits during the mating period may account for selection on SWS1 opsin genes. Selection pressures acting for typical forest species like *Plethodon* are less obvious. Whether UV sensitivity correlates with communication or any other biological function remains to be investigated in this group of vertebrates.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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