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 perceive and respond 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 many 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 genomes 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 tests of positive selection and clade models of selection divergence through comparing giraffe and okapi vision genes and orthologous sequences from other mammals.

Results. Signatures of selection were 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*. 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 adaptations. At least some of the genes that exhibit signature of selection may reflect adaptive response to differences in giraffe and okapi habitat. We hypothesize that requirement for long distance vision associated with predation and communication with conspecifics likely played an important role in the adaptive pressure on giraffe vision genes.

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2 **differences between giraffe and okapi**

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Abstract

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Introduction

62

63 Visual cognition is critical to health, survival and evolutionary success of terrestrial
64 vertebrates. Visual perception in mammals is attributable to various aspects such as
65 visual acuity, contrast sensitivity, motion perception, depth perception and color
66 discrimination (Osorio & Vorobyev, 2005; Kohn, 2007; Heesy & Hall, 2010). These
67 visual properties are inextricably linked to species evolutionary fitness. They
68 contribute to species competitiveness at food acquisition, predator avoidance, suitable
69 mate recognition, intra-specific communication and locating suitable habitat (Horth,
70 2007; Tisdale & Ferna´ndez-Juricic, 2009; Dimitrova & Merilaita, 2010).

71 Vision and ecological studies appear to show that considerable differences in visual
72 perception exist between giraffe and other artiodactyls including its close relative, the
73 okapi. Giraffes predominantly rely on visual communication due to their excellent
74 aerial vision (Young & Isbell, 1991; VanderWaal et al., 2013; Veilleux & Kirk, 2014).
75 This is partly mediated by their uniquely long legs and neck in comparison to other
76 ruminants (Mitchell et al., 2013). But okapi exhibit poor eyesight and mostly rely on
77 acute sense of smell and hearing to exploit their low light environment (Lindsey,
78 Green & Bennett, 1999; Greive & Iwago, 2003). Since giraffes inhabit the open
79 Savannah while okapi are restricted to dense Congo forests, differences in vision
80 between giraffe and okapi could be a function of their ecologies. However, the basis
81 of giraffe's uniquely excellent vision even among other artiodactyls with overlapping
82 ecological niches remains enigmatic.

83 Although gross morphological and cellular aspects of the eye can contribute to vision
84 differences between species (Pettigrew, 1986; Jeon, Strettoi & Masland, 1998;
85 Lindsey, Green & Bennett, 1999; Sivak, Andison & Pardue, 1999; Cepko, 2014),
86 adaptive variation in vision traits will ultimately be determined by changes at the gene
87 level. Vision in vertebrates is mediated by photoreceptors (rods and cones) which
88 contain visual pigments (opsins) bound to a retinal chromophore (Wald, 1935). Five
89 classes of opsins are expressed in vertebrate photoreceptors: a rod class of pigment
90 RH1 (rhodopsin) enables animals to see in dim light, and four classes of cone
91 pigments which enable perception of distinct color wavelengths. The cone class of
92 pigments include short wavelength sensitive type 1 opsin (SWS1/OPN1SW/L)
93 sensitive in the ultraviolet/violet range of the spectrum at 355-440 nm, SWS type 2
94 opsin (SWS2) sensitive in the blue range from about 410-490 nm, RH2 opsin (close
95 homolog of RH1) sensitive in the green range from about 480-535nm, long
96 wavelength opsin (LWS/OPN1LW/L) and middle wavelength-sensitive opsin
97 (MWS/OPN1MW/M) sensitive in the red range from about 490-570nm (Yokoyama,
98 2002; Bowmaker, 2008).

99 The cloning of bovine rhodopsin gene by Nathans & Hogness (1983) inspired efforts
100 to understand variations in molecular genetic mechanisms underlying vision in
101 mammals and vertebrates in general. Comparison of different classes of opsin genes
102 among vertebrates reveals gene loss, gene duplication and nucleotide substitutions to
103 play a fundamental role in the evolution of color vision in vertebrates (Yokoyama &
104 Radlwimmer, 1998; Yokoyama, 2002; Horth, 2007; Jacobs, 2009). All mammals have
105 apparently lost RH2 cones while SWS2 is retained only in some egg-laying mammals
106 (Hunt et al., 2009). The *LWS* gene duplication and diversification has resulted into
107 primates possessing both *LWS* and *MWS* genes which, respectively, express L-cone
108 pigment maximally sensitive at around 563nm and M-cone pigment maximally
109 sensitive at around 535nm (Bowmaker 2008). This provides some primates with
110 trichromatic vision due to presence of three spectrally distinct cone pigments
111 expressed by S*WS1*, *MWS* and *LWS* genes (Bowmaker, 2008). But most eutherian
112 mammals remain dichromatic with *SWS1* and either *MWS* or *LWS* genes (Collin et al.,
113 2009).

114   With respect to the functional mechanism of opsins, spectral tuning in vertebrates is
115   mainly determined by particular amino acids in the opsin protein structure. Two
116   decades ago, Yokoyama & Radlwimmer (1998) proposed the "five-sites" rule by
117   demonstrating that sequence changes at sites 180, 197, 277, 285 and 308 were very
118   important in determining variation in LWS spectral sensitivity among mammals. Such
119   sequence variations in visual pigments also occur naturally within species, resulting in
120   spectrally variant subtypes of cone pigments among populations with normal color
121   vision. For example, normal color human subjects show 4-5 nm variations based on
122   whether they possess a Serine or Alanine at position 180 of LWS (Merbs & Nathans,
123   1992; Kraft, Neitz & Neitz, 1998). Consequently, there is an interest to determine
124   whether these inter- and intra-species spectral variations in cone pigments confer
125   visual adaptations in species. Several studies on cichlids and a recent work on New
126   World primates suggest that changes in coding sequence of visual pigments may be
127   associated with matching photoreceptor spectral sensitivity to the visual environment
128   of the respective species (Hofmann et al., 2009; Sabbah et al., 2010; Matsumoto et al.,
129   2014).

130   While the evolution of opsins and other proteins in the visual phototransduction
131   system has been studied extensively (Larhammar, Nordström & Larsson, 2009;
132   Invergo et al., 2013), little attention has been given to proteins involved in other
133   processes that impact on whole vision process. Before reaching photoreceptors, light
134   must pass through the ocular media, consisting of sclera, cornea, lens and the vitreous,
135   and these serve to modify and focus light toward the retina. The structure,
136   transparency and light adjustment ability of the ocular media depends on specific
137   constituent proteins (Pierscionek & Augusteyn, 1993; Winkler et al., 2015). For
138   instance, the sclera and the cornea are packed with collagen fibrils and proteoglycans
139   which provide structural integrity of cornea. An example is lumican (LUM), a low
140   molecular weight leucine-rich proteoglycan with keratan sulfate side chain first
141   identified in the cornea as a regulator for organizing collagen fibers in the cornea
142   (Blochberger et al., 1992; Meek & Knupp, 2015), but is also present in the sclera and
143   other tissues (Ying et al., 1997). The crucial role of LUM in visual functions is further
144   demonstrated by growing evidence implicating LUM in various ocular defects such as

145   corneal opacity and high myopia (Chakravarti et al., 1998, 2000, 2003; Austin et al.,
146   2002). The eye lens contains high concentrations of proteins known as crystallins
147   which determine lens transparency and refractive power, but α-crystallin (CRYAA) is
148   a major type (Nagaraj et al., 2012). Mutations in *CRYAA* are associated with
149   development of cataracts in humans (Litt et al., 1998; Richter et al., 2008). With the
150   increasing number of whole-genome sequences of many vertebrates, we can now study
151   a broad range of genes enabling functionally integrated traits.

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

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

173

Materials and Methods

Identification of candidate genes

To obtain vision genes, multiple strategies were utilized to identify proteins with linked roles in vision. The initial step involved downloading all cattle protein sequences from ENSEMBL (Flicek et al., 2012) and screening for proteins annotated with gene ontology (GO) terms "phototransduction" (GO: 0007601), and "visual perception" (GO: 0007602). We used PANTHER (Mi et al., 2013) to first functionally annotate cattle proteins and then screen for those annotated with GO vision terms. The corresponding cattle nucleotide sequences for cattle vision protein were also obtained from ENSEMBL. Since some GO assignments to proteins are not necessarily based on direct experimental evidence but also rely on other evidences such as sequence similarity, we intended to obtain only those genes with proved functions in vision. To achieve this, 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 vision disease-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 based on the above criteria were selected. Orthologous mapping of cattle vision proteins to giraffe and okapi genomes returned sequences corresponding to 36 genes. These were the genes that were ultimately used in the 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 36 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

204   2). Also through TBLASTN searches with cattle vision proteins queries, orthologous

205   nucleotide sequences for all 36 vision genes for the target species were downloaded

206   from NCBI RefSeq mRNA or non-redundant nucleotide database. In case of existence

207   of multiple isoforms for a single gene, the isoform with length similar or closest to

208   giraffe and okapi sequences was selected. This is in recognition of the fact that

209   isoforms with similar or equivalent length are likely evolutionarily conserved with

210   similar function among species (Villanueva-Canas, Laurie & Alba, 2013). The final

211   list of species, ENSEMBL identity for cattle sequences, RefSeq accession numbers for

212   sequences/isoforms obtained from NCBI and corresponding length for each coding

213   sequence are provided in Supplemental File 3.

214   The coding DNA sequences for each gene were translated to the corresponding protein

215   sequence and sequences with internal termination codons were discarded. The protein

216   sequences were then aligned using MUSCLE release 3.8 (Edgar, 2004). Subsequently,

217   the protein sequence alignments were used guides for the generation of coding

218   sequence alignments for each gene. This procedure was implemented using RevTrans

219   (Wernersson & Pedersen, 2003). Phylogenetic trees for each gene were constructed

220   using the HKY85 substitution model of nucleotide evolution and maximum likelihood

221   framework implemented in PhyML Version 3.0 (Guindon & Gascuel, 2003).

222   Bootstrapping with 100 replicates was performed to be certain of the robustness of the

223   resulting phylogenies.

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

225   In order to examine if overall rates of evolution in vision genes contributed to

226   divergence in vision capabilities between giraffe and okapi, the rates of non-

227   synonymous substitutions per non-synonymous sites (dN) and synonymous

228   substitutions per synonymous sites (dS) were estimated for each branch of the tree

229   using the free ratio model of the codeml program in the PAML package (Yang, 2013).

230   The free-ratio model independently estimates dN, dS and dN/dS for each branch by

231   assuming that every branch in a tree has a different evolutionary parameter. This is

232   not a robust statistical test for positive selection but the key parameters obtained may

233   provide important information on the relative strengths of selection among species.

234

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 episodic, where few species in a phylogeny and few sites in a protein are affected by selection, it is required to hypothesize *a priori* a "foreground" branch expected to have evolved under positive selection (Zhang, Nielsen & Yang, 2005). The likelihood scores of branch-site alternative and null selection models based on dN/dS as implemented in CODEML of 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 that were predicted by Bayes empirical Bayes (BEB) approach (Yang, Wong & Nielsen, 2005) to belong to the codon class of positive selection on the foreground lineages were identified.

Clade models analyses of selection divergence

It has recently been observed that visual adaptations can also be contributed by divergent selective pressure on homologous visual genes of ecologically divergent species (Weadick & Chang, 2012; Schott et al., 2014). To explore whether giraffe and okapi differences in vision could partially be 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). The 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 (ω_2

264 > 0) and foreground ($\omega_3 > 0$) branches. The recently developed M2a_rel (Weadick &

265 Chang, 2012) serves as a useful null model for the CmC. In this analysis, category of

266 genes that contribute to the structural properties of cornea and lens and those that are

267 known to play a direct role in the light signaling function were investigated. Of 36

268 genes, 20 proteins were identified to belong in that category (Supplemental File 4). In

269 the genes which showed significant selection divergence, potential significance of

270 selection divergence was assessed by examining sites which had significant Bayes

271 posterior probability (> 0.75) in the divergent site class between target species

272 (giraffe or okapi) and other ruminants. We assessed these sites for possible functional

273 consequences based on literature review of functional studies.

274

275 **Results**

276 **Positive selection pressure within the visual genes of giraffe and okapi**

277 Vision genes, as prescribed by gene ontology (GO) and functional information from

278 literature, were identified in giraffe and okapi that were homologous to other

279 mammals. This screen yielded 36 vision genes which were subjected to a series of

280 analyses to determine selection pressure acting on these genes. Based on dN, dS and

281 dN/dS parameters as estimated by the free-ratio model, no significant differences of

282 the three evolutionary parameters were observed between giraffe and okapi. In both

283 species, overall dN, dS and dN/dS for each of the 36 vision genes were lower than

284 0.005, 0.05 and 0.1, respectively, suggesting that vision genes have evolved under

285 strong purifying selection as expected.

286 The branch-site test of positive selection was performed on all 36 genes and identified

287 lumican gene (*LUM*) as the only candidate exhibiting positive selection among the 36

288 vision genes in the okapi lineage (Figure 1A). Aside from *PRPH2* and *CYP27B1*

289 which had previously been shown to be candidates for adaptive evolution in giraffe

290 (Agaba et al., 2016), no additional candidates of positive selection were identified in

291 the giraffe lineages by the present analysis. Substitution analysis shows that the

292 majority of sites (> 80%) are conserved between okapi and closely related

293   cetartiodactyl mammals (Figure 1B). Positive selection in okapi's *LUM* is predicted to

294   occur at a single codon site, GCG, at position 36 which encodes Alanine (A). This site

295   exhibit strong posterior probability (0.94). The corresponding codon position in

296   giraffe is AGA while in other species is AGG both of which encode Arginine (R).

297   Besides the strong BEB posterior probability associated with codon position 36, there

298   is also a peculiar observation that R36A substitution seems to have required at least

299   two substitutions in the lineage leading to okapi.

300   To further evaluate the mechanisms of LUM's codon 36 evolution and obtain context

301   of the changes leading to 36A in okapi, ancestral LUM sequences at the interior nodes

302   were reconstructed using a broader vertebrate phylogeny. Apparently, only five

303   unique amino acid replacements at codon 36 were found in the whole vertebrate tree

304   (Figure 1B). Two of these changes occur deep within the phylogeny while three

305   involve terminal nodes and their immediate ancestors. The common ancestor of all

306   vertebrates appears to possess Proline (P, encoded by CCA) at this position. The most

307   ancient nonsynonymous change leads to Glutamine (Q, CAA) in the common ancestor

308   of mammals, followed by another nonsynonymous step leading to R (CGA) in the

309   ancestor of ungulates. For the terminal taxa, convergent replacements R36A and

310   Q36A are respectively observed in okapi and the Malayan colugo (*Galeopterus*

311   *variegatus*) (Figure 1C). Closer inspection of okapi and colugo protein sequences did

312   not reveal other convergent changes (Zhang & Kumar, 1997). But using a branch-site

313   test, a hypothesis of adaptive evolution on convergent site possibly due to similar

314   selective pressure in both lineages was found to be significant ($P < 0.001$).

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

317   The branch-site model assumption that adaptive evolution is determined by the

318   presence of positive selection in foreground branches ignores the possibility that

319   significant variation in ω-ratios among all branches and sites may also be indicative of

320   adaptive pressure (Schott et al., 2014). For the vision genes studied here, it is possible

321   that specific branch(es) on the tree exhibit sites variation due to selection divergence.

322   Upon examination of selection divergence using clade model C of Bielawski and Yang

323 (2004) comparing giraffe or okapi and other ruminants in twenty genes critical to light

324 transmission and light signaling processes, significant results were obtained for three

325 genes while the rest were found to be not significant. Significant genes were *SAG*

326 (found divergent between okapi and other ruminants), *CRYAA* and *OPN1LW* (found

327 divergent between giraffe and other ruminants) (Table 1).

328 In all three significant cases, vast majority of the sites (about 95%) were under strong

329 purifying selection in both foreground and background lineages to keep their

330 functions, while the proportion of divergent site classes was about 5%. The

331 proportions of neutrally evolving sites were negligible. Notably, divergently evolving

332 sites were under stronger purifying selection in *SAG* and *CRYAA* in the foreground

333 lineages than in the background lineages (Figure 2A and Figure 2B). Furthermore,

334 several residues in *SAG* and *CRYAA* at some divergently evolving sites are observed to

335 be shared between giraffe and okapi (Figure 2A and Figure 2B).

336 For the divergent site class in *OPN1LW,* a remarkable case of rate acceleration was

337 observed in the foreground lineage (ω = 339.6) compared with the background

338 lineages. Because it is theoretically possible for novel functions to be associated with

339 selection divergence in orthologous genes, we next identified sites predicted to have

340 high (> 0.75) posterior probability score as determined by PAML's Bayesian

341 computation. According to the five-sites rule, substitutions involving Serine (S),

342 Alanine (A), Tyrosine (Y), Histidine (H), Phenylalanine (F) and Threonine (T) at sites

343 180, 197, 277, 285 and 308 of the L-opsin exert cumulative change in spectral shifts.

344 In particular, the S180A, H197Y, Y277F, T285A and A308S substitutions modulate

345 absorption spectrum by decreasing 7, 28, 7, 15 and 16 nm, respectively, from the

346 maximum wavelength in an additive manner (Yokoyama & Radlwimmer, 1999). The

347 reverse substitutions increase the maximum absorption spectrum by the same

348 measures. Significant posterior probability scores in OPN1LW were found at amino

349 acid site 180 and 233, which correspond with A180S and S233T. These substitutions

350 are unique in giraffe when compared with other ruminants (Figure 2C). Except for the

351 A180S substitution, the other residues in the context of five-sites rule are identical

352 between giraffe and other ruminants. The second giraffe specific substitution (S233T)

353   also occurs at another spectrally important site within transmembrane domain 5 of the

354   red pigment (Figure 2D), where the A233S substitution has been observed to shift the

355   wavelength by 1 nm (Winderickx et al., 1992).

356   To gain further insight into the functional significance of giraffe's OPN1LW selection

357   divergence, we examined OPN1LW across broad range of mammals for possible

358   functional convergence associated with the five critical sites. It can be observed that

359   the entire *OPN1LW* gene tree is faithfully concordant with species phylogeny (Figure

360   3). An inspection of codons corresponding to sites 180, 197, 277, 285 and 308 of

361   OPN1LW shows similarities in the allelic combination of giraffe amino acids at the

362   respective sites with pinnipeds, bats and some primates (Table 2). In these taxa, the

363   overrepresented allele at the five sites is S, H, Y, T and A (henceforth denoted here as

364   SHYTA) for sites 180, 197, 277, 285 and 308, respectively. Giraffe's SHYTA allele,

365   although unique to ruminants, is observed in common with some humans, some old-

366   world primates, walrus and vesper bats.

367   The pattern of allelic combination shows sites 197, 285 and 308 to be invariant in

368   these taxa while site 180 shows some degree of S/A variation between- and within-

369   species. In particular, humans are known to be polymorphic for the S/A allele at site

370   180 of OPN1LW (Winderickx et al., 1992). The SHYTA and AHYTA forms of human

371   OPN1LW have their respective λ_{max} experimentally determined by Merbs & Nathans

372   (1992) at 557 nm and 552 nm. If the identity of residues at the five sites is a reliable

373   predictor of spectral tuning (and several experiments suggest it is (Table 2)), then a

374   5nm variation in the λ_{max} of LWS pigments between giraffe and other ruminants is

375   expected.

376

Discussion

378   The development of distinct attributes between species for a given trait is typically

379   complex involving a large number of genes. The visual system is well known to be

380   comprised of the actions of many genes, some of which exhibit tissue- and/or cell-

381   type restricted functions (Siegert et al., 2012). In this study, we performed an

382   evolutionary analysis on several genes participating in wide variety of visual process.

383   The ultimate aim is to determine molecular genetic basis and evolutionary factors

384   underlying giraffe excellent vision and its apparent disparity with that of okapi.

385   We discovered positive selection and selection divergence in genes with predominant

386   roles in corneal, lens and retinal functions in both organisms. This suggests that the

387   focal point of selection on vision phenotype may not be limited to a single functional

388   unit. Rather, the interplay of different elements in the visual pathway appears to be

389   mirrored by the operation of natural selection on functionally diverse vision genes,

390   possibly to adjust species' vision to the myriad of complexity of their visual

391   environment. This potentially concerted adaptive strategy is likely partly due to the

392   intricate functional matching between ocular and retinal aspects, a phenomenon

393   perhaps well demonstrated in teleost fish and birds' visual systems. For example, in

394   cichlids and birds, the degree by which the lens of particular species filters ultra-

395   violet (UV) light is reflected by the levels of expression or possession of their UV

396   pigments (Hofmann et al., 2010; Lind et al., 2014). Recent findings also suggest

397   widespread differential capacities in ocular transmittance of UV light among

398   mammals (Douglas & Jeffery, 2014). The fundamental question is whether a pattern of

399   selection signatures detected in okapi and giraffe vision genes is specifically an

400   adaptive response imposed by their visual environment, or whether it is a pattern

401   specific for other purposes. Our study can only provide insight on the likely role of

402   environment influences on the evolution of vision genes in giraffe and okapi.

403   Among okapi vision genes, *LUM* is shown to have undergone positive selection. The

404   irradiance spectrum at the floor of the low-light forest environment in which the

405   okapis are confined is expected to be dominated by shorter wavelengths in the blue-

406   green, blue and even ultraviolet ranges (Honkavaara et al., 2002; Warrant & Johnsen,

407   2013). Recently, Douglas & Jeffery (2014) shows okapis to possess a higher degree of

408   UV transmission through their ocular media than closely related artiodactyls living in

409   the open environment. Although specific sites required for LUM interaction with

410   collagens are not yet identified (but see Kalamajski & Oldberg, 2009)), the probable

411   site under positive selection shows signal of convergent adaptation with the nocturnal

412 Malayan colugo (Figure 1B). Okapi and colugo are evolutionarily diverse organisms
413 but one thing that they have in common is their low light forest habitat and nocturnal
414 lifestyles. In general, nocturnal mammals tend to have eyes adapted to transmit and
415 process UV (Zhao et al., 2009; Douglas & Jeffery, 2014). It is therefore likely that the
416 convergence of positive selection in *LUM* in okapi and Malayan colugo may be
417 associated with ocular adaptations related to spectral transmittance and survival in
418 reduced light environment.

419 Another gene found to exhibit signature of selection in okapi is *SAG*. Recently, *SAG*
420 was found to show strong evidence of convergent evolution in species adapted to dim-
421 light vision (Shen et al., 2012). Arrestin binds to photoactivated and phosphorylated
422 rhodopsin, a process that desensitizes rhodopsin and regulates the signaling process.
423 The mutation in the gene causes congenital stationery night blindness and other retinal
424 diseases (Kuhn, Hall & Wilden, 1984; Fuchs et al., 1995; Nakazawa, Wada & Tamai,
425 1998). It might be that evolutionary changes in *LUM* (associated with corneal
426 transparency) and *SAG* (important in rod mediated vision) could confer okapi with
427 concerted adaptive mechanisms associated with requirement for low light vision and
428 exploitation of the deep forest niche.

429 Besides *PRPH2* and *CYP27B1* (Agaba et al., 2016), this study further identifies
430 selection divergence in *CRYAA* and *OPN1LW* between giraffe and other ruminants.
431 Observed amino acid changes associated with selection divergence in giraffe's *CRYAA*
432 are shared with okapi and in some cases with other ruminant species (Figure 2B). This
433 may suggest shared functional variation or common phylogenetic signal. But a
434 comparison of giraffe's *OPN1LW* with those of other ruminants identifies two unique
435 changes that could provide giraffe with unique color-based tuning (Figure 2D).

436 The first change is the S233T which occurs in TM5 of the receptor (Figure 2D). The
437 location of the change is potentially important for the "chloride effect", a phenomenon
438 observed to be widespread in vertebrate L-cone pigments (Wang, Asenjo & Oprian,
439 1993; Hirano et al., 2001). In vertebrates, the binding of chloride ion at H197 in both
440 L- and M- cone pigments contributes to the red-shift of the chromophore's absorption
441 in the visual pigments. Whatever shift caused by S233T in the L-cone spectral tuning,

442   it is most likely to be subtle as both Serine and Threonine are hydroxyl-bearing amino

443   acids (Merbs & Nathans, 1993). Establishing actual impact of S233T in the spectral

444   tuning of the L-cone pigment requires functional studies.

445   The second change is the A180S substitution at one of the five functionally significant

446   sites of the red opsin. This confers giraffe with an SHYTA allele compared with an

447   AHYTA allele observed in okapi and other ruminants in the study. Based on the five-

448   sites rule, this is expected to provide giraffe with at least 5 nm spectral-shift toward

449   red when compared with other ruminants (Yokoyama & Radlwimmer, 1998, 1999). It

450   should be noted, nevertheless, that the two giraffe individuals sequenced in the project

451   are insufficient to conclude that A180S substitution is fixed in the giraffe populations.

452   Hence, the site may as well be polymorphic in giraffes as in humans.

453   If sequence changes observed in giraffe's *OPN1LW* confer giraffe with adaptive

454   benefits over other ruminants, these advantages can only be speculated upon based on

455   some common environmental challenges with other species with which it shares the

456   SHYTA genotype. This is based on the key assumption that distantly related species

457   facing common or related problems are likely to respond by developing convergent

458   solutions (Stern, 2013). It is, therefore, notable that giraffe share the SHYTA

459   genotype with distantly related mammals such as pinnipeds, bats and some primates

460   (Table 2). This may reflect some functional convergence associated with LWS color

461   discrimination among these species.

462   Pinnipedia (seals and walruses) are marine mammals whose existence depends upon

463   switching their lives between land and shallow coastal waters. In coastal waters,

464   where seals and walruses spend majority of their time in, are dominated by longer

465   wavelength colors such as green and red (Wozniak & Dera, 2007). Because of their

466   amphibious lifestyle, seals and walruses are presented with the need to adapt to the

467   dominant spectral irradiance when in water and also maintain some color vision when

468   on land (Griebel & Peichl, 2003). Apparently, pinnipeds, in their course of evolution,

469   have become cone monochromats after losing functional S-cone pigment and retaining

470   only L-cone pigment (Crognale et al., 1998). Presence of five-sites SHYTA genotype

471   in pinniped L-cone pigment could provide optimal ability to contrast rod and cone

472   signals necessary for residual color vision they may possess (Crognale et al., 1998;

473   Griebel & Peichl, 2003).

474   The importance of red color vision in bats is not clear but some bat species, including

475   fruit-eating bats, possess intact, functionally constrained *OPN1LW* gene that, on its

476   own, may provide bats with spectral mechanism for increased sensitivity during

477   navigation and foraging (Wang et al., 2004). Alternatively, since bats are nocturnal

478   mammals, their red color sensitivity could depend on the Purkinje shift. The Purkinje

479   effect is the apparent reduction in brightness of red object under dim light conditions

480   as a result of comparison of signal generated by the λ_{max} of rhodopsin and that of the

481   MWS/LWS opsins (Trezona, 1970). Under conditions where bats might benefit from

482   signal inputs of both cone and rod systems, for example under twilight at dusk or

483   dawn (Pavey et al., 2001; Melin et al., 2014), the SHYTA genotype may provide a

484   spectral mechanism to compensate for the Purkinje effect.

485   Anthropoid primates (consisting of New World and Old World monkeys) are usually

486   regarded as visual specialists due to their keen visual acuity and color perception

487   (Kirk & Kay, 1990; Kawamura, 2016). Indeed, the best example in higher mammals

488   linking sequence variation in L/M opsin to visual behavior in an ecological context is

489   observed in primates. In ateline New World primates which exhibit allelic variation at

490   three of the five spectrally important sites in the L-opsin (where SYT, SFT, AFT and

491   AFA at site 180, 277, and 285, respectively, are common variants), possession of SYT

492   is likely advantageous among primates in identifying ripe fruits in the background of

493   green leaves (Matsumoto et al., 2014).

494   As a general rule, matching of cone pigments spectral characteristics to spectral

495   reflectance of the visual environment (Lythgoe, 1984), should provide clues to

496   ecological factors driving visual genetic adaptations in species. For example, all

497   woolly lemurs (*Avahi spp*) endemic to Madagascar forests uniformly possess cone

498   pigment allele with λ_{max} precisely tuned to the spectral reflectance of the preferred

499   diet of young leaves (Veilleux et al., 2013). Likewise, diversification in the cone

500   pigment spectral sensitivity in fish species influences detection of prey and

501   communication with conspecifics (Sabbah et al., 2010). But the suite of ecological

502   factors more likely to play an important role in vision adaptations is shared among

503   ruminants and other artiodactyls of Savannah. This makes the sequence changes in

504   giraffe LWS opsin rather interesting and poses challenge in applying conventional

505   ecological arguments to the finding. Although the expected magnitude of λ_{max}

506   variation caused by the A180S is quite small (~5nm), the presence of an additional

507   change at site 233 located in TM5 of L-opsin suggests that the gene is under

508   differential selective pressure in giraffe. Since polymorphisms in human OPN1LW are

509   likely adaptive (Verrelli & Tishkoff, 2004; Verrelli et al., 2008), it is reasonable to

510   also postulate on selection drivers acting on *OPN1LW* in the giraffe.

511   Mitchell et al. (2013) and others have speculated that giraffe height and extraordinary

512   visual capacity may have co-evolved. Gradual acquisition of long necks may have

513   provided giraffes with selective ability to see predators from afar, besides the feeding

514   advantage for nutritious and top placed foliage (Mitchell & Skinner, 2003; Williams,

515   2016). Indeed, because of their excellent aerial vision, adult giraffes are rarely killed

516   by lions (Periquet et al., 2012; Strauss & Packer, 2013). But giraffe height advantage

517   to see lions from afar likely presents challenges in identifying camouflaged lions in

518   the background of tall dry grass of the semi-arid Savannah. This probably explains the

519   overrepresentation of giraffes among lion kills during the arid season (Owen-Smith,

520   2008; Davidson et al., 2013). Therefore, sequence changes in giraffe's L-cone pigment

521   could be an adaptive response to predation pressure that provides giraffes with

522   differential spectral mechanisms for enhanced ability to discriminate between dry

523   savannah vegetation and lions.

524   The alternative explanation is intra-specific communication among giraffe

525   populations. Among major giraffe populations, the characteristic pelage color is

526   reddish-brown spots separated by variable network of fine white lines (Brown et al.,

527   2007; Fennessy et al., 2016). In fish, avian and other mammal species, patches of

528   color on the face or tails are clearly for communication purpose with conspecifics

529   (Caro, 2005; Price et al., 2008; Stoddard & Prum, 2011). It is also thought that one of

530   the ways giraffes maintain reproductive isolation in the wild is through pelage-based

531   mate discrimination (Brown et al., 2007). One might think that spectral tuning of the

532 giraffe visual pigments should also match with characteristic color of conspecifics and
533 allow for easy recognition among giraffes. Accordingly, giraffe's L-cone pigment
534 might possess a slightly red-shifted λ_{max} to enable sharp detection of conspecifics for
535 several purposes, including preferred mate choices.

536 At the present time there shouldn't be *a priori* reasoning that one or the other
537 argument is a correct explanation of the actual significance of adaptive evolution in
538 giraffe or okapi vision genes. First, the fixation status of the sequence changes needs
539 to be clarified among giraffe and okapi populations. Secondly, a combination of
540 divergence-based and population genetics approaches is potentially suited to ascertain
541 the adaptive consequences of selection divergence (Verrelli et al., 2008). Finally,
542 establishing causal link between sequence changes and adaptation may require
543 functional studies on gene candidates in model species and comparative field studies
544 on giraffe and okapi visual behaviors.

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

559 **Figure 1**. Positive selection in *LUM* is predicted to have occurred in okapi (adapted to deep-
560 forest) when compared to other ruminants inhabiting light illuminated environment. (**A**) PhyML
561 generated maximum likelihood *LUM* gene tree that was used in branch-site test for positive
562 selection setting okapi as a foreground lineage. (**B**) LUM protein alignment showing positions at
563 which okapi differ with species within ruminant, cetacean, equine and pig families. Conserved
564 positions are omitted from the alignment. The codon position predicted to have undergone
565 positive selection is color highlighted at site 36 which encodes a unique amino acid in okapi
566 compared with other species in the alignment. (**C**) Vertebrate-wide evolution at codon 36 shows
567 signature of convergent evolution between okapi and Malayan colugo (*Galeopterus variegatus*).
568 The identity of codon at the predicted positive selection site and the respective amino acid (in
569 bracket) are shown for each of the terminal species and for some ancestral lineages.

570

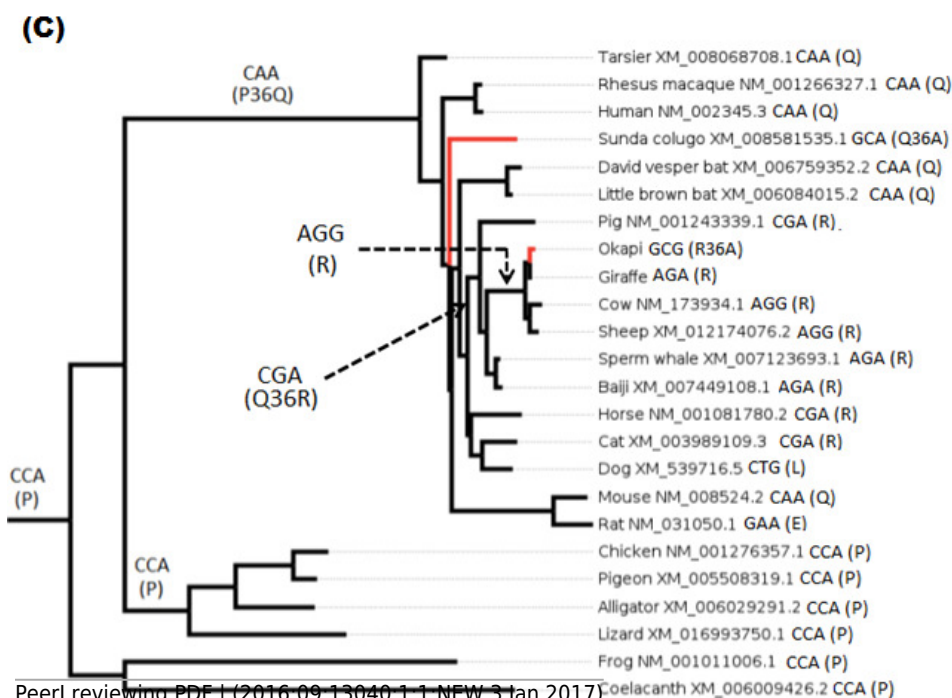
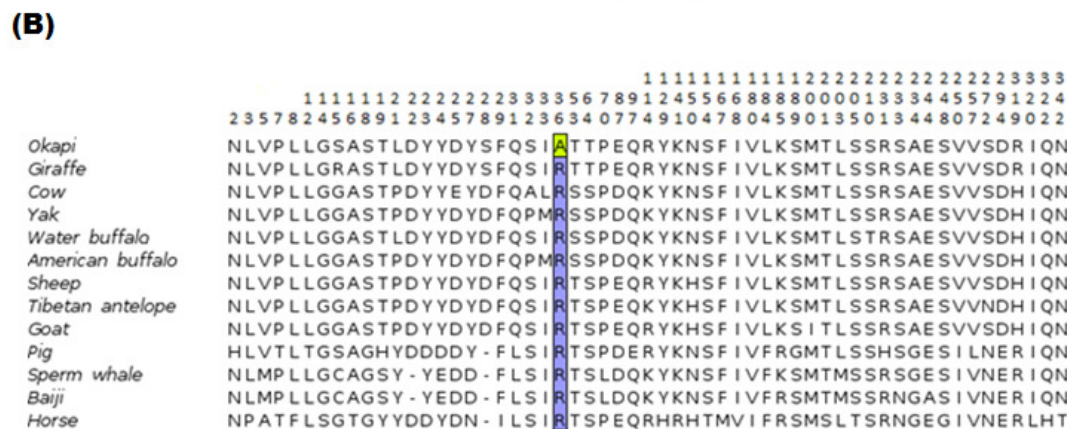
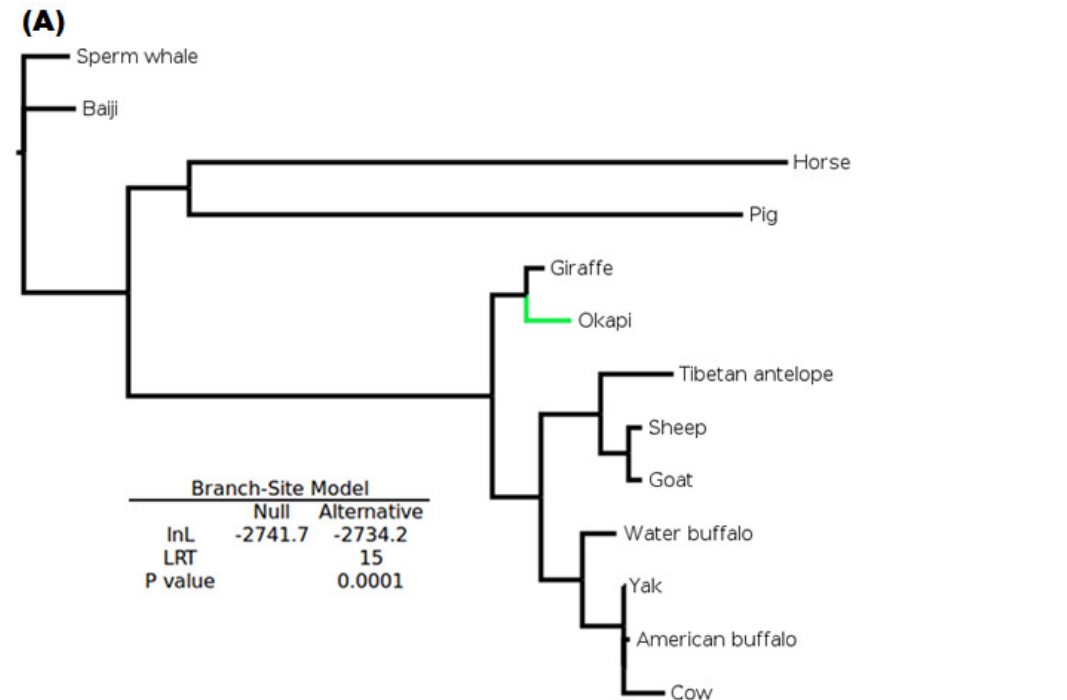
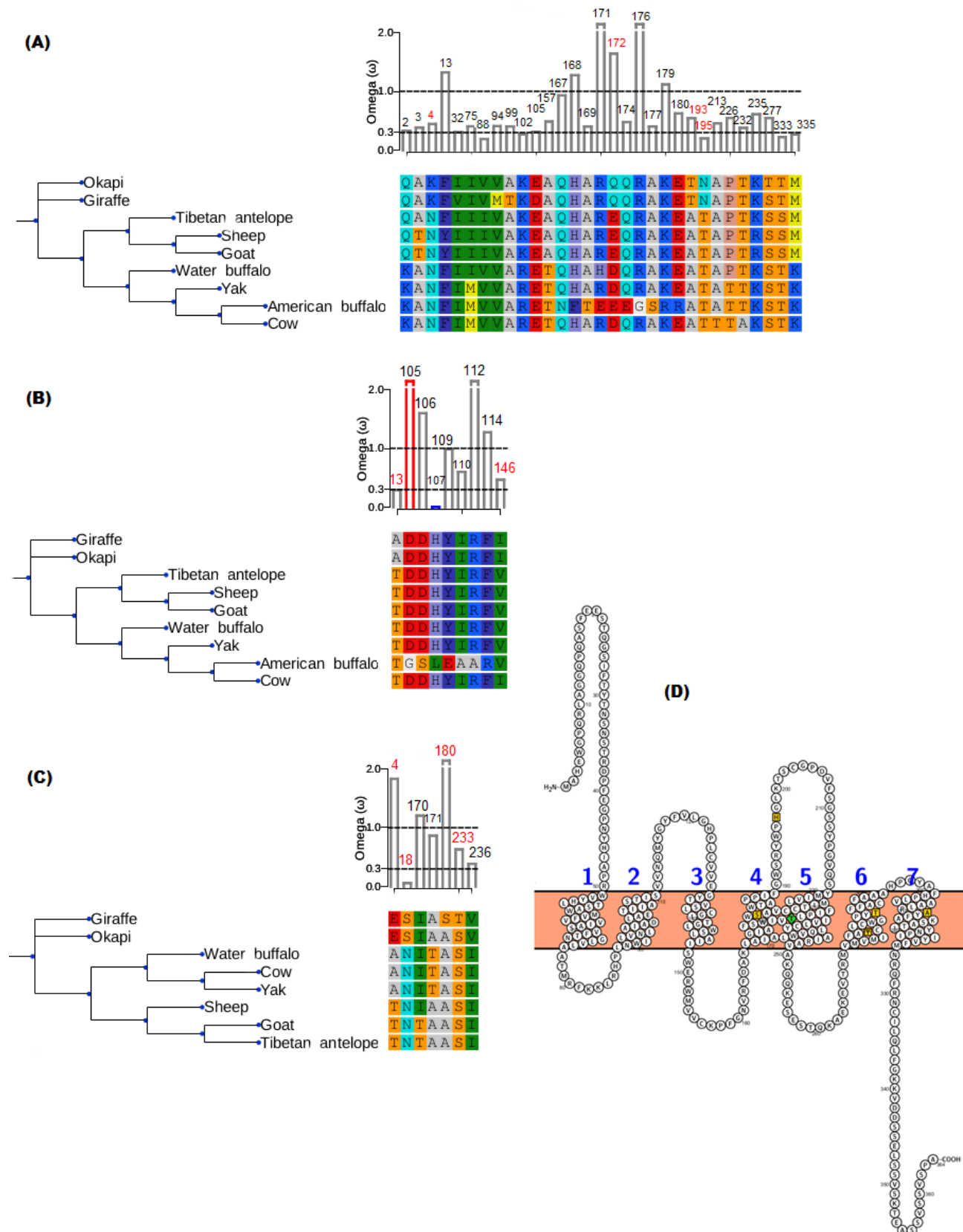



Figure 2. Gene trees and substitution analysis indicating sites (highlighted in red) potentially contributing to selection divergence in *SAG, CRYAA* and *OPN1LW*. The ω ratio for each variant site in the three genes was computed using the site-wise likelihood ratio analysis (Massingham & Goldman, 2005). (**A**) *SAG* has five sites which show signature of differential selection between okapi and other ruminants. (**B**) *CRYAA* has several sites which exhibit signature of selection but the associated sequence changes in giraffe are shared with okapi. (**C**) 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. All giraffe's sequences were found to be identical between NZOO and MA1 (Agaba et al., 2016) verifying that the identified substitutions are not artifacts. (**D**) The ribbon diagram of giraffe L-opsin highlighting important sequence changes relative to its secondary structure. Its spectrally important amino acids based on the five-sites rule are highlighted in yellow. A threonine at amino site 233 with respect to its unique S233T among ruminants is highlighted in green. The ribbon plot for the *OPN1LW* was generated by Protter (Omasits et al., 2013).

(A)

(B)

(C)

(D)

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591 **Figure 3**.The evolutionary relationship in mammals based on *OPN1LW* as revealed by coding
592 gene sequences. For species whose sequences were obtained from public database Refseq or
593 Genbank accession numbers for the respective sequences are shown.

Green monkey XM_007993132.1
Rhesus macaque XM_015128550.1
Crab-eating macaque AF158968.1
Baboon NM_001168797.1
Human NM_020061.5
Squirrel monkey XM_003943902.1
Tarsier XM_008071543.1
Tupaia XM_006166677.1
Flying lemur XM_008578409.1
Galago XM_003802556.1
Sifaka XM_012662045.1
Mouse lemur XM_012757188.1
Black-fruit bat XM_006915297.1
Long-fingered bat XM_016215131.1
Big brown bat XM_008159259.1
David vesper bat XM_006766920.2
Little-brown bat XM_006087664.2
Brandt vesper bat XM_005886120.2
Alpaca XM_006218499.1
Bactrian camel XM_010964076.1
Baiji XM_007456663.1
Killer whale XM_004286442.2
Bottlenose dolphin AF055457
Long-finned whale AY228446.1
Short-beaked dolphin AY228451.1
Giraffe
Okapi
Sheep XM_004022260.3
Goat XM_005701738.1
Tibetan antelope XM_005957735.1
Water buffalo XM_006043645.1
Yak XM_005909080.1
Cow NM_174566.1
Horse NM_001081845.1
Rhinocerus XM_004443325.1
Cat NM_001009871.1
Dog NM_001197072.1
Seal AY228448.1
Walrus XM_004409188.1
Giant panda XM_002928811.2
Polar bear XM_008709226.1
Ferret XM_004780711.2
Elephant NM_001280862.1
Rabbit NM_001322264.1

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595 **Table 1**. Significant selection divergence in three vision genes between giraffe or okapi (Clade 1) against the background of ruminant
596 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

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602 **Table 2.** Identity of amino acids at the functionally important sites in the L-opsin of giraffe and other mammals based on the five-sites
603 rule. For each species, the expected λ_{max} based on the five-site rule is shown. Where the actual λ_{max} of the pigment has been
604 determined, the value is indicated.

Species	Site/amino acid					Predicted λ_{max} (nm)	Experimental λ_{max} (nm)	References
	180	197	277	285	308			
Human (*Homo sapiens*)	S	H	Y	T	A	560	557	Merbs & Nathans, 1992
Human (*H. sapiens*)	A	H	Y	T	A	553	552	Merbs & Nathans, 1992
Rhesus macaque (*Macaca mulatta*)	S	H	Y	T	A	560	561.5	Bowmaker et al., 1991
Crab-eating macaque (*Macaca fascicularis*)	S	H	Y	T	A	560	561	Baylor, Nunn & Schnapft, 1
Baboon (*Papio anubis*)	S	H	Y	T	A	560	560	Bowmaker et al., 1991
Green monkey (*Chlorocebus sabaeus*)	S	H	Y	T	A	560	-	
Seal (*Phoca vitulina*)	S	H	F	T	A	552	510 (?)	Crognale et al., 1998; Lever
Walrus (*Odobenus rosmarus*)	S	H	Y	T	A	560	-	
Brandt's vesper bat (*Myotis brandtii*)	S	H	Y	T	A	560	-	
David's vesper bat (*Myotis davidii*)	S	H	Y	T	A	560	-	
Big brown bat (*Epistesicus fuscus*)	S	H	Y	T	A	560	-	
Little brown bat (*Myotis lucifugus*)	S	H	Y	T	A	560	-	
Cow (Bos taurus)	A	H	Y	T	A	553	555	Jacobs et al., 1998
Sheep (Ovis aries)	A	H	Y	T	A	553	552	Jacobs et al., 1998
Goat (*Capra hircus*)	A	H	Y	T	A	553	553	Jacobs et al., 1998
Giraffe (*Giraffa camelopardalis*)	S	H	Y	T	A	560	-	
Okapi (*Okapia johnstonii*)	A	H	Y	T	A	553	-	

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