Genomic evidence for rod monochromacy in sloths and armadillos suggests early subterranean history for Xenarthra

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Rod monochromacy is a rare condition in vertebrates characterized by the absence of cone photoreceptor cells. The resulting phenotype is colourblindness and low acuity vision in dim-light and blindness in bright-light conditions. Early reports of xenarthrans (armadillos, sloths and anteaters) suggest that they are rod monochromats, but this has not been tested with genomic data. We searched the genomes of *Dasypus novemcinctus* (nine-banded armadillo), *Choloepus hoffmanni* (Hoffmann’s two-toed sloth) and *Mylodon darwini* (extinct ground sloth) for retinal photoreceptor genes and examined them for inactivating mutations. We performed PCR and Sanger sequencing on cone phototransduction genes of 10 additional xenarthrans to test for shared inactivating mutations and estimated the timing of inactivation for photoreceptor pseudogenes. We concluded that a stem xenarthran became a long-wavelength sensitive-cone monochromat following a missense mutation at a critical residue in *SWS1*, and a stem cingulate (armadillos, glyptodonts and pampatheres) and stem pilosan (sloths and anteaters) independently acquired rod monochromacy early in their evolutionary history following the inactivation of *LWS* and *PDE6C*, respectively. We hypothesize that rod monochromacy in armadillos and pilosans evolved as an adaptation to a subterranean habitat in the early history of Xenarthra. The presence of rod monochromacy has major implications for understanding xenarthran behavioural ecology and evolution.

1. Introduction

Electrophysiological, molecular and genetic techniques have greatly increased our knowledge of the retinal basis for vision in mammals [1–4]. Cone photoreceptors—responsible for high acuity, colour vision in bright light—typically possess one of four spectral classes of photopigment called opsins. The presence of multiple cone opsins allows for the comparison of different wavelengths of light, whereas the dim-light sensitive rod photoreceptors possess a single type of opsin, precluding hue discrimination. The common ancestor of therian mammals probably possessed dichromatic colour vision (two cone classes: short-wavelength sensitive opsin 1 (*SWS1*) and long-wavelength sensitive opsin (*LWS*)) following the loss of two of four vertebrate cone types during a hypothesized ‘nocturnal bottleneck’ in the Mesozoic [5]. The loss of additional cone classes is relatively common and has evolved independently in assorted nocturnal, aquatic and subterranean mammals [3,6,7]. These losses presumably are a consequence of inhabiting dim-light niches in which colour discrimination is limited and provide well-documented cases of convergent, regressive evolution [2,3,6,7].

Xenarthrans (armadillos (Cingulata), sloths (Folivora) and anteaters (Vermilingua)) have been overlooked in vision research, despite being an ancient and evolutionarily distinct lineage of mammals [8–11]. Most xenarthran species do not occupy dim-light niches [12], but all three groups of xenarthrans are reported in behavioural [13–18] and anatomical studies [19–22] to have vision consistent with rod monochromacy wherein the retina lacks cones entirely. Rod monochromacy is characterized by low acuity and a complete lack of colour discrimination in dim-light, and blindness during the day (hemeralopia), as rod cells become saturated in bright light. Though pure-rod retinæ have long been described in mammals [20], these reports have typically been refuted by the results of
molecular and genetic studies (e.g. contrast [20], p. 216 with [7,23–25]). Only recently have genomic studies confirmed rod monochromacy in mammals [6,7], suggesting that this is a plausible phenotype for xenarthrans.

Using genomic and phylogenetic methods, we tested the hypotheses that xenarthrans are rod monochromats and that this condition was inherited from a common ancestor. Our results suggest that xenarthrans have a long history of rod monochromacy and that the most recent common ancestor of Xenartha was at most an LWS-cone monochromat. These findings indicate that xenarthrans inhabited an extreme dim-light niche early in their evolution, which we suggest was a subterranean habit given fossorial adaptations in fossil and many living xenarthrans.

2. Material and methods

(a) Data collection

We used BLASTN to search the publically available genomes of Dasypus novemcinctus (nine-banded armadillo) and Choloepus hoffmanni (Hoffmann’s two-toed sloth) for DNA sequences of cone and rod phototransduction genes, other cone- and rod-specific genes and genes expressed in both rods and cones (electronic supplementary material, dataset S1). We used mRNA transcripts from GenBank for reference sequences. We also mined NCBI’s Sequence Read Archive (SRA) for sequences from the genome of an extinct ground sloth, Mylodon darwinii. The SRA sequences were converted into FASTA format and imported into Generous v. 7.0.5 [26]. In Generous, we gathered sequences with BLASTN using exons and at the UCR Core Instrumentation Facility. Contig assembly was both directions using an automated DNA sequencer (ABI 3730xl) Gel Purification kit. Cleaned PCR products were sequenced in excised with razor blades and cleaned with a Bioneer AccuPrep (extension), followed by an extension at 72°C (denaturation) for 10 min. Genomic DNA (500–750 ng) was used as the template for the initial PCR reaction, and 1–1.5 μl of the PCR product was used in the nested PCR reactions. PCR products were assayed on 1% agarose gels, excised with razor blades and cleaned with a Bioneer AccuPrep Gel Purification kit. Cleaned PCR products were sequenced in both directions using an automated DNA sequencer (ABI 3730xl) at the UCR Core Instrumentation Facility. Contig assembly was performed in Generous using the Muscle alignment tool [28].

(b) Inactivating mutations and pseudogene dating

Three general types of inactivating mutations were searched for manually in Se-Al: splice donor/acceptor mutations, premature stop codons and frameshift indels. Owing to the relatively high frequency of GC as an alternative splice donor in mammals [29], this variant was not considered an inactivating mutation. Sequences with splice site mutations alone were not considered pseudogenes owing to the possibility of functional splice variants. All putative mutations were compared to outgroups to determine whether they were uniquely derived. Inactivation times of pseudogenes were estimated using previously described methods [7,30]. The alignments used for the analyses can be found in the electronic supplemental material, dataset S2. We assumed phylogenetic relationships and divergence time (global means) from [31] for these calculations.

3. Results and discussion

Dasypus novemcinctus has seven inactivated cone-specific genes (SWS1, LWS, GNAT2 (cone transducin alpha subunit), PDE6C (cone phosphodiesterase 6C), PDE6H (cone phosphodiesterase 6H)), CNGB3 (cone cyclic nucleotide-gated channel beta subunit), GRK7 (cone G-protein-coupled receptor)) and two pseudogenic rod and cone genes (GUC1A1B (guanylate cyclase activator 1B) and GUCY2F (guanylate cyclase 2F)) (figures 1 and 2). By contrast, all rod-specific genes are intact (electronic supplementary material, table S1). In theory, inactivating mutations in both cone opsins (SWS1 and LWS) and/or any of the subsequent genes in the cone phototransduction cascade (CNGA3 (cone cyclic nucleotide-gated channel alpha subunit), GRK7 and GUCY2F) should result in non-functional or absent cones. The exceptions are PDE6H and GRN3 (cone transducin gamma subunit) [43], which lead to partial rod monochromacy and reduced light-sensitivity in their respective absence. The inactivation of both cone opsins (SWS1 and LWS), GNAT2, PDE6C and CNGB3 all indicate that D. novemcinctus is a rod monochromat. For C. hoffmanni, SWS1, PDE6C, PDE6H, GNIGT2, GRK7 and GUCY2F are pseudogenic (figures 1 and 2; electronic supplementary material, table S2). The rod monochromats D. novemcinctus and C. hoffmanni both have inactivated CNGA3 and PDE6H pseudogene and/or pseudogene owing to the possibility of functional splice variants. [45,46] pseudogene and/or pseudogene. The rod monochromats D. novemcinctus and C. hoffmanni both have inactivated CNGA3 and PDE6H pseudogene and/or pseudogene.
LWS-cone monochromats (figure 2). Rod monochromats show a mosaic of pseudogenization with most of the phototransduction genes inactivated in multiple lineages, including SWS1, LWS, CNGB3, GNT2, GNT2, PDE6C and PDE6H. The pleiotropic GNB3 [46,47] is the only gene that has remained functional in all rod monochromats examined (figure 2).

Dasypus novemcinctus, C. hoffmanni and M. darwini share a large deletion in the cone-specific GRK7 (figure 1; electronic supplementary material, table S3). We estimate that this was inactivated in a stem xenarthran approximately 95 Ma (figure 3; electronic supplementary material, table S4). Dasypus novemcinctus and C. hoffmanni also share a unique missense mutation in SWS1, possessing a leucine at residue 23 rather than a proline (bovine RH1 numbering; figure 1; electronic supplementary material, table S3). A proline is present in all opsin across vertebrates [49], with missense mutations in rod opsins (RH1) resulting in a non-functional pigment in vivo [50], progressive photoreceptor degeneration in vivo [51,52], reduction in chromophore yield in vivo [53], unless otherwise noted. Luciates near the Cretaceous–Palaeogene (K-Pg) boundary on August 27, 2017 http://rspb.royalsocietypublishing.org/ Downloaded from

method to date gene inactivations [27], we estimated that SWS1 was pseudogenized approximately 80 Ma in a stem xenarthran (figure 3; electronic supplementary material, table S4), rendering the earliest crown xenarthrans at most LWS-cone monochromats. This provides evidence of the earliest acquisition of LWS-cone monochromacy in mammals (electronic supplementary material, table S5). Though LWS-cone monochromacy is frequently associated with nocturnality [3], the nocturnal bottleneck hypothesis posits that placental mammals were nocturnal through the end of the Mesozoic [5,20,55,56] owing to competition and/or predation pressures from diurnal sauropsids. Yet xenarthrans represent the only extant lineage of mammals that appear to have disposed of SWS1 prior to the end of the Mesozoic approximately 65.5 Ma (electronic supplementary material, table S5) suggesting that factors other than nocturnality may explain SWS1 and GRK7 inactivation in this lineage (see §4).

Dasypus novemcinctus, C. hoffmanni and M. darwini share one premature stop codon in exon 1 of PDE6C (TGA), and the former two share a stop codon in exon 5 (TGA; no BLAST results for M. darwini). As inactivated PDE6C leads to rod monochromacy in vertebrates [41–43], these shared mutations suggest that rod monochromacy originated in an ancestor to Xenarthra. To test this hypothesis, we performed PCR and successfully sequenced exons 4 and 5 in 10 and 9 xenarthrans, respectively. PDE6C is inactivated in both Choloepus species, B. tridactylus, D. novemcinctus, E. sexcinctus, C. villosus, P. maximus and T. mutatus (figure 1; electronic supplementary material, table S2), indicating rod monochromacy is present in all of these species. However, the putative shared mutations appear to be convergent as they are absent in all armadillos that were examined except D. novemcinctus (electronic supplementary material, table S2). Nonetheless, four sloth species share stop codons in PDE6C (figure 1; electronic supplementary material, table S3), and we estimate this gene was inactivated in the common ancestor of Pilosa (anteaters + sloths) shortly after this lineage diverged from circulates near the Cretaceous–Palaeogene (K-Pg) boundary

Figure 1. Examples of inactivating mutations for all retinal genes found to be inactivated in one or more xenarthrans. Green, frameshift deletion; yellow, frameshift insertion; blue, premature stop codon; purple, splice site mutation; red, P23L missense mutation. The numbering of the nucleotide positions corresponds to those in the electronic supplementary material, table S3. A proline is present rather than a proline (bovine RH1 numbering; figure 1; electronic supplementary material, table S3). A proline is present rather than a proline (bovine RH1 numbering; figure 1; electronic supplementary material, table S3). A proline is present rather than a proline (bovine RH1 numbering; figure 1; electronic supplementary material, table S3). A proline is present rather than a proline (bovine RH1 numbering; figure 1; electronic supplementary material, table S3).
This estimate predates the earliest unambiguous pilosan fossils (31.5 Ma, Pseudoglyptodon spp. [57]) and suggests that all known extinct and extant sloths and anteaters were/are rod monochromats (figure 3). However, exon 4 of PDE6C is intact in Cyclopes didactylus and M. tridactyla (electronic supplementary material, dataset S1), so complete sequences from anteaters will be required to test this hypothesis. No inactivating mutations in exons 4 and 5 of PDE6C were shared by all armadillos (electronic supplementary material, table S3), but our estimates for the inactivation of SWS1 (80.06 Ma), LWS (65.43 Ma), GNAT2 (59.55 Ma), PDE6C (45.7 Ma) and CNGB3 (43.65 Ma; electronic supplementary material, table S4) all predate crown armadillos (41.4 Ma [48]) and the earliest fossils of the two major extinct cingulate lineages: pampatheres (16 Ma, Scirrotherium; reviewed in [58]) and glyptodonts (48.6 Ma, Glyptatelus [10], except PDE6C and CNGB3). This suggests that rod monochromacy was/is present in all of these taxa (figure 3).

Rod monochromacy is characterized by the complete absence of cones and results in poor visual acuity in dim-light and total blindness in cone pathway

cone trichromats & dichromats

LWS-cone monochromats

all mammals

rods pathway

Figure 2. Patterns of protein loss in the phototransduction cascades of various mammals. Black symbols correspond to rod phototransduction proteins. All mammals investigated so far retain the entire rod pathway (though see note about sloth PDE6B in §3). Blue and red symbols correspond to intact and inactivated cone phototransduction genes, respectively. Arrows indicate the directionality of the phototransduction cascade beginning with the absorption of light by the opsins (RH1, SWS1 and LWS), activation of transducin (GN proteins), activation of phosphodiesterase (PDE proteins) and hyperpolarization of the photoreceptor by cGMP-gated channels (CNG proteins). The absence of an arrow indicates the predicted disruption of that portion of the cascade. Species not reported on in this paper are from references [7,32]. All paintings by Carl Buell (copyright John Gatesy) except star-nosed mole and naked mole-rat (Michelle S. Fabros).
4. The xenarthran subterranean bottleneck

Rod monochromacy represents an extreme retinal adaptation to dim-light conditions because rods, not cones, are activated when very few photons are available. Consistent with this hypothesis, it has only been discovered in deep-sea fishes [59], deep diving whales [6] and subterranean vertebrates [7,60]. Therefore a long history of extreme dim-light conditions is predicted to eliminate the function of cones via directional selection for a higher density of rods or relaxed selection on the maintenance of cones. We propose that the loss of SWSI and GRK7 in stem xenarthrans, and the subsequent, independent loss of cones in pilosans and armadillos, respectively, is a consequence of early xenarthrans passing through a subterranean bottleneck.

To our knowledge, Simpson [61] was the first to suggest that the last common ancestor of xenarthrans (‘edentates’) was fossorial. Molecular timetrees suggest that xenarthrans last shared a common ancestor near the K–Pg boundary [31,48]. Fossoriality in Mesozoic mammals is not without precedent and several lineages of Mesozoic synapsids are inferred to have exhibited burrowing behaviour [62–65]. Robertson et al. [66] suggested that mammals survived the mass extinction event at the K–Pg boundary, in part, by sheltering themselves from stressful conditions (e.g. infrared radiation resulting from the Chicxulub impact) in underground burrows. The earliest xenarthran fossils (Middle Palaeocene) show fossorial limb adaptations [67] and extensive xenarthran display(ed) fossorial behaviours and/or adaptations [68–74]. Though extant anteaters and sloths are terrestrial to arboreal, xenarthran synapomorphies include features that reflect a fossorial ancestry: strongly curved claws; a secondary scapular spine, allowing for a stronger retraction of the humerus [75]; plus a synsacrum and lateral accessory articulations of the lumbar vertebrae, which help stabilize the body while digging [76,77]. Besides xenarthrans, the latter character is present only in the subterranean Mesozoic mammal Fruitafossor [64].

The morphological and palaeontological evidence of ancestral fossoriality, coupled with the loss of SWSI and GRK7 in a stem xenarthran and rod monochromacy in early cingulates and pilosans, argues for a subterranean lifestyle in the earliest xenarthrans. We suggest that passage through this hypothesized subterranean bottleneck is a historical contingency that constrained xenarthran evolution. Specifically, rod monochromacy and modifications to the postcranium related to fossoriality probably prevented diversification into various locomotory types (e.g. gliding, flying, running) and feeding habits (e.g. active predation), and canalized tree sloths to convergently adopt a suspensory posture [77]. The presence of rod monochromacy in xenarthrans should be taken into account in future behavioural, ecological and conservation studies involving this enigmatic lineage of mammals.

Data accessibility. New DNA sequences were deposited in GenBank (KP096697–KP096713). Accession numbers for all sequences can be found in the electronic supplementary material, table S6. DNA alignments uploaded as electronic supplementary material, dataset S1. Acknowledgements. We thank four anonymous reviewers for comments on an earlier stage of our manuscript. Chloebus hoffmanni, M. penta- alyla and P. macrocephalus genomes were generated by Richard K. Wilson, Wesley Warren and The Genome Institute, Washington University School of Medicine.

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