Seeing red to being red: conserved genetic mechanism for red cone oil droplets and red coloration in birds and turtles supports the existence of colour vision in dinosaurs

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Abstract

Avian ketocarotenoid pigments occur in both the red retinal oil droplets that contribute to colour vision and bright red coloration used in signalling. Turtles are the only other tetrapods with red retinal oil droplets, and some also display red coloration. Recently, the $CYP2J19$ gene was strongly implicated in ketocarotenoid synthesis in birds. Here, we investigate $CYP2J19$ evolution in relation to colour vision and red coloration in reptiles using genomic and expression data. We show that turtles, but not crocodiles or lepidosaurs, possess a $CYP2J19$ orthologue, which arose via gene duplication before turtles and archosaurs split, and which is strongly and specifically expressed in the ketocarotenoid-containing retina and red integument. We infer that $CYP2J19$ initially functioned in colour vision in archelosaurs and conclude that red ketocarotenoid-based coloration evolved independently in birds and turtles via gene regulatory changes of $CYP2J19$. Furthermore, results strongly suggest the presence of colour vision in dinosaurs and pterosaurs.

Keywords: Carotenoid coloration, turtles, birds, $CYP2J$, colour vision, retinal oil droplets
Introduction

Ketocarotenoids are red xanthophyll carotenoids whose terminal ketone groups, when on the C4 position on one or both end rings, lead to the absorption of light at longer wavelengths than in other xanthophylls. They have been extensively studied in birds where they are the predominant mechanism of saturated red colours that are frequently sexually or socially selected signals. In addition, ketocarotenoids perform another key function in birds: the ketocarotenoid astaxanthin is the red pigment found in the oil droplets in longwave sensitive (LWS) cones of the avian retina. The red retinal oil droplets act as cut-off filters to enhance colour discrimination in colour vision. Ketocarotenoids in most landbirds are not obtained directly from the diet but instead synthesised by ketolation (addition of double-bonded oxygen at the C4 position of the end ring) of dietary yellow carotenoids such as zeaxanthin. The mechanism was for a long time obscure, but recent studies have strongly implicated a locus encoding a cytochrome P450 monooxygenase (CYP2J19) as the main or only avian ketolase enzyme that catalyses the conversion of dietary xanthophylls to the ketocarotenoids that are deployed in both red coloration and red retinal oil droplets. This raises the intriguing question of the origin of CYP2J19 function in birds, and its ancestry in vertebrates more generally.

Turtles (Testudines) are the only group of tetrapods apart from birds that possess red retinal oil droplets, and these contain ketocarotenoids. Like birds, turtles have excellent tetrachromatic colour vision. In contrast, other extant reptile lineages either lack retinal oil droplets (crocodiles, snakes) or have coloured oil droplets varying from clear to yellow and green, but not red (many lizards).

Red coloration of the integument is uncommon in turtles, but red coloration due to ketocarotenoids has been reported and shown to be related to sexual selection. Importantly, aquatic turtles, unlike most landbirds, have the potential to obtain red ketocarotenoids directly from their diet, and whether turtles have the capacity for carotenoid ketolation remains unknown.

Several interesting questions arise: Is there a shared genetic basis for ketocarotenoid synthesis in birds and turtles? Alternatively, was ketocarotenoid synthesis independently derived in the two
groups, or even absent in turtles? If ketocarotenoid synthesis does have a shared genetic basis, was the
original function of *CYP2J19* for colour vision or coloration?

The phylogenetic position of turtles within the reptiles has long been a source of debate mainly due to the highly derived morphologies of turtles and the somewhat contradictory molecular evidence. More recently, a number of large scale molecular studies using genomic data support a sister relationship between turtles and the archosaurs, forming the turtle-archosaur clade (Archelosauria) independent from the lepidosaurs (lizards, snakes and tuatara). With turtles now placed as an outgroup to archosaurs, the evolution of a locus related to colour vision and coloration in turtles has the potential to inform the occurrence of these traits in the dinosaurs and pterosaurs.

Here we address the genetic basis of ketocarotenoids in the retina and integument of turtles and birds using two approaches. First, we mine recent genomic data and perform phylogenetic analyses to reconstruct the evolutionary history of *CYP2J19* within reptiles and birds. Second, we examine expression of *CYPJ*-like loci in the Western painted turtle (*Chrysemys picta bellii*), which possesses both red retinal oil droplets and red ketocarotenoid-based integumentary coloration, to obtain evidence for functional involvement of *CYP2J19* in ketocarotenoid synthesis in different tissues in Testudines. We find that *CYP2J19* is conserved in birds and turtles but has apparently been lost in crocodiles, and that patterns of *CYP2J19* expression are consistent with a function in both colour vision and coloration in turtles, as in birds. We interpret these results to suggest that *CYP2J19* likely originally functioned in colour vision in the Testudines-archosaur clade ancestor, and has since been independently and repeatedly recruited for red integumental coloration by changes in gene regulation in turtles and birds. Finally, our results provide indirect evidence for the presence of good colour vision in lineages related to birds, including dinosaurs and pterosaurs.
Results

Evolution of CYP2J genes in amniotes

BLASTn searches revealed the presence of two CYP2J family loci in the three most complete turtle genomes out of the four available (Western painted turtle, Chinese soft-shell turtle, and green sea turtle). In contrast, we found at most a single CYP2J sequence in crocodilians (American alligator, Chinese alligator) and lepidosaurs (green anole, Schlegel’s Japanese gecko). Strikingly, phylogenetic analyses revealed that one of the CYP2J loci in turtles is orthologous to avian CYP2J19, since turtle sequences form a well-supported (100% bootstrap support) monophyletic clade with CYP2J19 sequences from birds (Figure 1). No CYP2J19 orthologues were identified in either crocodilians or lepidosaurs. The remaining reptile and avian CYP2J loci group together by order but support for other relationships is variable. Reconstructions suggest that the crocodilian and second turtle CYP2J locus are related to avian CYP2J40, but the bootstrap support is weak. The single CYP2J locus in lepidosaurs is placed as an outgroup to the Testudines and archosaur (crocodile + bird) sequences, with moderate bootstrap support (60%). The single human CYP2J locus (CYP2J2) is clearly an outgroup to all of the reptile and avian sequences (99% bootstrap support).

Overall therefore, we find strong evidence for the conservation of the CYP2J19 locus in avian and turtle (Testudines) lineages, but no evidence for the presence of CYP2J19 in crocodilians or lepidosaurs. Furthermore, our phylogenetic analysis indicates that CYP2J19 arose from a duplication event that occurred after the divergence of reptiles and mammals and before the split between Testudines and archosaurs and CYP2J19 was subsequently lost in crocodilians.
Specific expression of CYP2J19 in tissues containing red ketocarotenoids in the Western painted turtle

Preliminary experiments revealed high CYP2J19 expression in the retina and red plastrons of Western painted turtles of both sexes, and lower, variable expression in the liver, black plastron, yellow tail, black tail, yellow neck and black neck regions (not shown). In contrast, CYP2J40 expression was detected in all tissue types (not shown).

Quantification of expression via qRT-PCR confirmed the presence of high CYP2J19 expression in the ketocarotenoid-containing tissues (retina, red plastron) of both sexes, and low expression in other tissues (Figure 2). There was significant heterogeneity of CYP2J19 expression level among different tissue types ($F_{4,25} = 35.83, \ p <0.001$) but not among sexes ($F_{1,28} = 0.4524, \ p = 0.51$) (Table 1). As no sex-biased expression was detected, a comparison between all tissue types pooled for both sexes was carried out. The red plastron had the highest CYP2J19 expression relative to all other tissues ($p<0.05$), while the retina had higher expression when compared to all tissues except for the red plastron ($p<0.001$) (Table 1).
Discussion

We provide compelling evidence for the presence of a CYP2J19 orthologue in three divergent Testudines lineages that suggests conservation of CYP2J19 function in turtles and birds, despite ~250 million year divergence between these lineages. Expression patterns of CYP2J19 in painted turtles are consistent with this gene playing a role in ketocarotenoid synthesis in both retina and red-coloured integument, as recently described in birds. This raises the question of whether the original function of CYP2J19 was for colour vision or coloration. Consideration of the likely ancestral traits, given the extant distributions, strongly favours colour vision as the original function, as we now discuss (Figure 3).

Colour vision involving ketocarotenoid-containing oil droplets in LWS cones appears to be a pervasive feature in all birds and turtles, implying that red oil droplets were present in both ancestral birds and ancestral turtles. In contrast, red ketocarotenoid coloration has a patchy distribution in both groups. In turtles, red coloration is sparsely distributed amongst both the pleurodirans and cryptodirans. Redness appears to be rare or absent in most turtle families except for two cryptodiran families, Emydidae and Geomydidae, where red coloration is more common, as well as several taxa within the pleurodian family Chelidae. In addition, the pigmentary basis for redness in turtles has not been widely characterised. In birds, reconstruction of carotenoid-based plumage coloration has shown that this trait is not ancestral but was acquired multiple times during avian evolution. Evolutionary reconstructions of carotenoid coloration in bare body parts (such as bills) in birds are still awaited. An independent line of evidence suggesting that carotenoid coloration in the integument was not ancestral in birds concerns their lack of specific pigment cells containing carotenoids. Reptiles, like amphibians and teleosts, express carotenoids (along with pteridines) in specialised xanthophores/erythrophores, whereas birds express integumentary carotenoids in keratinocytes. Although studies have found pigment cells in the avian iris that resemble reptilian xanthophores and iridophores, the absence of specialised pigment cells for carotenoids in the integument of birds seems to imply a period in their ancestry when carotenoids were not used for coloration.
Since tetrachromatic colour vision involving red retinal oil droplets was likely present in the ancestral turtle and avian lineages, but red coloration was likely absent in both, we conclude that the ancestral function of CYP2J19 was for ketolation in red oil droplets and that the role of CYP2J19 in red coloration evolved independently in the two lineages (Figure 3). Many examples of phenotypic convergence via similar genetic changes have been observed across the vertebrates. Several examples involve melanin pigmentation, and include the MC1R locus which has been involved in convergent evolution of pale/dark coloration in birds and reptiles. Here, we highlight a remarkable example of convergent evolution whereby CYP2J19 would have been independently recruited in the birds and turtle lineages, through changes in gene expression, to function in ketocarotenoid-based coloration from an ancestral retina specific function. The ecological driver for this convergence in many instances may have been sexual selection.

Our results imply that red oil droplets themselves were present in the common ancestor of the archelosaurs (archosaurs and turtles), so that red oil droplets had a single origin in tetrapods (Figure 3). An alternative would be if CYP2J19 originally arose for a different function than red oil droplets, but this is unlikely since no other functions for ketocarotenoids have been described. The inferred timing of the gene duplication that produced CYP2J19 after the split with lepidosaurs is therefore concordant with the absence of red oil droplets in this clade. The apparent absence of CYP2J19 in crocodilian genomes could be explained by the derived loss of all oil droplets from the retina that has occurred in crocodilians. Although we cannot completely rule out the presence of a divergent CYP2J19 locus in crocodiles and/or lepidosaurs which was not identified in our genome searches, it is notable that our methods did successfully identify one CYP2J locus, as well as CYP2D6 and CYP2R1 in two crocodilian and two lepidosaur genomes (Figure S1). The function of CYP2J40 is unknown, but the observed expression amongst all the tissues studied (in both turtles and birds) does not suggest any direct involvement of this locus in red coloration.

As birds are phylogenetically nested within the saurischian dinosaurs, the continuous presence of red oil droplets in the avian lineage since the split with Testudines implies the presence of red oil droplets in the dinosaurs and other lineages (including pterosaurs) that are more closely related to the living birds than crocodiles. This inference is consistent with previous reports where the
reconstruction of the complements of opsin visual pigments and coloured oil droplets in general implied that dinosaurs had tetrachromatic colour vision\textsuperscript{41-43}. Thus, our study provides an independent line of evidence for good colour vision in terrestrial dinosaurs. Good colour vision was presumably a precondition for the proposed use of coloration in intraspecific signalling in dinosaurs, where putative melanosomes have been used to infer melanin-based coloration and iridescence\textsuperscript{43-45}. Our data indicate that co-option of \textit{CYP2J19} for red ketocarotenoid coloration in dinosaurs, as has occurred in birds and turtles, would also have been possible.

The implied presence of a functional carotenoid ketolase in turtles, which are primarily aquatic, is interesting in view of the availability of ketocarotenoids in potential prey items in aquatic environments, e.g. crustaceans, which means that in principle turtles might have lost ketolase activity. If true, this may be a legacy of their terrestrial ancestry in basal archelosaurs. Another possibility is that tight control of carotenoid concentration in retinal oil droplets is required for good colour vision, and that there is too much variation in dietary ketocarotenoids to rely on this source. In this context it is interesting to note that dietary supplementation of carotenoids in birds does not affect the carotenoid content in oil droplets used for colour vision\textsuperscript{46,47}.

In birds, the anatomical site of ketolation for red coloration has long been contentious but the pattern of \textit{CYP2J19} expression has now shown that it is variable among taxa. Some species, e.g. zebra finch, perform ketolation in the peripheral tissues such as beak where ketocarotenoids are deposited\textsuperscript{10,48}, whereas in other species such as ploceids, ketolation occurs centrally in the liver, and ketocarotenoids are transported in blood to sites of deposition (Twyman et al., in prep). In turtles, the presence of \textit{CYP2J19} expression in red integument, but not in liver, is indicative of the peripheral conversion model in this group.

Apart from birds and turtles, the only other vertebrate in which red retinal oil droplets have been reported is the lungfish \textit{Neoceratodus}\textsuperscript{49}. Acquisition of red oil droplets in this lineage was likely independent to that of Testudines/archosaurs since \textit{CYP2J19} arose a long time after the split between lungfish and tetrapods, and this is supported by our failure to find a \textit{CYP2J19-like} sequence in the \textit{Neoceratodus} genome using BLAST searches. It should also be noted that the presence of ketocarotenoids in lungfish red oil droplets has not, to our knowledge, been established.
In summary, we have uncovered a remarkable case where a gene with a strongly conserved function in colour vision has been independently co-opted for red coloration in turtles and birds via changes in patterns of gene expression. Since CYP2J19 arose within reptile evolution, the genetic basis for red ketocarotenoid coloration in amphibians and ray-finned fish warrants further research. Notably, our findings in turtles and their phylogenetic position in the vertebrate tree of life strengthen the evidence that the ever charismatic dinosaurs were able to see colour.
Methods

Data mining and phylogenetic analysis of amniote CYP2J loci

BLASTn searches were conducted in all available reptilian genomes, belonging to the testudine [4], crocodilian [4] and lepidosaur [9] lineages (see Supplementary Table 1). We also performed BLASTn searches in the common ostrich (Struthio camelus) (Palaeognathae), which together with chicken (Galloanserae) and zebra finch (Neoaves) represent all three major extant avian lineages. Searches were conducted using CYP2J, CYP2R1 and CYP2D6 sequences taken from five Ensembl release 83 genomes: zebra finch (Taeniopygia guttata), chicken (Gallus gallus), human (Homo sapiens), anole lizard (Anolis carolinensis) and Chinese softshell turtle (Pelodiscus sinensis) 50. Nomenclature for avian and reptile CYP2J genes was taken from a study on avian CYPs 51. Previous work has shown that there are two lineages of avian CYP2J loci, CYP2J19 and CYP2J40 (31), whereas humans and other mammals have a single CYP2J locus, CYP2J2. CYP2R1 and CYP2D6 are single copy CYP families that are closely related to CYP2J and were used as outgroups (31).

Nucleotide sequences were aligned in MEGA version 6 52 using MUSCLE 53. Phylogenetic reconstructions of protein sequences derived from the nucleotide alignment were performed by maximum likelihood in PhyML-SMS (Smart Model Selection), using model selection based on Bayesian Information Criterion (http://www.ebi.ac.uk/Tools/sfc/emboss_seqret/) 54. The selected model was JTT+G6 (fixed at 1.997) +I (fixed at 0.089). Branch support was assessed with 1000 bootstrap pseudoreplicates.

Laboratory methods

Captive-bred male (N=3) and female (N=3) 3 month old hatchlings of the Western painted turtle were obtained by artificial incubation of eggs at constant temperatures that produce a single sex (26°C and 31°C, respectively), as previously described 55. At this life stage, red coloration is present
in the plastron but has yet to develop on the neck. Individuals were euthanized by an overdose of the 
anaesthetic propofol, and stored in RNAlater. All procedures were approved by the IACUC of Iowa 
State University. The following tissues were studied: eye, neck skin, neck muscle, plastron, tail skin 
and liver.

Total RNA was extracted using QIAGEN RNeasy Mini kits. Separate extractions were 
performed on red and black regions of the plastron, yellow and black regions of the neck, and grey tail 
skin. Dissected tissues were individually placed in 1.5ml Eppendorf tubes and submerged in liquid 
nitrogen before manual homogenization using an Eppendorf homogenizer and addition of Buffer RLT. 
The lysate was centrifuged for 2 minutes at 13,000rpm in QIAshredder spin columns before 
proceeding with subsequent full speed centrifugation step for 3 minutes. DNase digestion was 
performed using the RNase-Free DNase Set. All primers were designed in Primer3Plus using the 
Western painted turtle genome.

First strand synthesis was performed with 10µl total RNA and N6 primer (0.5µM) using 
SuperScriptII RT (Life technology Invitrogen) according to the manufacturer’s instructions. RT-PCR 
reactions contained 1 x NH4 Buffer, MgCl2 (1.5mM), each dNTP (2.5mM), each primer (0.4µM), 
BioTaq DNA polymerase (Bioline) (0.5U) and cDNA (~50ng). Reactions were run in a G-Storm GS1 
Thermal Cycler (Life Science Research) under the following conditions: 2 minutes at 94°C followed 
by 35 – 50 cycles of heating for 30 seconds at 94°C, 45 seconds at 60°C and 90 seconds at 72°C with 
a final extension of 5 minutes at 72°C. The amplified full length fragment was purified using ExoSap-
IT (Affymetrix) and sequenced on both strands via Sanger sequencing (see Supplementary 
Information) to confirm gene identity.

Quantitative real-time RT-PCR was carried out in an MJ Opticon2 (Research Engines) 
thermal cycler using the Quantitech SYBRGreen kit (Qiagen) for male and female retina, liver, red 
plastron, black plastron and yellow neck regions. We used three technical replicates for each 
condition, and three reference loci (TBP, GAPDH and HPRT1). The geNorm application for the 
evaluation of expression stability in the control genes was applied to assess the suitability of the 
reference loci. M values (denoted as the average pairwise variation of a control gene with all other 
control genes) for TBP, GAPDH and HPRT1 were 1.051, 1.085 and 0.991 respectively, indicating
suitability for their use. Differences in gene expression among tissues were assessed via Analysis of Variance using the “car” package in RStudio version 3.2. The Box-Cox power transformation for normality was applied, and lambda was fixed at 0.3 for subsequent statistical analysis. The assumptions of ANOVA were verified (see Supplementary Information).
Data Accessibility
DNA sequences: Genbank accessions XXXX

Author Contributions
HT designed the experiments, carried out molecular lab work, analysed the data and drafted the manuscript; NV, RL supplied the samples and helped edit the manuscript; SA helped to conceive the study and helped edit the manuscript; NM conceived the study, designed the experiments and drafted the manuscript. All authors gave final approval for publication.

Competing Interests
We have no competing interests.

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References


Figure 1. JTT model-based protein phylogeny based on all CYP2J, CYP2R1 and CYP2D6 sequences obtained. Bootstrap values represent 1000 pseudoreplicates. CYP2J19-like sequences and CYP2J40-like sequences are outlined in red and blue respectively. CYP2R1 and CYP2D6 clades have been collapsed. For fully expanded phylogeny, see Supplementary Fig. 1.

Figure 2. Results of qRT-PCR experiments quantifying male and female tissue-specific expression for CYP2J19 normalised against TBP, GAPDH and HPRT1. Error bars represent SEM from three individual males and females.

Figure 3. Reconstructed scenario for evolution of CYP2J19 function in red retinal oil droplets and red coloration in reptiles. Red lines denote presence of red retinal oil droplets. The inferred presence of ketocarotenoid-containing retinal oil droplets coincides with the inferred duplication of CYP2J2 prior to the turtle-archosaur split, followed by subsequent loss of oil droplets and CYP2J19 in the crocodilian lineage. Red highlighted branches show the independent gain of red ketocarotenoid based coloration in certain turtle and bird lineages associated with co-option of CYP2J19 expression for coloration.
### Table 1

Tukey’s pairwise tests of $\textit{CYP2J19}$ expression

<table>
<thead>
<tr>
<th>Pairwise tissue comparisons</th>
<th>p adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver – Retina</td>
<td>0.0000420 ***</td>
</tr>
<tr>
<td>Yellow neck – Retina</td>
<td>0.0000284 ***</td>
</tr>
<tr>
<td>Black plastron - Retina</td>
<td>0.0000188 ***</td>
</tr>
<tr>
<td>Red plastron – Retina</td>
<td>0.0434566 *</td>
</tr>
<tr>
<td>Yellow neck – Liver</td>
<td>0.9998532</td>
</tr>
<tr>
<td>Black plastron – Liver</td>
<td>0.9975022</td>
</tr>
<tr>
<td>Red plastron – Liver</td>
<td>0.0000000 ***</td>
</tr>
<tr>
<td>Black plastron – Yellow neck</td>
<td>0.9998199</td>
</tr>
<tr>
<td>Red plastron – Yellow neck</td>
<td>0.0000000 ***</td>
</tr>
<tr>
<td>Red plastron – Black plastron</td>
<td>0.0000000 ***</td>
</tr>
</tbody>
</table>
Figure 1

The figure shows a phylogenetic tree comparing various species' CYP2J-like enzymes. The tree includes species such as human, western painted turtle, Chinese soft-shell turtle, Chinese alligator, common ostrich, chicken, zebra finch, American alligator, green anole, and Schlegel's Japanese gecko. Specific branches are labeled with support values (80, 93, 100). The tree illustrates the evolutionary relationships and similarities among these species' CYP2J-like enzymes.