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IRON METABOLISM GENES IN BROWSING AND GRAZING RHINOCEROSES: IMPLICATIONS FOR IRON OVERLOAD DISORDER

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Abstract

Iron overload disorder is a serious condition that affects many animals of conservation interest, including rhinoceroses. Iron overload disorder is only found in browsing rhinos (African black, *Diceros bicornis,* and Sumatran, *Dicerorhinus sumatrensis*) but not in grazing species (African white, *Ceratotherium simum*, and greater one-horned, *Rhinoceros unicornis*). Iron overload is connected with many of the other health issues seen in captive browsing rhinoceroses, so it is vitally important that the iron metabolism process is studied to improve the existing husbandry procedures of these critically endangered animals. The objective of this study was to characterize genes related to iron metabolism to determine if a genetic pattern exists that could help to describe the genetic basis of iron overload disorders in browser rhinoceroses. We amplified and sequenced the regions around the candidate mutations then analyzed those sequences for evidence of rapid evolution. We found derived mutations in the candidate genes are present in all black rhino subspecies, but not present in Sumatran rhinos. We did not find any evidence of positive selection on any site in any of the genes that we investigated. No mutations were conserved between black and Sumatran rhinos, which supports the idea that these two species likely have a different genetic basis for iron overload disorder. A better understanding of iron metabolism from a genetic perspective will improve diagnostic tools and preventative treatments for iron overload disorder in these endangered species.

Table of Contents

Page

Acknowledgments 2

Abstract 3

List of Tables 5

List of Figures 6

Introduction 7

Materials and Methods 15

Results 19

Discussion 22

References 26

Tables 30

Figures 42

List of Tables

Page

Table 1: Sample summary 30

Table 2: Information on candidate genes 32

Table 3: Information on other researched genes 33

Table 4: Primer information 34

Table 5: PCR protocls 35

Table 6: Mutations observed on EPB41 36

Table 7: Mutations observed on Slc28a2 37

Table 8: Mutations observed on STEAP4 38

Table 9: Mutations observed on HFE 39

Table 10: Mutations observed on Slc40a1 40

Table 11: Selection Analysis 41

List of Figures

Page

Figure 1: Summary of iron metabolism 42

Figure 2: Derived mutations observed on the EPB41 gene 43

Figure 3: Derived mutations observed on the Slc28a2 gene 44

Figure 4: Derived mutations observed on the STEAP4 gene 45

Figure 5: Derived mutations observed on the HFE gene 46

Figure 6: Derived mutations observed on the Slc40a1 gene 47

Introduction

There are many issues facing rhinoceros populations in recent decades. Habitat loss, fragmentation, over exploitation, and poaching for rhino horn are major threats to wild populations of both Asian and African species of rhinoceros (Metrione & Eyres 2014). In 2014 in Africa alone, there were approximately 3.55 rhinos poached every day (Save the Rhino International 2015). Bringing individuals into human care is one conservation strategy to protect and manage these animals. In 2013, it was estimated that there are only 29,000 rhinos in the wild: 5055 African black, *Diceros bicornis*; 100 Sumatran, *Dicerorhinus sumatrensis*; 20,400African white, *Ceratotherium simum*; 3345 greater one-horned, *Rhinoceros unicornis*; 58 Javan, *Rhinoceros sondaicus.* (Because there are only 58 Javan rhino individuals left in the world and there were no Javan DNA samples in the Frozen Zoo, Javan rhinos were excluded from this study.) One strategy to protect these animals is to house them in institutions under human care. There are approximately 865 rhinos housed in zoological institutions worldwide. There are many conservation benefits to bringing rhinoceroses into captivity, such as having more *ex situ* research opportunities, having an insurance population, attracting attention and support for rhinoceros conservation, and using these individuals to educate communities about conservation issues (Metrione & Eyres 2014). However, there are still problems facing rhinoceroses under human care. We need a better understanding of rhinoceros biology in order in increase survivorship and reproductive success of our captive populations (Dennis *et al.* 2007). A misunderstanding or inability to provide the ideal husbandry, like the proper diet specific to each species, can result in diseases only seen in animals under human care. Iron overload disorder is one such issue affecting captive rhinoceros populations (Dennis *et al.* 2007; Clauss & Paglia 2012; Paglia & Tsu 2012; Ganz & Nemeth 2012).

Iron is an essential trace element and is important in red blood cell formation, in oxygen transport, in the production of ATP, and in enzyme systems (Molenaar 2005). Iron is important in mitochondrial metabolism and the production of ATP (Adenosine triphosphate), the cellular energy molecule (Napier *et al.* 2005). Iron is initially absorbed in the intestines and is then released into the blood plasma through ferroportin. Once in the blood, it is bound to the protein transferrin and then delivered throughout the body (Figure 1) (Arezes & Nemeth 2015). Iron is highly redox reactive and has an important role in electron transfer, so it is important that the pathways that metabolize this trace mineral be strictly regulated (Gulec & Collins 2014). Excess iron can create reactive oxygen species which cause damage to DNA, proteins, and organelles, which leads to secondary issues and organ failure (Arezes & Nemeth 2015).

Iron overload disorder occurs when there is more iron than normal circulating throughout the body or when too much iron is being deposited within cells (Clauss & Paglia 2012). Hemosiderin are iron storage molecules, but when too many hemosiderin are deposited within cells, a disease called hemochromatosis, or iron overload disorder, occurs. In humans, primary hemochromatosis is caused by a genetic defect (Pietrandgelo 2010). Secondary, or acquired, hemochromatosis has multiple potential causes including the consumption of too much iron (Molenaar 2005). High concentrations of iron can also catalyze hydroxyl free-radical production, which can cause damage to the lipid membrane and to DNA as well as altering certain enzyme functions (Molenaar 2005).

In rhinoceroses, iron overload disorders are linked to other medical conditions that browsing rhinoceroses often develop in captivity (Dennis *et al.,* 2007). Excess iron can lead to necrosis, or cell injury, and thus increases the susceptibility of infection (Courtois 2015; Olias *et al.* 2012). This can result in potentially fatal conditions like necrotizing dermatitis, which is

extremely painful and causes ulcers and lesions all over the body (Courtois 2015; Paglia & Tsu 2012). Iron overload is also often seen with hemolytic anemia, the premature destruction of red blood cells (Mylniczenko *et al.* 2012). Hemolytic anemia is one of the leading causes of death in captive rhinos (Dennis *et al.*, 2007). In humans, iron overload has been linked to insulin resistance, resulting in obesity and diabetes, which are other conditions often seen in captive rhinoceroses (Solanas-Barca *et al.* 2009; Wrede *et al.* 2006; Venn-Watson *et al*. 2012).

Iron overload disorder can be diagnosed and monitored by measuring concentrations of iron deposits in tissues and by monitoring levels of iron analytes in blood work (Molenaar 2008). Monitoring blood work in species prone to iron overload disorder is important because sometimes there are no observable symptoms and iron overload disorder is discovered only during the necropsy, after the animal has already died from the disease (Clauss & Paglia 2012). However, determining normal reference ranges for normal blood iron metabolites is difficult because there is no published data about wild rhinoceros ranges and the limited data that is available is highly variable (Mylniczenko *et al.* 2012; Miller *et al.* 2012). The best way to diagnose this disorder is liver biopsy, but this involves very invasive surgery, so this procedure is very rarely performed (Mylniczenko *et al.* 2012).

Current therapeutic treatments for iron overload disorder include phlebotomy and chelation therapy (Beutler 2007; Molenaar 2005). Phlebotomy is the process of drawing large amounts of blood with the purpose of removing excess iron from the blood stream and having the bone marrow regenerate normal red blood cells (Casanova-Esteban *et al.* 2011; Molenaar 2005). For rhinos, phlebotomy can require frequent sedation, special facilities, and trained technicians, so it is not always a viable option for all institutions (Mylniczenko *et al.* 2012). Chelation therapy is adding compounds into a diet that help metabolize the iron so that it can be

excreted (Beutler 2007). Chelation therapy is not widely utilized because it is expensive, especially for such large animals. Also, there are known negative side effects to chelation such as pain at the injection site, fever, gastro-intestinal distress, seizures, and hypotension (Mylniczenko *et al.* 2012). These procedures only help to maintain a good quality of life and neither cures this disease nor prevents this disease from occurring.

Although this disorder is seen in humans and a variety of other species, only certain species of rhino seem to be predisposed to this condition. Iron overload disorder is only found in virtually every browser rhinoceros (African black, *Diceros bicornis*, and Sumatran, *Dicerorhinus sumatrensis*) but not in grazers (African white, *Ceratotherium simum*, and greater one-horned, *Rhinoceros unicornis*) (Clauss & Paglia 2012; Paglia 2015; Linzmeier *et al*. 2013). This disease is also only found in captive animals and is not seen in wild rhinos (Kock *et al.* 1992; Candra *et al*. 2012; Clauss & Paglia 2012; Paglia 2015). The exact cause of iron overload disorder in rhinoceroses is unknown. However, there are a few proposed hypotheses that attempt to explain why this disease is so prevalent in browser rhinoceroses in captivity.

One hypothesis attributes the predisposition of certain rhino species to iron overload disorder to their red blood cells (Weber *et al.* 2004; Harley *et al.* 2004). Rhinoceroses have very unusual erythrocytes when compared to other mammals. Rhinoceros erythrocytes have lower concentrations of cellular energy (ATP) and lower concentrations of certain enzymes, including catalase (Paglia & Tsu 2012). Such concentrations in a human would cause hemolytic anemia or immunodeficiency, but are normal for rhinoceroses (Paglia & Tsu 2012). In rhinoceroses, these differences reduce their ability to neutralize oxidants, making their cells more likely to overload iron (Weber *et al.* 2004; Molenaar 2005). A possible explanation for this apparent metabolic disadvantage is that browsing animals evolved when the availability of many essential metals,

including iron, was low and it was not necessary to process a large amount of iron (Paglia & Tsu 2012).

Another hypothesis is that the diet fed to browser rhinoceroses in zoos could contribute to the development of iron overload disorder. In the wild, browsers choose food sources with natural chelators that bind to iron to help them process and excrete it (Lavin 2012; Paglia & Tsu 2012). Typically, diets fed to browser rhinoceroses in captivity include grass, alfalfa hay, and pellets which all contain more iron than a wild diet of only browse (Candra *et al*. 2012). Diet with reduced amounts of iron have been shown to reduce blood iron levels in captive populations of black rhinos (Mylniczenko *et al.* 2012). However, this can be difficult to implement in zoos because it goes against traditional husbandry, and low-iron diets are often more expensive (Clauss & Paglia 2012; Mylniczenko *et al.* 2012). It is also logisticallydifficult to imitate natural mineral availability because there are many seasonal and geographical variations in dietary iron content in wild browse (Helary *et al.* 2012). This hypothesis could explain why iron overload disorder is not observed in grazer rhinoceroses, since they need to be able to process the iron and other metals that they ingest from the soil when they are consuming grasses (Clauss & Paglia 2012).

However, there are some inconsistencies with the hypothesis that these dietary adaptations alone lead to increased iron levels. For example, one would expect to see iron overload disorder in browsing ruminants, like goats and deer, but they seem to be unaffected by the disease (Clauss & Paglia 2012). This idea also does not explain why some lemur species are more susceptible to iron overload disorder than others since the diets between lemur species are very similar (Clauss & Paglia 2012). However, the iron overload seen in other species is likely due to malfunctions in different parts of the metabolic pathway than rhinos, so the mechanism of

this disorder is likely very different, so comparisons should be performed with caution. Despite these inconsistencies in other species, for captive browsing rhinoceroses, implementing a low-iron diet may be a good first step in preventing iron overload disorder.

Former studies predict that genetic predispositions could be a cause of iron overload disorder in certain species of rhinoceros (Paglia & Tsu 2012; Miller *et al*. 2012). A genetic approach to studying this disease is useful because many of the advances made in studying human iron storage disease arose from genetic research. Mutations on certain genes (HFE, TfR2, HJV) are the most common cause of issues with iron regulation and absorption in humans (Andrews 2008; Ganz & Nemeth 2012; Mylniczenko *et al.* 2012). We hypothesize that a mutation on a gene related to iron metabolism in browsing rhinos relative to grazing rhinos will affect the function of this pathway and explain why browsing rhinos appear to be predisposed to this disorder.

Although there are many genes involved in iron metabolism, previous research using genomic data (RNAseq) and analysis detecting likely deleterious mutations (SIFT approach) identified three candidate mutations on three genes that appear to be important to iron metabolism in rhinoceroses (Lowe 1999; Linzmeier *et al*. 2013). The EPB41 gene is responsible for erythrocyte shape and stability (Nunomura *et al.* 2011), and the G111E mutation on this gene could be linked to possible morphological differences between the erythrocytes of different rhinoceros species or help characterize susceptibility to oxidative stress in different species of rhinoceros (Ganz *et al.* 2012). The Q173K mutation on the SLC28a2 gene is connected to the low ATP concentrations in rhinoceros erythrocytes and this mutation could affect membrane expression (Young *et al.,* 2013; Pastor-Anglada *et al.,* 2008). The I433S mutation on the STEAP4 gene could be linked to insulin resistance, which is a common metabolic issue in

captive and wild rhinoceroses (Schook *et al.* 2015). In humans, there is a connection between insulin resistance and iron storage issues, so this is an important gene to study in rhinoceroses (Wrede *et al.* 2006; Gauss *et al.* 2013; Venn-Watson *et al.* 2012). This connection has also been supported in an equine model (Nielsen 2012). STEAP4 is expressed in the intestine and is therefore involved in dietary iron absorption in the intestines (Andrews 2008). One objective of this study was to analyze these candidate genes on a larger sample set from four species and eight subspecies of rhinos to determine how conserved the derived mutations in the selected genes are at the intraspecific level. Learning more about these mutations will help us to better understand the mechanism of this disorder and to propose methods to improve the treatment of susceptible rhinoceroses in zoos.

The other genes investigated in this study were genes that have been shown to contain mutations that increase iron overload, or hemochromatosis, in humans. Multiple mutations on the HFE, or hemochromatosis, gene affect iron metabolism in humans. The HFE gene regulates hepcidin (a peptide hormone that modulates iron availability) expression, and binds to the transferrin receptor (Andrews 2008). Mutations on this gene can affect this binding and reduce this receptor's affinity for iron-loaded transferrin (Bennett *et al.* 2000). The HFE gene is also linked to the major histocompatibility complex which affects iron absorption (Barton *et al*. 2015). Another gene relation to iron overload in humans is the Slc40a1 gene which codes for ferroportin (Andrews 2008). Mutations on this gene are linked with hyperferritinemia (an excess of the iron storage molecule, ferritin, in the blood) and iron overload in humans (Aguilar-Martinez *et al*. 2005). The objective of studying these genes was to see if the genes that are involved in iron storage disease in humans share a similar mutation in browsing rhinoceroses.

This research seeks to support our hypothesize that a mutation on a gene related to iron metabolism in browsing rhinos, relative to grazing rhinos, will affect those species' ability to metabolize iron. We predict that there is a mutation on EPB41, Slc28a2, STEAP4, HFE, or Slc40a1 in only a browser species of rhino. This would suggest that there could be a structural or functional difference in the proteins coded for by these genes that would affect some part of the iron metabolism pathway and offer an explanation of why browser rhinos seem to be predisposed to this condition.

Materials and Methods

Sampling

Samples from 52 rhinoceros individuals belonging to four species and eight subspecies were used in this study: south-western black rhino, *Diceros bicornis bicornis* (2 individuals), eastern black rhino, *Diceros bicornis michaeli* (10), south-central black rhino, *Diceros bicornis minor* (10), Sumatran rhino, *Dicerorhinus sumatrensis sumatrensis* (6), Bornean rhino, *Dicerorhinus sumatrensis harrissoni* (4), greater one-horned rhino, *Rhinoceros unicornis* (3), northern white rhino, *Ceratotherium simum cottoni* (10), and southern white rhino, *Ceratotherium simum simum* (7). All DNA samples were obtained from the DNA collection of the Frozen Zoo ® at the San Diego Zoo Institute for Conservation Research in Escondido, California.

The samples of DNA were quantified using a Nanodrop (Thermo Fisher Scientific) to determine concentration and quality through absorbance. From that information, samples that appeared to be of good quality were selected. The samples chosen also represented individuals from as many different zoological institutions and as many wild-born individuals as possible to create a genetically diverse sample (Table 1). When necessary, the samples were diluted with dH_2O to reach the desired concentration of 5ng/µl.

Five genes were analyzed in this study: (1) Erythrocyte membrane protein band 4.1 (EPB41), (2) Sodium/nucleoside cotransporter 2 (Slc28a2), (3) Six-transmembrane epithelial antigen of prostate 4 (STEAP4), (Table 2), (4) Hereditary hemochromatosis protein (HFE), and (5) Solute carrier family 40 member 1 (Slc40a1) (Table 3).

The domestic horse, *Equus caballus*, genome was obtained from Genbank (NCBI) and Ensembl (Yates *et al.* 2016) genome browsers. The partially annotated white rhino genome from

the University of California Santa Cruz genome browser was also utilized. Alignments of these reference genes were used to design primers. Primers were designed in conserved regions of the alignments. (Table 4).

PCR Conditions

Polymerase chain reaction (PCR) amplifications were performed in a 20µl volume using T100 thermal cyclers (Bio-Rad). Each reaction included 2.0µl 10X ATGold buffer (Applied Biosystems), 1.0µl dNTP (2.5mM), 0.6µl forward and reverse primers (10µM), 0.2µl AmpliTaq Gold polymerase (Applied Biosystems), 2.0µl template DNA, and enough ddH_2O to bring the volume to 20µl. The PCR cycling conditions were 95°C for 6 minutes followed by 34 cycles of denaturation at 94°C for 1 minute, annealing at a primer-dependent temperature for 1 minute, and extension at 72°C for 1 minute, with a final extension at 72° for 7 minutes (Table 5). For PCR that required troubleshooting, Bovine Serum Albumin (BSA) was added to reduce inhibition from contaminating proteins (Kreader 1996). Dimethyl sulfoxide (DMSO) was added in certain PCR reactions to increase the yield of replication of GC nucleotide rich exons (Farell & Alexandre 2012). All PCR products were verified in a 1% agarose gel electrophoresis to confirm proper DNA amplification in all rhinoceros subspecies.

Products were purified using ExoSAP cleanup (Thermo Fischer Scientific). PCR primers and annealing temperatures were also used for cycle sequencing reactions. The genes were sequenced using the Sanger method using an Applied Biosystems 3031 Genetic Analyzer (Sanger & Coulson 1975).

Sequence Annotation and Alignment

DNA sequences from all rhino subspecies were edited and aligned with Sequencher 3.1.1 (Gene Codes, Ann Arbor, MI). The sequences were validated through nBLAST (NCBI). Alignments were visualized using Geneious v1.2.1 (Kearse *et al.* 2012).

Geneious v1.2.1 was also used to visualize multi-species alignments that include rhinoceros sequences with more distantly related taxa (rat, *Rattus norvegicus*, gorilla, *Gorilla gorilla gorilla*, marmoset, *Callithrix jacchus*, mouse lemur, *Microcebus murinus*, dolphin, *Tursiops truncates*, and horse, *Equus caballus*). Other species nucleotide sequences were obtained from Ensembl.

Selection Analysis

Phylogenetic trees were inferred for all genes studied using a Bayesian approach in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). Analyses for positive selection were performed. The Bayesian inference consisted of two concurrent runs with four Markov chains (one cold and seven heated chains with a temperature of 0.2), twenty million generations (sampled every 1,000 generations), and a 10% burn-in. We verified that potential scale reduction factors were near to 1.0 for all parameters, and that the average standard deviation of split frequencies was below 0.01. Analyses for positive selection were performed among rhino species/subspecies. The codeml program from PAML (Yang 2007) was used to identify significant differences in likelihood values between nearly neutral (model 7) and positive selection models with unconstrained omega (model 8) and omega constrained to 1.0 (model 8a). Amino acid sites under positive selection and P-values were inferred using the Bayes-Empirical-Bayes dN/dS

approach in the unconstrained model 8. Branch-site models were also tested to detect positive selection affecting a few codon sites along particular rhino lineages.

Results

EPB41

The 5'UTR – exon 2 region of the EPB41 gene was amplified and sequenced in all rhino species studied and 11 mutations were observed. Three of these mutations were synonymous (did not produce any amino acid changes in the protein) and 8 were non-synonymous (produced an amino acid change). The derived candidate mutation G111E was present in all black rhino subspecies, but neither present in the Sumatran rhinos nor any of the grazer species. This result supports that the G111E mutation is conserved (fixed) among black rhino subspecies (Table 6, Figure 2). Other non-synonymous mutations, S104F and T120P, appear to be linked to each other, in only two subspecies of black rhino, *Diceros bicornis bicornis* and *Diceros bicornis minor*. Mutation V102I was only seen in Sumatran rhinos. When comparing rhinos with other not-so closely related mammalian species, the G111E mutation was only found in black rhinos, and no other non-synonymous mutations were found at that amino acid site in the EPB41 gene, suggesting that this site is highly conserved across mammals (Figure 2).

Slc28a2

The exon 6 region of the Slc28a2 gene was amplified and sequenced in all rhino species studied, and 5 mutations were observed. Two of these mutations were synonymous and three were non-synonymous. The derived candidate mutation Q173K on Slc28a2 was present in all black rhino subspecies, but not present in the Sumatran rhinos. This result supports that this mutation is conserved (fixed) among black rhino subspecies (Table 7, Figure 3).The V184I mutation was only seen in Sumatran rhinos. When comparing rhinos with other not-so closely

related mammalian species, the Q173K mutation was only found in black rhinos, and no other non-synonymous mutations were found at that amino acid site in the Slc28a2 gene, suggesting that this site is highly conserved across mammals (Figure 3).

STEAP4

The exon 5 region of the STEAP4 gene was amplified and sequenced in all rhino species studied, and 10 mutations were observed. Seven of these mutations were synonymous and three non-synonymous. The derived mutation I433S on STEAP4 was present in all black rhino subspecies, but not present in the Sumatran rhinos. This result supports that this mutation is conserved (fixed) among black rhino subspecies (Table 8, Figure 4). When comparing rhinos with other not-so closely related mammalian species, the I433S mutation was only found in black rhinos, but other non-synonymous mutations were also found at that amino acid site in the Slc28a2 gene, in rat (I433M) and mouse (I433S) suggesting that this site is not as conserved in mammals as showed before in other candidate mutations (Figure 4).

HFE

The exon 2 region of the HFE gene was amplified and sequenced in all rhino species studied, and 7 mutations were observed. Six of these mutations were synonymous. The S88T mutation was present in all black rhino subspecies, but this mutation had been previously observed in black rhinos (Beutler *et al.* 2001). The alignment produced supports that this mutation is conserved among black rhino subspecies (Table 9, Figure 5). When comparing rhinos with other not-so closely related mammalian species, the S88T mutation is found only in black rhinos and no other non-synonymous mutations were found at that amino acid site in the

HFE gene, suggesting that this site is highly conserved across mammals (Figure 5). The H63H mutation identified in the human HFE gene was present in both black and white rhinos (Table 9) (Barton *et al.* 2015).

Slc40a1

The exon 3 and exon 5 regions of the Slc40a1 gene were amplified and sequenced in all rhino species studied, and two mutations were observed on exon 3 and one mutation was observed on exon 5. Both mutations on exon 3 were synonymous. Alignments were generate and these exons are well conserved between taxa (Table 10, Figure 6). The A77D and N114H mutations identified in the human Slc40a1 gene were not present in any rhino species (Table 10) (Jones *et al.* 2002).

Selection Analysis

No signature of positive selection (measured as the ratio of nonsynonymous versus synonymous mutations) was found in any of the genes investigated after testing site and branch-site models (Table 11).

Discussion

Using a sample set that included all four species and all eight subspecies of extant rhinoceroses, we supported our predictions and confirmed that all derived mutations initially identified in the candidate genes (EPB41, Slc28a2, and STEAP4) were shared only between black rhino subspecies using a larger sample size than previous studies (Figures 2,3,4) (Linzmeier *et al.* 2013). This suggests a single origin of the derived mutations in the black rhino lineage. No mutations were conserved between black and Sumatran rhinos. This supports the hypothesis that these two species have a different genetic basis for iron overload, which is expected, due to the phylogenetic relationships between these two species. Molecular data shows the greater one-horned rhinos separating first, followed by the African species (black and white) diverging from the Sumatran rhinos (Steiner & Ryder 2011). Since black rhinos are more closely related to white than Sumatran, it makes sense that these two species may have evolved different mechanisms for this apparent predisposition to iron overload. Future studies should focus on looking for a mutation on the Sumatran rhino genome that may explain their mechanism of iron overload since the candidate genes used in this study focus on black rhinoceroses. It would also be helpful to sequence and compare extinct rhinoceros species, like the woolly rhino, *Coelodonta antiquitatis*, to further investigate the evolution of these genes.

The non-synonymous mutations that we observed on the candidate genes alter the amino acid sequence which may change the structure or function of the protein. EBP41 codes for structurally important proteins in the erythrocyte membrane skeleton and is vital for regulating structure and stability of red blood cells (Nunomura *et al.* 2011). Slc28a2 is also related to red blood cells, so changes in the function of the transporter proteins coded for by this gene could possible explain the very low ATP concentrations in rhino erythrocytes (Young *et al.* 2013;

Pastor-Anglada *et al.* 2008). Therefore, interspecific differences in these two genes could be related the abnormalities within rhinoceros erythrocytes which increase their susceptibility to oxidative stress and make them more likely to overload iron (Paglia & Tsu 2012; Molenaar 2005). The third candidate gene, STEAP4, codes for enzymes that reduce iron and are involved metabolic homeostasis (Gomes *et al.* 2012). Mutations on this gene may alter the function of these enzymes and result in inflammatory responses that can lead to insulin-resistance and obesity (Gauss *et al.* 2013). These are conditions have been connected to iron overload in humans and equines, and they are frequently seen in captive browsing rhinos (Haap *et al.* 2011; Nielsen *et al.* 2012).

There were also non-synonymous mutations conserved between all black rhino subspecies on the genes involved in human hemochromatosis, which supported our prediction (Figures 5,6). There was one mutation that was unique to black rhinos on the HFE gene. Although this mutation had been previously described, we confirmed that this mutation is present in all black rhino subspecies and is not present in any other rhino subspecies (Beutler *et al*. 2001). The HFE gene codes for a protein than binds and regulates the transferrin receptor, so mutations on this gene might affect this binding ability and increase the transferrin receptor's affinity to load iron (Barton *et al.* 2015). The C282T mutation on the HFE is one of the most common causes of iron overload in humans (Feder *et al.* 1997). However, even in humans, the way that these genes affect iron metabolism is not very well known, and there are people with this mutation who do not develop iron overload, and there are many people who develop iron overload from different mutations (Beutler 2003). Slc40a1 is another gene related to human hemochromatosis (Aguilar-Martinez *et al.* 2005). It also codes for a transporter protein that exports iron from the intestinal cells into circulation, so a mutation on this gene might increase

absorption of iron which could explain why browsing rhinos are so sensitive to dietary iron (Aguilar-Martinez *et al.* 2005). However, our prediction was not supported for this gene and the only non-synonymous mutation observed on the Slc40a1 gene was only in the greater one-horned rhinos, so it does not explain the browser species' predisposition to iron overload.

The genes that we looked at in this study had low genetic variation and there were no positively selected sites on any of the genes (Table 11). This shows that these genes are likely not undergoing rapid evolution. Evidence of rapid evolution would indicate an accelerated rate of speciation and a strong selection towards mechanisms that would increase iron absorption (Herbert *et al.* 2003). This would have supported the hypothesis that browsing rhinos adapted a high sensitivity to iron in response to wild diets with low mineral availability (Paglia & Tsu 2012). The high number of fixed alleles conserved between multiple, distantly related mammalian species suggests evolutionary constraints on these genes because of their important functional roles in iron metabolism (Camaschella 2005).

There are many other genes involved in iron metabolism, so more genes need to be sequenced and more mutations need to be investigated in order to describe the genetic mechanism of iron overload in rhinos. Future studies should create functional assays to look at any phenotypical differences caused by the mutations that were observed. Another possible direction would be to compare the microbiome genetic variability in captive and free-ranging rhinoceroses since gut flora are involved in mineral absorption (Bian *et al.* 2013; Cheralyil *et al.* 2011).

It is vitally important that iron overload disorder is studied and that husbandry, nutritional, and medical procedures in zoos are improved to help preserve these critically endangered animals. Rhinoceroses in the wild are threatened by poaching and habitat loss and

rhinoceroses in captivity are threatened by iron overload disorder. More research into iron metabolism in rhinoceroses may also lead to better treatments for rhinos and the wide range of other species affected by iron overload, such as birds (Klasing *et al*. 2012), bottlenose dolphins, *Tursiops truncates*, (Venn-Watson *et al*. 2012), and humans. A better understanding of iron metabolism from a genetic perspective will hopefully improve husbandry procedures, diagnostic tools, and preventative treatments for iron overload disorder.

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Tables

Table 1: Summary of DNA samples used. KB number refers to the location of the sample in the Frozen Zoo. Species 360 is an online database of animals in zoological institutions. Dilution of a sample is denoted by a (d). A captive born individual is denoted by CB.

Scientific Name	KB#	Location when sampled	Species360 ID	Date Collected	Orig DNA Conc ng/ul	Birth Type
Black (n=22)						
Diceros bicornis bicornis	6718	ETOSHA	--	16-Sep-99	94.9	CB
Diceros bicornis bicornis	6719	ETOSHA	--	Oct-98	176 (d)	CB
Diceros bicornis michaeli	4524	SD-WAP	682065	11-Aug-86	152 (d)	CB
Diceros bicornis michaeli	5994	GARDENCTY	00456	--	285.6	CB
Diceros bicornis michaeli	5995	GARDENCTY	00458	--	146 (d)	CB
Diceros bicornis michaeli	6066	ST LOUIS	85437	25-Apr-86	145 (d)	CB
Diceros bicornis michaeli	7160	DENVER	00457	--	186.8	CB
Diceros bicornis michaeli	7972	SAN FRAN	1564	--	132 (d)	CB
Diceros bicornis michaeli	10649	KANSASCTY	102863	23-Jul-99	169 (d)	Wild Born
Diceros bicornis michaeli	12005	CALDWELL	104130	1-Aug-01	69.2	CB
Diceros bicornis michaeli	16787	SD-WAP	687485	14-Jul-87	132 (d)	CB
Diceros bicornis michaeli	7163	DENVER	11902	--	176 (d)	CB
Diceros bicornis minor	6119	LOSANGELE	001708	1-Dec-87	153.6	CB
Diceros bicornis minor	6232	LOSANGELE	001079	Jun-88	110 (d)	CB
Diceros bicornis minor	6944	SANDIEGOZ	589278	12-Jun-97	115 (d)	CB
Diceros bicornis minor	8717	ZIMBABWE	--	18-Nov-93	188.7	Wild Born
Diceros bicornis minor	9245	DALLAS	896577	12-Jul-90	250.2	CB
Diceros bicornis minor	Nikili	Mohammed Jama	--	--	100 (d)	CB
Diceros bicornis minor	Sinam	Mohammed Jama	--	--	179.8	CB
Diceros bicornis minor	Ubala	Mohammed Jama	--	--	166.9	CB
Diceros bicornis minor	Chirunda	Mohammed Jama	--	--	91.9	CB
Diceros bicornis minor	Siabuwa	Mohammed Jama	--	--	240.3	CB
Greater One-Horned (n=3)						
Rhinoceros unicornis	4723	SD-WAP	683352	17-Apr-86	139 (d)	CB
Rhinoceros unicornis	5412	SD-WAP	028842	17-Apr-86	124 (d)	CB
Rhinoceros unicornis	5850	MILWAUKEE	202	5-Oct-87	218 (d)	CB
Sumatran (n=10)						
Dicerorhinus sumatrensis sumatrensis	6196	MELAKA	--	1-Dec-87	160 (d)	Wild Born
Dicerorhinus sumatrensis sumatrensis	6197	MELAKA	--	--	49.50	Wild Born
Dicerorhinus sumatrensis sumatrensis	6198	MELAKA	--	--	133.90	Wild Born

Scientific Name	KB#	Location when sampled	Species360	Date Collected	Orig DNA Conc ng/ul	Birth Type
Dicerorhinus sumatrensis sumatrensis	OR1266	SANDIEGOZ	--	--	12.70	Wild Born
Dicerorhinus sumatrensis sumatrensis	8031	SANDIEGOZ	691738	10-Jan-02	176 (d)	Wild Born
Dicerorhinus sumatrensis sumatrensis	8126	CINCINNAT	189051	10-Jan-02	121 (d)	Wild Born
Dicerorhinus sumatrensis harrissoni	OR2142	SOS Rhino	--	10-Jan-02	146 (d)	Wild Born
Dicerorhinus sumatrensis harrissoni	20276	TABIN WILDLIFE RESERVE, MALAYSIA	--	--	60.01	Wild Born
Dicerorhinus sumatrensis harrissoni	20277	TABIN WILDLIFE RESERVE, MALAYSIA	--	--	45.6	Wild Born
Dicerorhinus sumatrensis harrissoni	20278	TABIN WILDLIFE RESERVE, MALAYSIA	--	--	116.4	Wild Born
White (n=17)						
Ceratotherium simum cottoni	3731	SD-WAP	100282	30-Jun-86	148 (d)	Wild Born
Ceratotherium simum cottoni	5763	DVURKRALV	059006	15-Jun-88	148.5	Wild Born
Ceratotherium simum cottoni	5764	SD-WAP	689610	2-May-88	235.8	Wild Born
Ceratotherium simum cottoni	5765	DVURKRALV	059009	--	244.6	CB
Ceratotherium simum cottoni	5766	DVURKRALV	059001	4-Apr-88	117.2	CB
Ceratotherium simum cottoni	8172	DVURKRALV	059010	--	160 (d)	CB
Ceratotherium simum cottoni	8173	DVURKRALV	059012	--	148 (d)	CB
Ceratotherium simum cottoni	8174	DVURKRALV	059007	--	271.4	Wild Born
Ceratotherium simum cottoni	8175	SD-WAP	689609	--	364.3	Wild Born
Ceratotherium simum cottoni	10529	--	--	--	67.74	CB
Ceratotherium simum simum	4558	SD-WAP	682447	17-Apr-86	192 (d)	CB
Ceratotherium simum simum	5892	SD-WAP	100264	10-Aug-87	152.8	Wild Born
Ceratotherium simum simum	6181	SANDIEGOZ	011623	--	145 (d)	Wild Born
Ceratotherium simum simum	8099	SD-WAP	026210	30-Jul-02	80.58	CB
Ceratotherium simum simum	10723	SD-WAP	100262	19-Aug-99	280 (d)	Wild Born
Ceratotherium simum simum	OR125	SD-WAP	682410	15-Apr-86	180 (d)	CB
Ceratotherium simum simum	OR502	SD-WAP	101661	7-Dec-79	180 (d)	CB

Table 2: Information on candidate genes. Candidate mutations proposed by: Linzmeier R, Paglia DE, Ganz N, Ganz T, Thompson R, LaMere S, Lee P. 2013. Current studies on molecular mechanisms of iron homeostasis in rhinoceroses. Proceedings of the 2013 International Elephant and Rhino Conservation and Research Symposium, 2013: 487-505.

Gene	**Mutation**	**Phenotypic Effect**
Slc28a2	Q173K	Erythrocyte ATP levels
EPB41	G111E	Hemolytic anemia
STEAP4	I433S	Insulin resistance

Table 3: Information on other researched genes. * Described in: Barton JC, Edwards CQ, Acton RT. 2015. HFE gene: Structure, function, mutations, and associated iron abnormalities. Gene 574: 179-192. ** Described in: Jones DC, Young NT, Pigott C, Fuggle SV, Barnardo MCNM, Marshall SE, Bunce M. 2002. Comprehensive hereditary hemochromatosis genotyping. Tissue Antigens 60: 481-488.

Gene	**Mutation (in humans)**	**Phenotypic Effect (in humans)**
HFE	70 described mutations*	Hemochromatosis
Slc40a1	A77D, N144H**	Hemochromatosis

Table 4: Primers used for DNA amplification.

Amplicon	Exon	Forward or Reverse		Primer Annealing Temp (*C)	PCR Temperature (*C)
EPB41	5'UTR-E2	F	5’ TTGACCAAGATCAAGGAGCGGACA 3’	60.3	54.0
		R	5’ GGTGTCTAACCTACCTTCATTA 3’	51.3	
Slc28a2	E6	F	5’ CACTTATCCTTGGGTGAACTAACT 3’	54.4	54.5
		R	5’ CAATACTTGACACATGCACTTTGTAATTA 3’	54.5	
STEAP4	E5	F	5’ TCACTTTAAAATGTAAATATG 3’	41.6	47.0
		R	5’ CATTATTCTTCTTTAAACATA 3’	41.1	
HFE	E2	F	5’ GTGGGCCCAGACACAGCTGGT 3’	64.6	60.0
		R	5’ GTCCAGAAGTCGACGATGAACA 3’	59.6	
Slc40a1	E3	F	5’ ATTGGGCAAGAATATTTTCCATTG 3’	52.4	48.0
		R	5’ TGAGTGGTGTTATAACTAAG 3’	46.4	
	E5	F	5’ CAGATGATACAGATTAGGAAGA 3’	48.9	48.0
		R	5’ TATATTAGGATTCAGTTTAAATC 3’	43.7	

Table 5: Specific PCR Protocols for each gene.

	EPB41	**Slc28a2**	**STEAP4**	**HFE**	**Slc40a1**
Amplified Region	5'UTR - Exon 2	Exon 6	Exon 5	Exon 2	Exon 6
ATGold buffer (µl)	2.0	2.0	2.0	2.0	2.0
dNTP (2.5mM) (µl)	1.0	1.0	1.0	1.0	1.0
Forward Primer (µl)	0.6	0.6	0.6	0.6	0.6
Reverse Primer (µl)	0.6	0.6	0.6	0.6	0.6
AmpliTaq Gold Polymerase (µl)	0.2	0.2	0.2	0.2	0.2
ddH2O (µl)	12.6	12.6	11.6	13.6	11.6
BSA (µl)	1.0	1.0	1.0	0.0	1.0
DMSO (µl)	0.0	0.0	1.0	0.0	1.0
Template DNA (µl)	2.0	2.0	2.0	2.0	2.0
Annealing Temp (ºC)	50.0	54.5	47.0	60.0	52.0

Table 6: Mutations observed on the 5'UTR – exon 2 region of the EPB41 gene. The mutation originally observed by Linzmeier *et al.* (2013) is shaded. Synonymous mutations are italicized. *C/T and *A/C mutations appear to be linked and all black rhinoceros subspecies except for *Diceros bicornis michaeli* contain the mutation at these sites.

			Nucleotides						Amino Acids			
	EPB41		Browsers		Grazers							
	Mutation	cDNA No.	Black	Sumatran	White	GOH	Horse	Human	Mutation	Codon	Ancestral	Mutations
	G/T	282	T	G	T	G	G	G	V94F	1st	V	F
	A/G	301	G	A	G	A	A	A	K100R	2nd	K	R
	G/A	306	G	A	G	G	G	G	V102I	1st	V	I
	G/C	309	G	G	C	G	G	G	E103Q	1st	E	Q
*	C/T	313	C/T; all Dbm C	C	C	C	C	C	S104F	2nd	S	F
	G/A	334	A	G	G	G	G	G	G111E	2nd	G	E
	T/G	349	T	T	T	G	G	T	I116R	2nd	I	R
*	A/C	360	A/C; all Dbm A	A	A	A	A	A	T120P	1st	T	P
	A/G	*425*	*A*	*A*	*A*	*G*	*C*	*A*	*T141T*	*3rd*	*T*	*T*
	A/G	*431*	*A*	*A*	*A*	*G*	*G*	*A*	*P143P*	*3rd*	*P*	*P*
	A/C	*449*	*A*	*A*	*A*	*C*	*A*	*A*	*S149S*	*3rd*	*S*	*S*

Table 7: Mutations observed on exon 6 of the Slc28a2 gene. The mutation originally observed by Linzmeier *et al.* is shaded. Synonymous mutations are italicized.

		Nucleotides						Amino Acids			
Slc28a2		Browsers		Grazers							
Mutation	cDNA No.	Black	Sumatran	White	GOH	Horse	Human	Mutation	Codon	Ancestral	Mutations
G/A	511	G	G	G	A	G	G	R170K	2nd	R	K
C/A	519	A	C	C	C	C	C	Q173K	1st	Q	K
C/T	*551*	*C*	*C*	*T*	*C*	*T*	*C*	*F183F*	*3rd*	*F*	*F*
G/A	552	G	A	G	G	G	A	V184I	1st	V	I
C/T	*584*	*C*	*C*	*C*	*T*	*C*	*C*	*H194H*	*3rd*	*H*	*H*

Table 8: Mutations observed on the exon 5 of the STEAP4 gene. The mutation originally observed by Linzmeier *et al.* is shaded. Synonymous mutations are italicized.

		Nucleotides						Amino Acids			
STEAP4		Browsers		Grazers							
Mutation	cDNA No.	Black	Sumatran	White	GOH	Horse	Human	Mutation	Codon	Ancestral	Mutations
G/A	*1169*	*G*	*A*	*G*	*G*	*G*	*G*	*L389L*	*3rd*	*L*	*L*
C/T	*1196*	*C*	*T*	*C*	*C*	*C*	*C*	*T398T*	*3rd*	*T*	*T*
C/T	*1205*	*C*	*C*	*C*	*T*	*T*	*C*	*Y401Y*	*3rd*	*Y*	*Y*
G/A	1225	G	G	A	G	G	G	S408N	2nd	S	N
T/C	1245	T	T	T	C	T	T	Y415H	1st	Y	H
C/T	*1262*	*C*	*T*	*C*	*C*	*C*	*C*	*Y420Y*	*3rd*	*Y*	*Y*
T/G	1300	G	T	T	T	T	T	I433S	2nd	I	S
A/G	*1322*	*G*	*A*	*G*	*A*	*A*	*A*	*P440P*	*3rd*	*P*	*P*
G/A	*1367*	*G*	*G*	*A*	*G*	*G*	*G*	*R455R*	*3rd*	*R*	*R*
G/A	*1388*	*A*	*G*	*G*	*G*	*G*	*-*	*S462S*	*3rd*	*S*	*S*

Table 9: Mutations observed on the exon 2 of the HFE gene. The mutation originally observed by Beutler *et al.* is shaded. Synonymous mutations are italicized. *C/T mutation is present in *Dicerorhinus sumatrensis sumatrensis*, but not found in *Dicerorhinus sumatrensis harrissoni*.

			Nucleotides					Amino Acids			
HFE		Browsers		Grazers							
Mutation	cDNA No.	Black	Sumatran	White	GOH	Horse	Human	Mutation	Codon	Ancestral	Mutations
C/T	*110*	*T*	*C*	*C*	*C*	*T*	*T*	*G36G*	*3rd*	*G*	*G*
C/T	*128*	*C*	*C*	*C*	*T*	*T*	*T*	*H42H*	*3rd*	*H*	*H*
C/T	*167*	*C*	*C*	*C*	*T*	*C*	*C*	*D55D*	*3rd*	*D*	*D*
C/G	*182*	*C*	*G*	*C*	*C*	*C*	*C*	*A60A*	*3rd*	*A*	*A*
* *C/T*	*191*	*C*	*C/T; all Dsh C*	*C*	*C*	*C*	*T*	*H63H*	*3rd*	*H*	*H*
G/A	*260*	*A*	*G*	*G*	*G*	*G*	*G*	*Q86Q*	*3rd*	*Q*	*Q*
G/C	265	C	G	G	G	G	G	S88T	2nd	S	T

Table 10: Mutations observed on exons 3 and 5 of the Slc40a1 gene. Synonymous mutations are italicized. *Sites were included because those are sites of hemochromatosis-linked mutations on the human genome.

			Nucleotides						Amino Acids			
Slc40a1		Browsers		Grazers							
Mutation	cDNA No.	Black	Sumatran	White	GOH	Horse	Human	Mutation	Codon	Ancestral	Mutations
Exon 3											
G/C	*547*	*G*	*G*	*G*	*A*	*G*	*G*	*R40R*	*3rd*	*R*	*R*
* C/C	657	C	C	C	C	C	C	none	cite of human mutation A77D		
A/C	*689*	*C*	*A*	*A*	*A*	*A*	*A*	*R88R*	*1st*	*R*	*R*
Exon 5											
C/G	840	C	C	C	G	C	C	T138S	2nd	T	S
* A/A	857	A	A	A	A	A	A	none	cite of human N144H mutation		

Table 11: Results from selection analysis. Tree length corresponds to number of substitutions. The dN/dS ratio represents non-synonymous versus synonymous mutations. The P values are the chi-squared distribution of the likelihood ratios from the two most conservative selection models (p=1 = neutral selection; p<1 = purifying selection; p>1 = positive selection)

Gene	**Number of sequences**	**Length of coding sequence**	**Tree length**	**dN/dS**	**P (M8 vs M7)**	**P (M8 vs M8a)**	**Positively selected site (M8)**
EPB41	45	201 (67 codons)	0.29099	0.64677	1.000	0.975	None
Slc28a2	44	138 (46 codons)	0.22323	0.24603	0.999	0.996	None
STEAP4	23	261 (87 codons)	0.25774	0.27484	1.000	1.000	None
HFE	45	219 (73 codons)	0.39477	0.09523	1.000	1.000	None

Figures

Figure 1: Summary of iron metabolism. Dietary iron is ingested and absorbed in through enterocytes in the intestines (A). The iron then travels to the bone marrow where it is incorporated with erythrocytes (B). The erythrocytes circulate iron throughout the body. Macrophages in the spleen recycle the iron (C) and the iron is then stored in tissues or in hepatocytes in the liver (D) or sent back to the bone marrow to be recirculated. (Adapted from Andrews 2008).

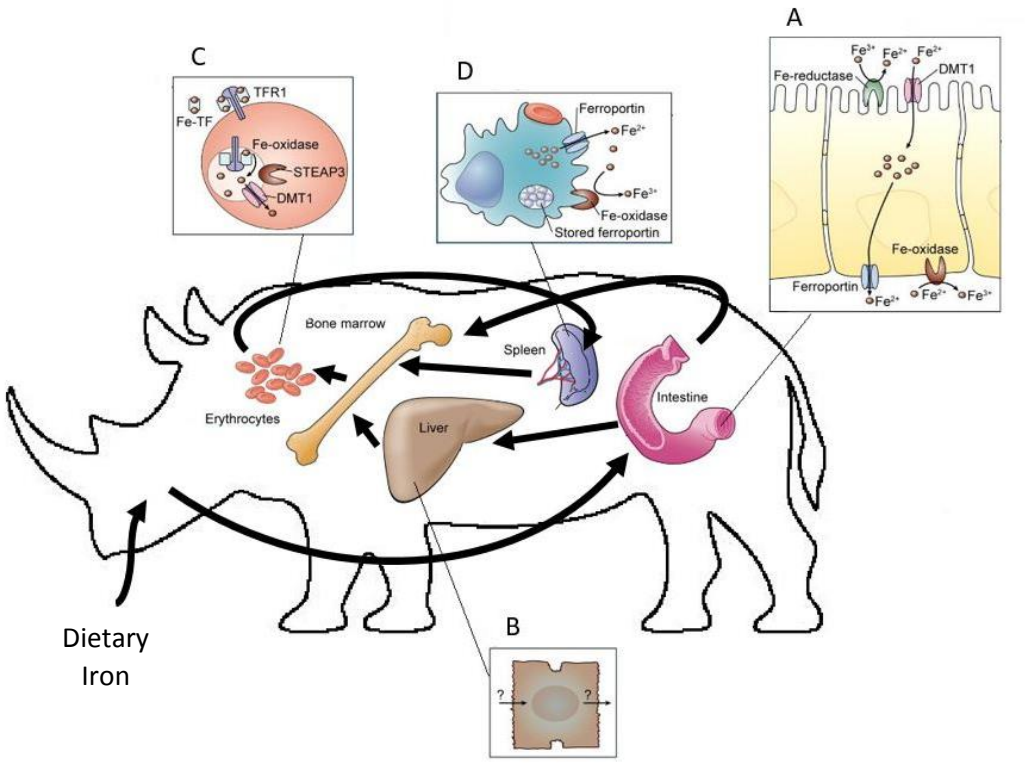

Figure 2: Derived mutations observed on the EPB41 gene. The highlighted derived mutations are present in all black rhinoceros subspecies.

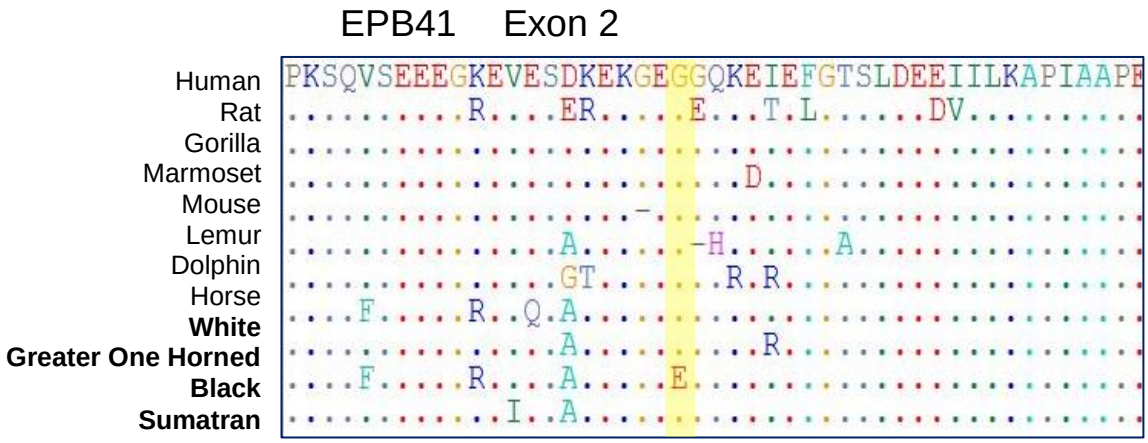

Figure 3: Derived mutations observed on the Slc28a2 gene. The highlighted derived mutations are present in all black rhinoceros subspecies.

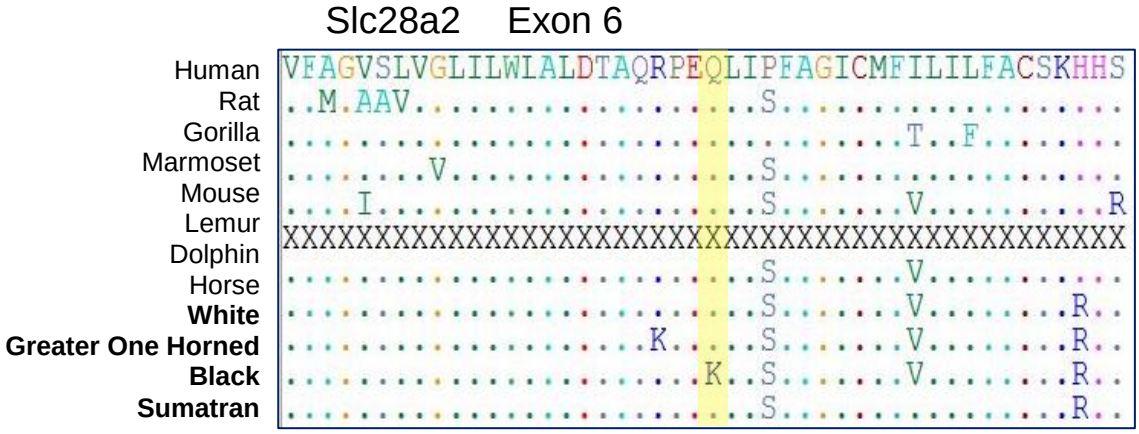

Figure 4: Derived mutations observed on the STEAP4 gene. The highlighted derived mutations are present in all black rhinoceros subspecies.

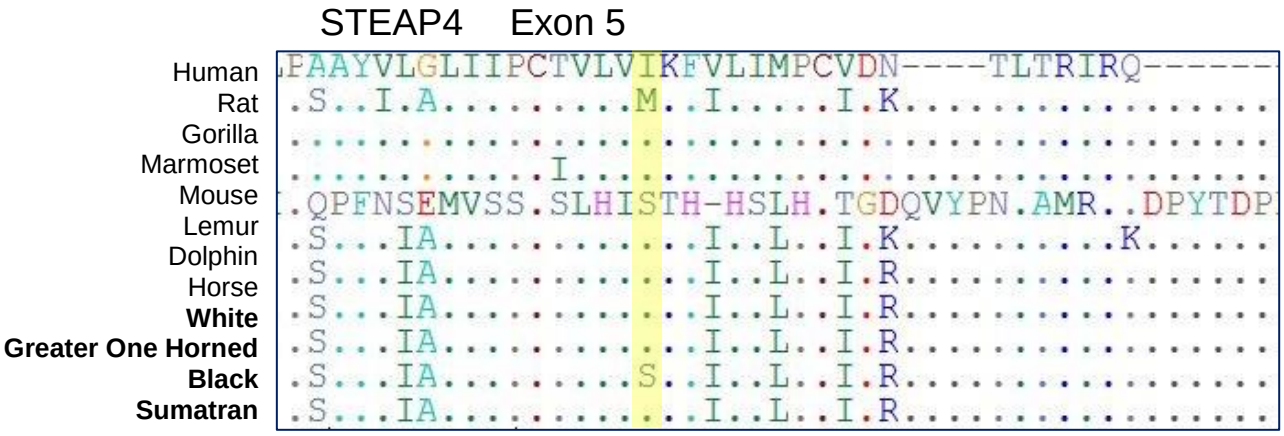

Figure 5: Derived mutations observed on the HFE gene. The highlighted derived mutations are present in all black rhinoceros subspecies.

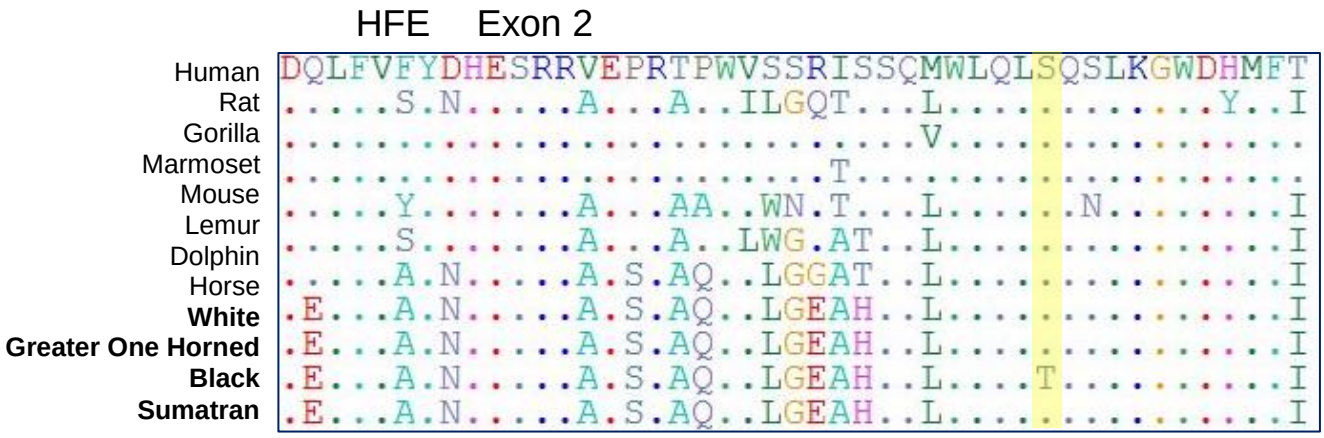

Figure 6: Derived mutations observed on the Slc40a1 gene. No mutations were present in all black rhinoceros subspecies.