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Evolutionary analysis of vision genes identifies potential drivers of visual differences between giraffe and okapi

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Background. The capacity of species to respond and perceive visual signal is integral to their evolutionary success. Giraffe is closely related to okapi, but the two species have broad range of phenotypic differences including their visual capacities. Vision studies rank giraffe's visual acuity higher than all other artiodactyls despite sharing similar vision ecological determinants with most of them. To what extent giraffe unique visual capacity and its difference with okapi is reflected by changes in their vision genes is not understood.

Methods. The recent availability of giraffe and okapi genome provided opportunity to identify giraffe and okapi vision genes. Multiple strategies were employed to identify thirty-six candidate mammalian vision genes in giraffe and okapi genomes. Quantification of selection pressure was performed by a combination of branch-site test of positive selection and clade models of selection divergence through comparing giraffe and okapi vision genes and their corresponding orthologous sequences from other mammals obtained from public gene banks.

Results. Signatures of selection was identified in key genes that could potentially underlie giraffe and okapi visual adaptations. Importantly, some genes that contribute to optical transparency of the eye and those that are critical in light signaling pathway were found to show signatures of adaptive evolution or selection divergence. Comparison between giraffe and other ruminants identifies significant selection divergence in *CRYAA* and *OPN1LW* in giraffe. Significant selection divergence was identified in *SAG* while positive selection was detected in *LUM* when okapi is compared with ruminants and other mammals. Sequence analysis of *OPN1LW* showed that at least one of the sites known to affect spectral sensitivity of the red pigment is uniquely divergent between giraffe and other ruminants.

Discussion. By taking a systemic approach to gene function in vision, the results provide the first molecular clues associated with giraffe and okapi vision adaptation. At least some of the genes that exhibit signature of selection may reflect adaptive response to differences in giraffe and okapi habitat. Moreover, requirement for long distance vision associated with predation likely played an important role in the adaptive pressure on giraffe vision genes.

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Abstract

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Introduction

Visual cognition is critical to health, survival and evolutionary success of terrestrial vertebrates. In mammals visual cognition manifests itself into several sub-responses arising from light signal processing: visual acuity which is the capacity for the eye to resolve closely spaced objects, contrast sensitivity, motion perception, depth perception which is the three dimensional view of the object, and color discrimination (Osorio & Vorobyev 2005; Kohn, 2007; Heesy & Hall, 2010). These visual elements are inextricably linked to species evolutionary success in terms of their competitiveness at food acquisition, predator avoidance, suitable mate recognition, intra-specific communication and finding suitable habitat□. Vision and ecological studies appear to show that considerable distinction in vision perceptiveness exists between giraffe and other artiodactyls including its close relative, the okapi. Giraffes have excellent aerial vision reinforced by their long necks, which is uniquely the highest among ruminants and predominantly rely on vision communication relative to other senses (Young & Isbell, 1991; Mitchell et al., 2013; VanderWaal et al., 2013; Veilleux & Kirk, 2014). By contrast, okapi have poor eyesight adapted to low-light environment and depend heavily on their smell and hearing acuities to exploit the environment (Lindsey, Green & Bennett, 1999; Greive & Iwago, 2003). Giraffe better visual acuity measured at 25–27 cycles per degree than okapi could be a function of their respective ecology, since giraffe inhabit the light illuminated Savannah habitat while okapi are specifically restricted to low-light environment in the deep forests of

Congo. However, the basis of giraffe's uniquely excellent vision even among other artiodactyls sharing the same environment remains enigmatic.

Adaptive evolution on vision can operate at three levels namely, at organ level, cellular and biochemical level. At the level of the organ, mammals have evolved specialized organ, the eye, to transmit and focus incident light on a photosensitive retina which convert the visual image into neural signals for onward transmission to the image processing optic centers in the brain via the optic nerve. The complex interaction of species and their environment with respect to the visual tasks they perform has resulted in different eye sight specialization among mammals. Broadly, mammals have evolved differential spatial positioning of the eyes relative to the head which enables either to use a single eye to focus on a single point (monocular vision) or use both eyes for the same purpose (binocular vision) (Pettigrew, 1986). Variations in the gross morphology of the eyes can also be found in closely related species. Giraffes, for example, have fairly round eye orbits which provide an increased binocular field of vision and depth perception while okapi orbits are more elongated laterally which could be advantageous in their peripheral vision (Lindsey, Green & Bennett, 1999).

Transmission of light to the neurosensory retina and transduction of light signal into neural information for eventual transmission to the brain is primarily accomplished by specialized tissue and cell types of the eye (Jeon, Strettoi & Masland, 1998; Sivak, Andison & Pardue, 1999; Purves, Augustine & Fitzipatrick, 2003; Cepko, 2014). Transparent cornea and lens combine to transmit and refract light towards the retina. The photoreceptors (rods and cones) detect light and pass it as electrochemical signal to the bipolar and horizontal cells. Bipolar and horizontal cells relay the signals from photoreceptors to amacrine and ganglion cells via synaptic contacts. Various species have different tissue-level and cellular adaptations to optimize for their vision requirements. Specific patterning of collagen fibrils and proteoglycans across the cornea stroma determines differences in corneal light transparency, refractive power and ability to filter out ultraviolet (UV) light among vertebrate species (Winkler et al. 2015). The eye lens is composed of various crystallins proteins which determine its

transparency and refractive power. The refractive index of the lens is associated with its shape and both parameters were shown to vary between species depending on their visual requirements (Pierscionek & Augusteyn 1993). Moreover, visual acuity tends to be higher in mammals with smaller relative cornea and lens sizes (Veilleux & Kirk, 2014). In the retina, between species variations in the number and relative distributions of rods and cones allow for variations in polychromatic vision and nocturnal or diurnal habits of mammalian species (Wikler & Rakic, 1990; Peichl, 2005; Perry & Pickrell, 2010). Also, topographic heterogeneity in ganglion cell density in the retinas of different species may provide differential capacities in transferring information to the brain. This is expected to contribute to variations in visual acuity in mammals. As demonstrated by anatomical and behavioral measurements of variation in visual performances in various species, species with higher ganglion cell density generally have increased visual acuity than species with lower ganglion cell density (Rolls & Cower, 1970; Pettigrew et al, 1988; Collin & Pettigrew, 1989; Coimbra et al., 2013).

Many biochemical processes involving several genes have roles in vision, the most widely studied process being the molecular genetic basis of the light signaling mediated by the light pigments, interacting proteins and other proteins downstream the signaling pathway. Photopigment rhodopsin, located on rod cells disk membranes, specifically mediate vision in the dark and its signaling desensitization requires direct interaction of phosphorylated rhodopsin with arrestin (Vishnivetskiy et al., 2007). Color vision is primarily mediated by cone cells through photopsins comprising of short-, middle- and long-wavelength sensitive opsins. Comparison of extant and ancestral vision genes reveals episodes of nucleotide substitutions that critically impact on spectral tuning of short- and long- wavelength light pigment to vary and coincide with fundamental differences in green-red color detection among mammals (Yokoyama & Radlwimmer, 1998; Yokoyama, 2002; Horth, 2007). The “five-site rule” proposed by Yokoyama & Radlwimmer (1998) which generally applies across mammals predicts that allelic variations at critical functional sites (i.e. sites 180, 197, 277, 285 and 308) of the long-wavelength sensitive opsin determines species-specific spectral sensitivity in the red range of the visible spectrum. More recently, it has been

shown that variations in specific allelic combination among some of the five sites of long-wavelength sensitive opsin could confer adaptive significance on ecologically relevant traits. For example, it has been observed that the amino acids variation at three of the five sites, that is sites 180, 277 and 285, influence the ability of some primates to distinguish different wavelengths in the red color range important for seeing the ripe fruit (Matsumoto et al., 2014).

For such an evolutionarily important trait as vision, genes associated with vision processes will often be subject to purifying selection and therefore are expected to be conserved over evolutionary timescales (Lamb, 2011). However, we recently published giraffe genome and detected few of its coding genes associated with vision to show signatures of adaptation (Agaba et al., 2016). These genes included *Peripherin-2* (*PRPH2*) and *Cytochrome P450 family 27* (*CYP27B1*). *PRPH2* encodes a protein intergral to rods and cones and mutations in this genes cause various forms of retininis pigmentosa, pattern dystrophies and macular degenerations (Keen & Inglehearn, 1996). *CYP27B1* codes for an enzyme that hydroxylate Vitamin D to modulate normal calcium and phosphorus homeostasis required for proper development and maintenance of bones. Recently, additional *CYP27B1* functions in relation to vision have been proposed. These include participating in pathways that counteract inflammation, angiogenesis, oxidative stress, and fibrosis that confer protection for various retinopathies such as age-related macular degenerations in mice and humans (Parekh et al., 2007; von Lintig et al., 2010; Morrison et al., 2011).

In order to elucidate on the evolutionary processes underlying disparity in giraffe and okapi vision, we take advantage of the availability of giraffe and okapi genomes to analyze thirty-six (36) candidate 'visual' genes through comparison with those of closely related species. The objectives are first to identify genes exhibiting signatures of adaptive evolution and/or divergent selection and secondly to relate sequence changes in giraffe and okapi vision proteins to possible change in visual functions.

Materials and Methods

Identification of candidate genes

To obtain vision genes multiple strategies were utilized to identify proteins with direct or probable roles in vision. The initial step involved downloading cattle protein sequences from ENSEMBL (Flicek et al. 2012) and screening for proteins annotated with gene ontology terms "phototransduction" (GO: 0007601), and "visual perception" (GO: 0007602). The corresponding cattle nucleotide sequences for cattle vision protein queries were also obtained from ENSEMBL. We used PANTHER (Mi et al., 2013) to screen for proteins functionally annotated with GO vision terms. Since GO annotation is a computational functional assignment, the reliability of gene function in vision was checked by a careful literature curation. Searches for the literature proof of gene involvement in vision was performed based upon at least one of the following criteria: (i) the presence of Ocular/Cortical Visual Impairement-associated mutations in human orthologue; (ii) expression in the eye since genes expressed in a given organ at high levels are likely vital in the development and function of that organ and, (iii) interaction with known visual genes and loss of vision in knockout or sporadic mutant mice. Only genes with at least two references linking to a role in vision were selected. Orthologous mapping of cattle vision proteins to giraffe and okapi genomes identified 36 genes which were used for further analysis (Supplemental File 1).

The lineages, gene sequence alignments and gene trees

Other mammalian taxa were selected on the basis of availability of sequences for the candidate vision genes in the refseq dataset of GENBANK (Benson et al., 2013) or ENSEMBL. Sequences with questionable protein coding quality status based upon having incomplete coding sequence or presence of internal stop codons were removed. The sequences for giraffe and okapi candidate vision genes were obtained by performing TBLASTN search using cattle proteins against giraffe and okapi genome sequences that were generated as part the giraffe genome project (Supplemental File 2). Also through TBLASTN searches with cattle vision proteins queries, orthologous nucleotide sequences for all 36 vision genes for the target species were downloaded from NCBI RefSeq mRA or non-redundant nucleotide database. In case of existence of multiple isoforms for a single gene, the isoform with length similar or closest to giraffe and okapi sequences was selected. This is in recognition of the fact that

isoforms with similar length are likely evolutionarily conserved with similar function among species (Villanueva-Cańas, Laurie & Alba, 2013). The final list of species, ENSEMBL identity for cattle sequences, RefSeq accession numbers for sequences/isoforms obtained from NCBI and corresponding length for each coding sequence are provided in Supplemental File 3.

The coding DNA sequences for each gene were translated to the corresponding protein sequence and sequences with internal termination codons were discarded. The protein sequences were then aligned using MUSCLE release 3.8 (Edgar, 2004), subsequently the protein sequence alignment was then used a guide for the production of coding sequence alignment for each gene. This procedure was implemented using RevTrans (Wernersson & Pedersen, 2003). Phylogenetic trees for each gene were constructed using the HKY85 substitution model of nucleotide evolution and maximum likelihood framework implemented in PhyML Version 3.0 (Guindon & Gascuel, 2003) and bootstrapping with 100 replicates was performed to be certain of the robustness of the resulting phylogenies.

Estimation of the average rates of non-synonymous and synonymous substitutions

In order to examine if overall rates of evolution in vision genes contributed to divergence in vision capabilities between giraffe and okapi, the rates of non-synonymous substitutions per non-synonymous sites (dN) and synonymous substitutions per synonymous sites (dS) were estimated for each branch of the tree using the free ratio model of the codeml program in the PAML package (Yang, 2013). The free-ratio model independently estimates dN, dS and dN/dS for each branch by assuming that every branch in a tree has a different evolutionary parameter. This is not an explicit statistical test for selection but the key parameters obtained may provide the first line of evidence in terms of relative strength of selection among species.

Identification of genes and amino acid residues under positive selection

To determine adaptive evolution on giraffe and okapi vision genes, signatures of positive selection acting across giraffe and okapi lineages against the background of broad range of mammals was independently assessed for each vision gene. The

branch-site test for positive selection was used to identify genes showing signatures of adaptive evolution. The test applies codon models of evolution using normalized nonsynonymous to synonymous substitution rate ratio (ω or dN/dS) by assuming that adaptive evolution is a rare event during evolution of species and only few sites along the proteins will be affected by positive selection (Zhang, Nielsen & Yang, 2005). As such, it is required to hypothesize *apriori* a branch expected to have evolved under positive selection termed as "foreground". The likelihood scores of branch-site alternative and null models based on dN/dS as implemented in CODEML in the PAML package were compared using the likelihood ratio test (LRT). Significant case of positive selection was only assumed if LRT yielded $p < 0.05$ using the chi-squared distribution at one degree of freedom. For genes that were identified to be under significant positive selection, amino acid residues in the protein sequences were identified that were predicted by Bayes empirical Bayes (BEB) approach to belong to the codon class of positive selection on the foreground lineages (Yang, Wong & Nielsen, 2005).

Clade models analyses of selection divergence

It has been recently observed that phenotypic adaptive evolution in vision can also be contributed by divergent selection in orthologous proteins of ecologically divergent species (Weadick & Chang, 2012; Schott et al., 2014). To explore whether giraffe and okapi differences in vision could be partly explained by divergent selection on their vision proteins, the two species were independently compared with other ruminants by applying PAML's Clade Model C (CmC) (Bielawski & Yang, 2004). CmC partitions different branches within the phylogeny as "background" and "foreground" as well as existence of three site categories, two of which experience uniform selection across the entire phylogeny (either purifying selection ($0 < \omega_0 < 1$) or neutral evolution ($\omega_1 = 1$)) while the third is allowed to vary between background ($\omega_2 > 0$) and foreground ($\omega_3 > 0$) branches. The recently developed M2a_rel (Weadick & Chang, 2012) serves as a useful null model for the CmC. In this analysis, since the cornea, lens and retina are central optical systems in animal vision, only genes that contribute to the structural properties of cornea and lens and those that are known to play critical role in the light signaling function were investigated. Twenty (20) proteins were identified in our total

vision gene list: *Cyclic Nucleotide Gated Channel Alpha 2* (*CNGA2)*, *Cyclic Nucleotide Gated Channel Alpha 4 (CNGA4), Crystallin Alpha A* (*CRYAA*), *Guanine nucleotide-binding protein G(t) subunit alpha-1* (*GNAT1*), *Guanine nucleotide-binding protein G(t) subunit alpha-2* (*GNAT2*), *Guanine nucleotide-binding protein subunit beta-1* (*GNB1*), *Guanine nucleotide-binding protein G(t) subunit gamma-T1* (*GNGT1*), *Guanylate Cyclase Activator 1A* (*GUCA1A*), *Guanylate Cyclase Activator 1B* (*GUCA1B*), *Lumican* (*LUM*), *Long-wave-sensitive opsin-1* (*OPN1LW*), *Short-wave-sensitive opsin-1* (*OPN1SW*), *Phosphodiesterase subunit delta* (*PDE6D*), *Phospholipase C beta 4* (*PLCB4)*, *Retinol dehydrogenase 11* (*RDH11*), *Retinol dehydrogenase 12* (*RDH12*), *RPE-retinal G protein-coupled receptor* (*RGR*), *Rhodopsin* (*RHO*), *Retinal Pigment Epithelium-Specific Protein 65kDa* (*RPE65*) and *S-antigen* (*SAG*). In the genes which showed significant selection divergence, potential significance of selection divergence was assessed by examining sites which had significant Bayes posterior probability (> 0.75) in the divergent site class between giraffe or okapi and other ruminants. We assessed these sites for possible functional consequences based on literature review of functional studies.

Results

Positive selection pressure within the visual genes of giraffe and okapi

Based on average rates of evolution as determined by dN, dS and dN/dS parameters as estimated by the free-ratio model, no significant differences of the three evolutionary parameters were observed between giraffe and okapi (Supplemental File 4). In both species, overall dN, dS and dN/dS were lower than 0.005, 0.05 and 0.1, respectively, suggesting that vision genes have generally evolved under strong purifying selection as expected. Since positive selection tend to be episodic by affecting few amino acid sites along particular lineage, the widely used branch-site models are robust means of discovering cases of positives selection in a gene for given species. Previously, we used the branch-site test in a genome-wide screen and detected positive selection in *PRPH2* and *CYP27B1* in the giraffe lineage. We have also used the branch-site test

here to further examine whether some okapi vision genes are also associated with adaptive evolution. The results show *LUM* as a candidate for positive selection among the 36 vision genes in the okapi lineage (Figure 1). Substitution analysis shows that the majority of sites (> 80%) are conserved between okapi, other ungulates and cetaceans (Figure 1B). In fact, positive selection in okapi's *LUM* is predicted to occur at a single codon site, GCG, at position 36 which encodes Alanine. The corresponding codon position in giraffe is AGA while in other species is AGG both of which encode Arginine. Clearly, the common ancestor of okapi and giraffe must have had Arginine at this LUM site. The peculiar observation is that R36A substitution seems to have required at least two substitutions in the lineage leading to okapi. Also, positive selection at this site is associated with strong BEB posterior probability (0.94) (Figure 1A).

Divergent selection pressure has shaped the evolution of giraffe and okapi important vision genes

We also examined among twenty genes critical to light transmission and light signaling pathway which genes exhibit signature of divergent selection. After setting giraffe and okapi as foreground lineages against the background of other ruminant species significant results were obtained for three genes: *SAG,* in okapi, and *CRYAA* and *OPN1LW,* in the giraffe lineage (Table 1). *SAG* binds to photoactivated and phosphorylated rhodopsin which desensitize the receptor and regulates the signaling process; the mutation in the gene causes congenital stationery night blindness and other retinal diseases (Kuhn, et al., 1984; Fuchs et al., 1995; Nakazawa et al., 1998). *CRYAA* is a structural protein in the lens that provides its structural integrity and contributes to the transparency and refractive index of the lens; mutations in the gene result into congenital cataract disorders (Litt et al., 1998; Horwitz, 2003; Nagaraj et al., 2012). *OPN1LW* is induced by light photons to change its conformation following isomerization of its 11-*cis*-retinal into all-*trans*-retinal triggering phototransduction cascade. In humans, the maximum sensitivity of *OPN1LW* is at 560 nm of the light spectrum which makes it more sensitive to the red color than any other opsin. Defects in the gene have been found to affect color blindness (Nathans et al., 1986; Nathans et al., 1993). □

In all three significant cases, vast majority of the sites (about 95%) were under strong purifying selection in both foreground and background lineages to keep their functional integrity while the proportion of divergent site classes were about 5%. The proportions of neutrally evolving sites were negligible. Notably, divergently evolving sites were under stronger purifying selection in the foreground lineages in the two genes, *SAG* and *CRYAA,* than in the background lineages. However, in divergent site class for *OPN1LW,* giraffe as a foreground lineage showed a remarkable case of rate acceleration (ω = 339.6) compared with other ruminant lineages. Because it is theoretically possible for novel functions to be associated with selection divergence in orthologous genes we next identified sites predicted to have high (> 0.75) posterior probability score as determined by PAML's Bayesian computation. According to the five-sites rule, substitutions involving Serine (S), Alanine (A), Tyrosine (Y), Histidine (H), Phenylalanine (F) and Threonine at five key sites (i.e. sites 180, 197, 277, 285 and 308 of the mature opsin encoded by *OPN1LW*) have been observed to exert cumulative change in spectral shifts. In particular, the S180A, H197Y, Y277F, T285A and A308S substitutions modulate absorption spectrum by decreasing 7, 28, 7, 15 and 16 nm from the maximum wavelength in an additive manner, respectively, while the reverse substitutions increases it by the same measures (Yokoyama & Radlwimmer, 1999). Significant posterior probability scores were found in *OPN1LW* at two sites, 180 and 233, the sites which are observed to be uniquely variant between giraffe and other ruminants (Figure 2). Except for A180S substitution, the residues at remaining critical sites of OPN1LW are identical between giraffe and other ruminants which apparently suggest that optimal detection in the red color range could be different between giraffe and other ruminants. The second giraffe specific substitution (T233S) occurs at another spectrally important site within the red pigment, where the A233S substitution has been observed to shift the wavelength by 1 nm (Winderickx et al., 1992). However, we do not think that this substitution is functionally consequential in terms of spectral tuning for color sensitivity between giraffe and other ruminants as both Serine and Threonine are hydroxyl-bearing amino acids (Merbs & Nathans, 1993).

To gain further insight into the functional significance of giraffe's OPN1LW selection divergence, we phylogenetically examined the long-wavelength sensitive opsin across broad range of mammals for possible functional convergence associated with the five critical sites. It can be observed that the entire *OPN1LW* gene tree is faithfully concordant with species phylogeny (Figure 3A). However, the resultant tree using only codons corresponding to sites 180, 197, 277, 285 and 308 of OPN1LW reveals interesting positional shifts and clustering. Apparently, giraffe is observed to cluster within an artificial clade together with pinnipeds, bats and some primates (Figure 3B). The overrepresented allele at the five sites is Serine, Histidine, Tyrosine, Threonine and Alanine (henceforth denoted here as SHYTA) for sites 180, 197, 277, 285 and 308, respectively, in this clade. The giraffe SHYTA allele is observed in common with some old-world monkeys, walrus and vesper bats. The similarity of SHYTA allelic combination may reflect species-specific evolutionary pressure resulting in functional convergence among evolutionarily distant species in color discrimination in the red range of the visible spectrum.

Discussion

The development of distinct attributes between species for a given trait is very complex and likely involves multiple genes. Vision is a typical trait that requires modulated actions of many genes, some of which with tissue- and/or cell-type restricted functions (Siegert et al., 2012). The involvement of many genes suggests that the evolutionary divergence of complex traits that require coordinated functions of multiple tissue/cell types constituting a complex organ, such as the eye, cannot be fully explained by a single gene or a single tissue. In this study, we examined several genes specific to different tissues of the eye that are involved in different aspects of vision function to determine general and specific factors underlying giraffe excellent vision and its disparity in vision with okapi.

Our approach of studying many genes with diverse functions in vision afforded us the opportunity to identify several genes that potentially underlie visual adaptations in

giraffe and okapi as well as providing insight into the extent of the action of natural selection on vision phenotype. In both species, we discovered positive selection and significant selection divergence in genes with predominant roles in corneal, lens and retinal functions suggesting that the focal point of selection on vision phenotype may not be limited to a single optical unit. Rather, the interplay of different functional elements in vision appears to be mirrored by the operation of natural selection on functionally diverse vision genes, possibly to adjust species' vision to their particular ecological settings.

Vision plays a fundamental role in the survival of most animals. Giraffes are the longest-necked mammals which depend heavily on their eyesight to feed, communicate and avoid predators. Interestingly, Mitchell et al. (2013) observed that giraffe features associated with good vision seemed to be correlated with its long neck. In addition to our previous finding of positive selection on *PRPH2* and *CYP27B1*, this study identifies selection divergence in *CRYAA* and *OPN1LW* between giraffe and other ruminants. Coordinated evolutionary changes on vision genes associated with skeletal physiology, lens transparency and color vision could provide insights into molecular basis of giraffe's long distant and acute vision. We compared giraffe's red opsin with other ruminants and observed changes that could provide giraffe with unique color-based tuning to match with spectral reflectance of the surrounding environment. The notable change is the A180S substitution at one of the five functionally significant sites of the red opsin, which confers giraffe with an SHYTA allele compared with an AHYTA allele observed in okapi and other ruminants. Based on the five-sites rule, this is expected to provide giraffe with at least 5 nm spectral-shift toward red when compared with other ruminants (Yokoyama & Radlwimmer, 1998; Yokoyama & Radlwimmer 1999; Matsumoto et al., 2014).

Adaptive significance for the OPN1LW difference between giraffe and other ruminants can only be speculated upon. Giraffes, just like all other wild ruminants, have lions as their most frequent predators (Bercovitch and Berry 2009; Periquet et al. 2012). However, giraffe height advantage to see lions from afar likely presents challenges in identifying camouflaged lions in the background of tall dry grass of the

semi-arid Savannah (Owen-Smith, 2008; Davidson et al., 2013). Perhaps the SHYTA genotype provides giraffes with enhanced ability to discriminate between dry savannah vegetation and lions. It is also notable to observe that the SHYTA genotype is possessed by other mammals including some bat species and some fruit-eating old-world primates which may signify convergent solution for similar or related problem. For fruit-eating primates, possessing SYT at three of the five spectrally important sites of OPN1LW is observed to be advantageous in helping identify ripe fruits at a distance (Matsumoto et al., 2014). The importance of red color vision in bats is not clear but some bat species including vespertilionid bats possess intact, functionally constrained *OPN1LW* gene that probably helps in hunting fruits or for other purposes (Wang et al., 2004).

Okapis, on the other hand, live in low-light environment and compared with other ruminants which live in the open environment of the Savannah, they are hidden from many predators such as lions. Our study showed that the genes *LUM* and *SAG* have undergone, respectively, positive selection and significant selection divergence in the okapi lineage. Recently, rod arrestin (SAG) was found to show strong evidence of signatures of convergent evolution in species adapted to dim-light vision (Shen et al., 2012). The evolutionary changes in a gene associated with corneal transparency (*LUM*) together with coordinated changes in a gene that is important in rod mediated vision (*SAG*) could confer okapi with complex mechanisms associated with requirement for low light vision and exploitation of the deep forest niche.

LUM is a low molecular weight leucine-rich proteoglycan with keratan sulfate side chain specifically expressed in the cornea as a regulator for organizing collagen fibers in the cornea (Blochberger et al., 1992; Meek & Knupp, 2015). Although functional studies to assess precise role of the site predicted to have been affected by positive selection are missing, it has been shown that *LUM* deficiency in mice leads to disruption in corneal transparency (Chakravarti et al., 1998). We speculate that positive selection in LUM is a result of okapi vision adaptation to maintain corneal transparency driven by their confinement in deep forests where ambient light levels are reduced. Alternatively, it's possible that positive selection in LUM could be linked

with okapi eye adaptations related to UV light transmission. Douglas & Jeffery (2014) shows okapis to possess a higher degree of UV transmission through their ocular media than closely related artiodactyls. What is particularly interesting to note is that mammals with a high degree of UV transmission have reduced visual acuity and more adapted to dim light vision (Douglas & Jeffery, 2014). This might point to the existence of evolutionary switch in a system of genes important for vision which simply work to adapt species to dim light environments and also expanding their spectral range to improve visual sensitivity.

Conclusions

Subset of genes known to play functional role in vision has been analyzed in order to identify if remarkable differences in vision between giraffe and okapi is associated with adaptive evolution. The finding that visual genes are highly conserved in their evolution signifies strong purifying selection in giraffe and okapi visual genes. However, putative evidence of significant positive selection and selection divergence is observed on some key vision genes in both giraffe and okapi. Signature of selection in genes functionally associated with important optical elements of the eye, such as the cornea, the lens and the retina, could be indicative of concerted, organ-level impact of natural selection in adjusting species' vision to their respective environment. This demonstrate the importance of system-level understand of molecular evolution associated with complex traits (Invergo et al., 2013). We believe that comparative evolutionary vision studies such as this could contribute to the understanding of the molecular genetic system underlying vision in mammals in general.

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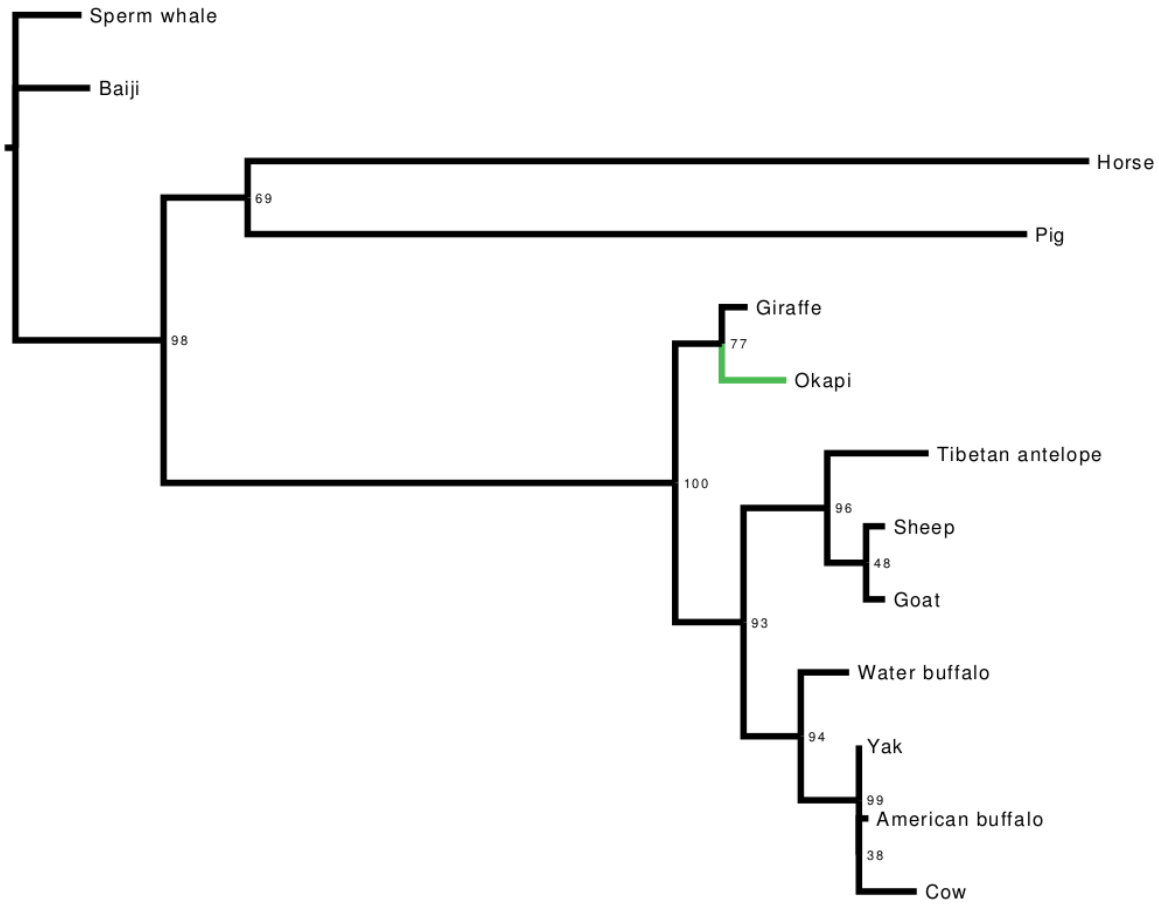
690 **Figure 1**. Positive selection in *Lumican* (*LUM*) is predicted to have occurred in okapi (adapted to
691 deep-forest) when compared to other ruminants inhabiting light illuminated environment. (A)
692 PhyML generated maximum likelihood *LUM* gene tree that was used in branch-site test for
693 positive selection setting okapi as a foreground lineage. The numbers adjacent to the nodes are
694 posterior probability bootstrap support. (B)LUM protein alignment showing positions at which
695 okapi differ with species within ruminant, cetacean, equine and pig families. Conserved positions

are omitted from the alignment. The (*) indicate identical amino acid with okapi's residue used as reference. The codon predicted to have undergone positive selection is at position 36 which encodes a unique amino acid in okapi compared with other species in the alignment.

(A)



Branch-Site Model		
	Null	Alternative
lnL	-2741.7	-2734.2
LRT		15
P value		0.0001

(B)

	2	3	5	7	8	12	14	15	16	18	19	21	22	23	24	25	27	28	29	31	32	33	36	53	64	70	87	97	114	129	141	150	154	167	168	180	184	185	189	200	201	203	205	210	231	233	234	244	248	250	257	272	294	319	320	322	34
Okapi	N	L	V	P	L	L	G	S	A	S	T	L	D	Y	Y	D	Y	S	F	Q	S	I	A	T	T	P	E	Q	R	Y	K	N	S	F	I	V	L	K	S	M	T	L	S	S	R	S	A	E	S	V	V	S	D	R	I	Q	N
Giraffe	*	*	*	*	*	*	*	R	*	*	*	*	*	*	*	*	*	*	*	*	*	*	R	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Cow	*	*	*	*	*	*	*	G	*	*	*	P	*	*	*	E	*	D	*	*	A	L	R	S	S	*	D	*	K	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	H	*	*	*
Yak	*	*	*	*	*	*	*	G	*	*	*	P	*	*	*	*	*	D	*	*	P	M	R	S	S	*	D	*	K	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	H	*	*	*
Water buffalo	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	*	D	*	*	*	*	R	S	S	*	D	*	K	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	H	*	*	*
American buffalo	*	*	*	*	*	*	*	G	*	*	*	P	*	*	*	*	*	D	*	*	P	M	R	S	S	*	D	*	K	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	H	*	*	*
Sheep	*	*	*	*	*	*	*	G	*	*	*	P	*	*	*	*	*	D	*	*	*	*	R	*	S	*	*	*	*	*	*	H	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	H	*	*	*
Tibetan antelope	*	*	*	*	*	*	*	G	*	*	*	P	*	*	*	*	*	D	*	*	*	*	R	*	S	*	*	*	K	*	*	H	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	N	*	H	*	*	*
Goat	*	*	*	*	*	*	*	G	*	*	*	P	*	*	*	*	*	D	*	*	*	*	R	*	S	*	*	*	*	*	*	H	*	*	*	*	*	*	*	I	*	*	*	*	*	*	*	*	*	*	*	*	H	*	*	*
Sperm whale	*	*	M	*	*	*	*	C	*	G	S	Y	-	*	E	*	D	-	*	L	*	*	R	*	S	L	D	*	K	*	*	*	*	*	*	F	*	*	*	*	M	*	*	*	*	G	*	*	I	*	N	E	*	*	*	*
Baiji	*	*	M	*	*	*	*	C	*	G	S	Y	-	*	E	*	D	-	*	L	*	*	R	*	S	L	D	*	K	*	*	*	*	*	*	F	R	*	*	*	M	*	*	*	N	G	A	*	I	*	N	E	*	*	*	*
Horse	*	P	A	T	F	*	S	G	T	G	Y	Y	*	D	*	*	N	-	I	L	*	*	R	*	S	*	*	*	*	H	R	H	T	M	V	I	F	R	*	*	S	*	T	*	*	N	G	*	G	I	*	N	E	*	L	H	T
Pig	H	*	*	T	*	T	*	*	*	G	H	Y	*	D	D	*	*	-	*	L	*	*	R	*	S	*	D	E	*	*	*	*	*	*	*	*	F	R	G	*	*	*	*	*	H	*	G	*	*	I	L	N	E	*	*	*	*

Figure 2. Selection divergence in long-sensitive opsin pigment (*OPN1LW*) between giraffe and closely related shorter ruminant species. Giraffe's *OPN1LW* sequences were found to be identical between NZOO and MA1 (Agaba et al., 2016) verifying that the identified substitutions are likely real. Substitution analysis shows seven variant sites (4, 8, 170, 171, 180, 233 and 236) which differ between giraffe and any ruminant species shown in the phylogeny. Variant sites 180 and 233 have Bayes posterior probability of 0.93 and 0.89 respectively. Of these two sites, site 180 is predicted to have ω ratio > 1 by site-wise likelihood ratio analysis (Massingham & Goldman, 2005).

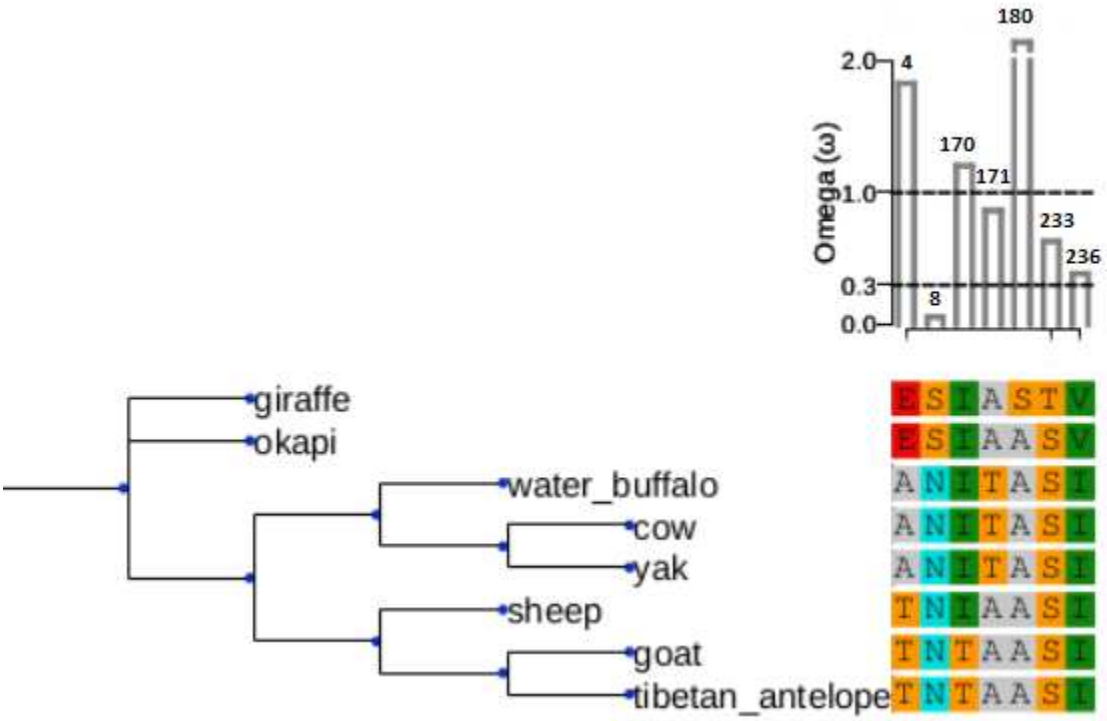


Figure 3. Evolutionary relationship in mammals as revealed by *OPN1LW* gene using (**A**) its entire coding sequence and (**B**) using codons 180, 197, 277, 285, and 308 coding for the mature peptide region of the long wavelength sensitive opsin. For species whose sequences were obtained from public database Refseq or Genbank accession numbers for the respective sequences are shown. * Humans are polymorphic at residue 180 with Serine and Alanine as common amino acids.

A:

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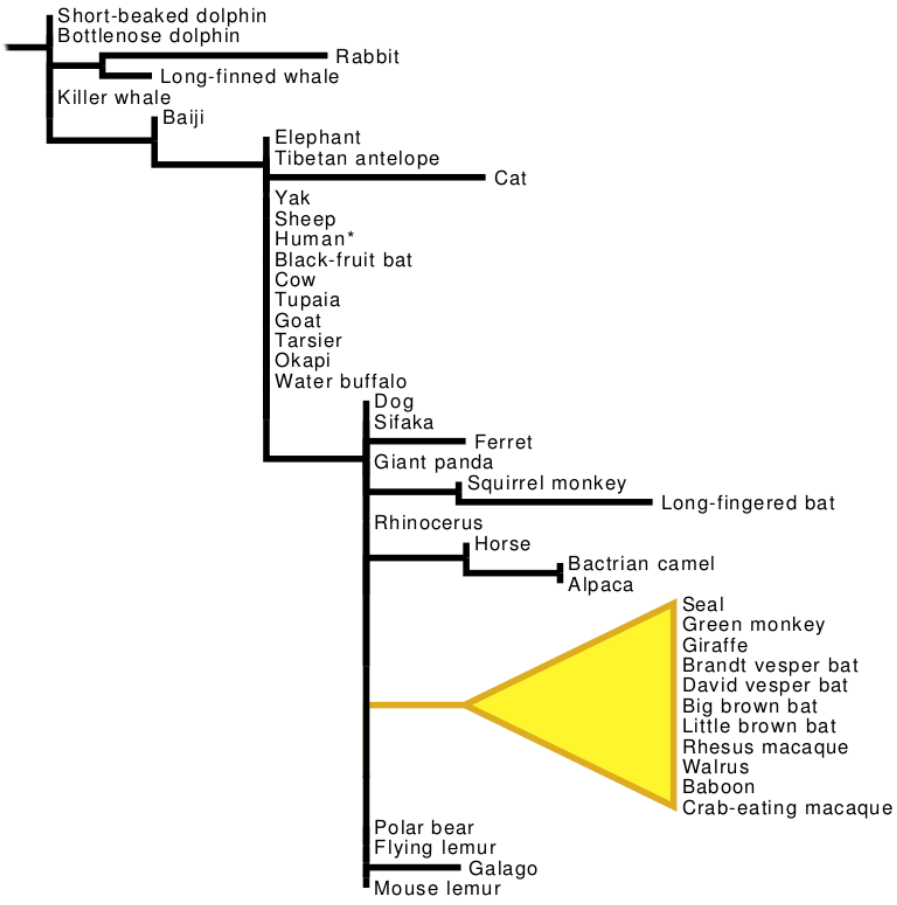
744 **B:**



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753 Table 1. Significant selection divergence in three vision genes between giraffe or okapi (Clade 1) against the background of ruminant
754 species (Clade 0)

	Giraffe							Okapi					
	lnL			Site classes				lnL		Site classes			
Gene	M2a_rel	CmC	LRT	0	1	2	*P*- value	CmC	LRT	0	1	2	P-value
CRYAA	-936.5	-933.8	5.3	$P_0 = 0.9$ $\omega_0 = 0.0$	$P_1 = 0.0$ $\omega_1 = 1$	$P_2 = 0.1$ $\omega_{Clade\ 0} = 1.4$ $\omega_{Clade\ 1} = 0.0$	0.02	-935.1	2.8	$P_0 = 0.9$ $\omega_0 = 0.0$	$P_1 = 0.0$ $\omega_1 = 1$	$P_2 = 0.1$ $\omega_{Clade\ 0} = 1.2$ $\omega_{Clade\ 1} = 0.0$	0.09
SAG	-2177.5	-2176.8	1.6	$P_0 = 0.4$ $\omega_0 = 0.05$	$P_1 = 0.1$ $\omega_1 = 1$	$P_2 = 0.5$ $\omega_{Clade\ 0} = 0.5$ $\omega_{Clade\ 1} = 0.2$	0.2	-2175.5	4.1	$P_0 = 0.95$ $\omega_0 = 0.08$	$P_1 = 0.0$ $\omega_1 = 1$	$P_2 = 0.05$ $\omega_{Clade\ 0} = 0.0$ $\omega_{Clade\ 1} = 2.3$	0.04
OPN1LW	-1780.6	-1778.2	4.7	$P_0 = 0.96$ $\omega_0 = 0.0$	$P_1 = 0.03$ $\omega_1 = 1$	$P_2 = 0.01$ $\omega_{Clade\ 0} = 0.0$ $\omega_{Clade\ 1} = 339.6$	0.03	-1780.2	0.7	$P_0 = 0.95$ $\omega_0 = 0.0$	$P_1 = 0.0$ $\omega_1 = 1$	$P_2 = 0.05$ $\omega_{Clade\ 0} = 0.9$ $\omega_{Clade\ 1} = 0.0$	0.4

755