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The *HFE* Gene of Browsing and Grazing Rhinoceroses: A Possible Site of Adaptation to a Low-Iron Diet

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ABSTRACT: When rhinoceros species that are browsers in the wild are fed in captivity they become iron overloaded. Presumably, their iron-absorptive mechanisms have evolved to become highly efficient. In humans, mutations of the *HFE* gene cause increased iron absorption. To determine whether the *HFE* gene of rhinoceroses has undergone mutation as an adaptive mechanism to improve iron absorption from iron-poor diets, we have sequenced the entire coding region of the *HFE* genes of four species of rhinoceros. Two of these were browsing species and two were grazing species. Although the *HFE* gene has been well preserved across species, numerous nucleotide differences were found between rhinoceros and human or mouse, some of which changed deduced amino acids. Of these mutations, only one found in the black rhinoceros appears to be a viable candidate mutation that might adversely affect HFE function. This mutation, S88T, is in a highly conserved region that is involved in the interaction between transferrin receptor and HFE.

INTRODUCTION

The regulation of body iron content is of critical importance. Too little iron results in iron deficiency anemia and depletion of some tissue enzymes, while too much iron produces hemochromatosis, a clinical syndrome characterized by cirrhosis, diabetes, bronzing of the skin and heart disease (1). Since there is no mechanism for actively excreting iron from the body, iron content is regulated by modulating absorption. Clearly, each species must adapt the efficiency of the absorptive mechanism to the content and chemical state of iron in its diet.

A number of animal species appear to develop iron overload in captivity (2–9). A striking example has recently been documented among rhinoceros species. Browsing species such as the African black rhinoceros (*Diceros bicornis*) and the Sumatran rhinoceros (*Dicerorhinus sumatrensis*), which have evolved in an environment where their diet consists largely of poor sources of iron, such as leaves and twigs become iron overloaded in captivity. In contrast, other species, such as the African white rhinoceros (*Ceratotherium simum*) and Indian rhinoceros (*Rhinoceros unicornis*), that have a diet that consists of grass from which iron is presumably more readily available, do not become iron overloaded in captivity (10–12).

In humans hereditary hemochromatosis is an autosomal recessive disorder that has long been known to be caused by mutation of a gene located in the HLA complex of chromosome 6. In 1996 this gene, *HFE* (13), was cloned. Three polymorphic mutations of *HFE* are known; two of these are clearly associated with the development of hemochromatosis (13–15) while the third appears, also, to result in increased iron accumulation (16, 17). The *HFE* knockout mouse also accumulates iron (18, 19). These

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findings indicate that *HFE* plays a role in the regulation of body iron content.

In the present study we sequenced parts of the introns and the entire coding region of the *HFE* genes of four rhinoceros species, two grazers and two browsers, to determine whether mutations in this gene were responsible for the higher iron uptake of the browsing species. By comparing the deduced amino acid substitutions found in the browser with sequences found in the mouse and human we conclude that it is possible that one of the *HFE* mutations identified could play a role in the increased iron accumulation that is found in one of these species.

MATERIALS AND METHODS

Cell culture and construction of a cDNA library. A primary fibroblast culture established from a skin biopsy of a black rhinoceros was obtained from Dr. Oliver Ruder, Zoological Society of San Diego. Total RNA was extracted from confluent fibroblast cultures and poly(A^+) RNA was purified on an oligo(dT) cellulose column (QuickPrep mRNA purification kit, Pharmacia, Piscataway, NJ). A cDNA library was constructed using the Librarian cDNA Library Construction System (InVitrogen, Carlsbad, CA). Briefly, cDNA was synthesized from mRNA using avian myeloblastosis reverse transcriptase primed with oligo (dT). cDNA was synthesized from RNase H-treated DNA/RNA duplexes using DNA polymerase I. After blunt end ligation of the cDNA to *Eco*RI linkers, the cDNAs were size-selected by agarose gel electrophoresis and packaged in the λ phage vector λgt10. The packaged library was amplified in the *E. coli* strain C600 *Hfl*.

Extraction of genomic DNA. Genomic DNA was prepared from an African black rhinoceros, an African white rhinoceros, an Indian rhinoceros and a Sumatran rhinoceros from either peripheral blood mononuclear cells isolated from heparinized blood samples by Ficoll–Hypaque gradient centrifugation or from tissues obtained during necropsy procedures at the San Diego Zoo and stored at −70°C. DNA was isolated by sodium dodecyl sulfate (SDS)–proteinase treatment overnight at 55°C, followed by phenol and chloroform extraction, ethanol precipitation, and resuspension in 10 mM Tris–HCl, pH 7.4, 0.1 mM EDTA.

Isolating rhinoceros HFE cDNA and gene fragments. Oligonucleotides 5′-gcctcagagcaggaccttg and 5′-cagtgagtctgcaggctgcgt were used to amplify the fragment of human *HFE* cDNA extending from nucleotide (nt) 85 in exon 2 to nt 1070 in exon 6 (just beyond the stop codon). This fragment was purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and 200 ng labeled with 100 μCi of [α-^{32}P]dATP (3000 C/mM) using the PCR primers and the Prime-It II kit, Stratagene (La Jolla, CA). A black rhinoceros *HFE* cDNA clone was isolated and purified by probing the rhinoceros cDNA library with this probe. Fifteen NZY plates each containing XL1-Blue infected with 50,000 pfu in a top agarose overlay were grown overnight at 37°C. Duplicate lifts were made on 0.45 micron Magna nylon transfer membranes (Osmonics Inc.), denatured in 0.5 N NaOH/1.5 M NaCl, neutralized with 0.5 M Tris–Cl, pH 7.5/1.5 M NaCl and UV crosslinked using a Stratolinker (Stratagene). The membranes were incubated at 42°C in 120 ml of hybridization mix containing 0.9 M NaCl, 50% formamide, 10% dextran sulfate, 1% SDS and 200 μg/ml salmon sperm DNA for 1 to 2 h. After adding 0.7 to 1.0 × 10^6 cpm per milliliter of the ^{32}P probe the incubation was continued overnight. The membranes were washed in three changes of 300 ml of 0.5× SSC, 0.1% SDS at 55°C for 15 min each and then in 300 ml of 0.2× SSC, 0.1% SDS at 55°C for 15 min. The membranes were visualized by exposure to XAR-2 X-ray film. The positive clones were plaque purified and phage DNA was isolated from a liquid culture using standard techniques (20).

Constructing genomic libraries. An African white rhinoceros genomic library was made by ligating a partial *Bam*HI digest of rhinoceros DNA into lambda Fix vector (Stratagene) following the Stratagene protocol. The library contained

TABLE 1

Comparison of the Structure of Human and Rhinoceros *HFE* Genes

	HFE intron size (nt)	
Intron No.	Human	Rhinoceros
1	3327	3116
2	209	203
3	1053	844
4	157	131
5	953	1000

approximately 0.25×10^6 primary clones. A more 5′ *HFE* fragment extending from the ATG in exon 1 to nt 591 in exon 3 was used to screen this library. The fragment was made by PCR of the human cDNA clone described above using a vector primer (pcalHC 5′) and an oligonucleotide in exon 3, 5′-cagctccagcaactgctgcag. The positive genomic clones isolated were plaque purified and phage DNA was prepared as described above.

Polymerase chain reactions (PCRs) contained 33.5 mM Tris–Cl, pH 8.8, 8.3 mM $(NH_4)_2SO_4$, 3.35 mM MgCl, 85 μg/ml BSA, 5% DMSO, 200 μM dNTPs, 250 ng of each oligonucleotide, 1.5 U *Taq* polymerase, and 0.5–1 μg genomic DNA or 100 pg of phage clone DNA per 100 μl of PCR mix. After denaturing for 4 min at 98°C, 30 cycles of PCR at 94°C, 30 s, 56–64°C, 30 s and 72°C, 0.5 to 2.5 min was carried out. Amplified fragments were purified for sequencing using QIAquick PCR purification kit, Qiagen.

Sequencing was carried out by a fluorescent-tagged dideoxy chain termination method using an ABI (Foster City, CA) Model 377 automated sequencer.

EXPERIMENTAL AND RESULTS

Intron sequences. An *HFE* clone, 1816 nucleotides in length was isolated from the black rhinoceros cDNA library. It had 87.5% identity with the human *HFE* nucleotide sequence. When the sequence of adjacent exons in the cDNA clone was obtained, primers were made to amplify the intervening sequences from rhinoceros genomic DNA. The black rhinoceros cDNA library was rescreened with a more 5′ human *HFE* probe but no positive clones were found. The white rhinoceros genomic library was then screened with the 5′ human *HFE* probe and 5 positive clones were isolated; the inserts all appeared to be the same size, about 9 kb. By using a reverse primer in exon 3 to sequence the phage clone DNA and making new primers from the sequence obtained, the remainder of the rhinoceros *HFE* intron and exon sequence was obtained. The exon sequences of the four species have been deposited in GenBank (Accession No. AY007541–AY007544); the sequences of exon and intron 1 of the white rhinoceros and of exons and introns 2–6 of the black rhinoceros are deposited in GenBank with Accession Nos. AF301581 and AF301582. The intron sizes are listed and compared to those of the human in Table 1. Differences between species in the intron sequences are summarized in the appendix.

Coding regions. The rhinoceros *HFE* intron sequence was then used to design intronic primers so that the *HFE* exons of the various rhinoceros species could be amplified, sequenced and compared. Table 2 lists the primers used to PCR

TABLE 2

Oligonucleotide Primers Used to Amplify Rhinoceros *HFE* Exons

	Sense primer (5′–3′)	Antisense primer (5′–3′)	Fragment size (nt)
Exon 1	gatcccactggccaggaag	gcatcccagtgcccggag	382
Exon 2	ttgattcagaaggtatgtggag	agggaccgaatgacctcaga	485
Exon 3	aggagtctgaggtcattcgg	ggattctgctactctgatcttg	769
Exon 4/5	agcttgctttgtctgaacagg	aagagaagacttctcatggatg	768
Exon 6	gtgatcagggttgagacgag	gtccttagcataacttaacgtag	356

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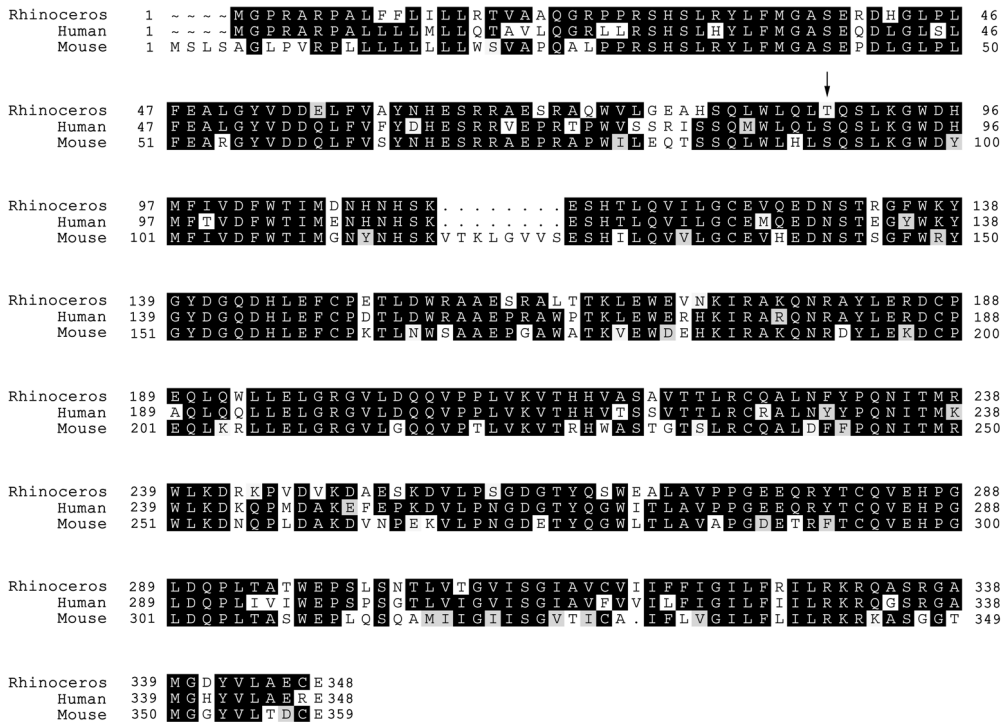

FIG. 1. Comparison of the amino acid sequences of the African black rhinoceros, human, and mouse. The arrow indicates the position of the amino acid change that may have functional significance.

amplify and sequence the *HFE* exons of the four rhinoceros species. The amino acid sequence of the black rhinoceros is compared with that of the mouse and the human in Fig. 1.

The nucleotide differences in the *HFE* coding region between the 4 rhinoceros species are listed in Table 3 and the deduced amino acids (AA) are compared with human and mouse AA at the same position. Rhinoceros and human *HFE* coding regions are the same length, 1047 nt, but the mouse is 33 nt or 11 AA longer. The African black rhinoceros HFE nt sequence has an 85% identity with the human sequence in the coding region by fasta analysis; the AA identity is 77.9%.

Sequence variability between rhinoceros species. There were 27 exonic nucleotides at which the four rhinoceros species are not identical. Twenty-one of these produced no coding change. Although occasionally such noncoding changes may be biologically significant, this is very rarely the case, and for this reason only the six mutations that produced an amino acid change are considered further here. Five of these substitutions were in an amino acid that is not conserved between human and mouse, implying that in these positions a specific amino acid is not required for normal function of the gene; these regions of the protein are somewhat permissive with respect to which amino acid is present. The glycine to methionine change at amino acid 137, the valine to methionine change in amino acid 246, and the alanine to glycine at amino acid 345 were found in a grazing species; defects in the *HFE* gene would be expected in the browsing species, in which iron absorption would need to be upregulated. In the case of the mutations at AA 268 and 321 the two genotypes were found both in browsers and grazers. The only change in a conserved amino acid was the mutation that caused substitution of a threonine for serine-88 in the HFE molecule. This mutation occurred in one browsing species, the Black African, but not in the other (Sumatran) and was not found in either of the two grazing species. This rather conservative amino acid substitution occurs in a 52-

TABLE 3

Coding Region Differences between Rhinoceros Species

				Nucleotides						
				Browsers		Grazers		Amino acids		
	Mutation	cDNA No.	Genomic No.	Black African	Sumatran	White African	Indian	Rhino	Human	Mouse
Exon 1	C → G	27	27	C/C	G/G	C/C	C/C	L9L	L	L
	G → A	48	48	G/G	G/A	G/G	G/G	R16R	Q	W
	A → G	72	72	A/A	G/G	A/A	G/G	P24P	L	P
Exon 2	T → C	108	3224	T/T	C/C	C/C	C/C	G36G	G	G
	C → T	126	3242	C/C	C/C	C/C	T/T	H42H	L	L
	C → T	165	3281	C/C	C/C	C/C	T/T	D55D	D	D
	C → G	180	3296	C/C	G/G	C/C	C/C	A60A	F	S
	C → T	189	3305	C/C	C/T	C/C	C/C	H63H	H	H
	A → G	258	3374	A/A	G/G	G/G	G/G	Q86Q	Q	H
	G → C	263	3379	C/C	G/G	G/G	G/G	S88T	S	S
Exon 3	T → C	393	3712	T/T	C/C	T/T	C/C	S131S	S	S
	T → G	402	3721	T/T	G/G	T/T	G/G	G134G	G	G
	A → T	410	3729	A/A	A/A	A/A	T/T	K137M	K	R
	T → C	423	3742	T/T	T/T	T/T	C/C	D141D	D	D
	C → G	486	3805	C/C	G/G	C/C	C/C	A162A	A	A
	G → A	555	3874	G/G	A/A	G/G	G/G	R185R	R	K
	T → G	573	3892	T/T	G/G	G/G	G/G	L191L	L	L
Exon 4	G → A	736	4899	G/G	G/G	A/A	G/G	V246M	M	L
	C → T	783	4946	C/C	T/T	C/C	T/T	D261D	D	D
	A → T	803	4966	A/A	T/T	A/A	T/T	E268V	I	L
	C → T	813	4976	C/C	C/C	C/C	T/T	A271A	A	A
	T → C	822	4985	T/T	T/T	T/T	C/C	P274P	P	P
	C → A	876	5039	C/C	C/C	A/A	C/C	P292P	P	P
Exon 5	C → T	915	5209	C/C	T/T	C/C	C/C	L305L	L	M
	T → C	961	5255	T/T	C/C	T/T	C/C	F321L	F	[a]
	A → G	1005	5299	A/A	G/G	A/A	A/A	S335S	S	S
Exon 6	C → G	1034	6328	C/C	C/C	G/G	C/C	A345G	A	T

[a] The best fit between human and mouse HFE amino acids shows a gap in this position.

residue region of the protein sequence (amino acids 81–132) in which with one exception, every amino acid in the rhinoceros sequence is the same as either mouse or human or both (Fig. 1). It seems possible that a substitution in this highly conserved region may have an effect on the function of the HFE protein. The mutation is located on the α_1 helix of the HFE protein (21) that is a key interaction site for the transferrin receptor (TfR) (22), but there is no direct contact between S88 and TfR (Fig. 2). However, nearby residues Q89 and L85 have van der Waals interactions with TfR residues T657 and S654, and Q86 forms two hydrogen bonds with T658. S88 makes van der Waals contact with neighboring K92 and V68 in the loop preceding the α_1 helix, and a hydrogen bond from its side-chain hydroxyl group to the carbonyl oxygen of W84. Threonine at position 88 does not contribute additional hydrogen bonding potential, but does occupy a greater volume than serine due to the extra methyl group. The additional space required by threonine may alter the close interactions of surrounding residues K92, V68, and W84 resulting in subtle changes in the local α_1 helix or nearby loop structures of HFE. Variations in the HFE structure at TfR contact sites can affect the kinetics and thermodynamics of complex formation and may attenuate the ability of HFE to inhibit the TfR–Tf interaction (23). If so, it could represent an adaptive change in the low iron environment in which

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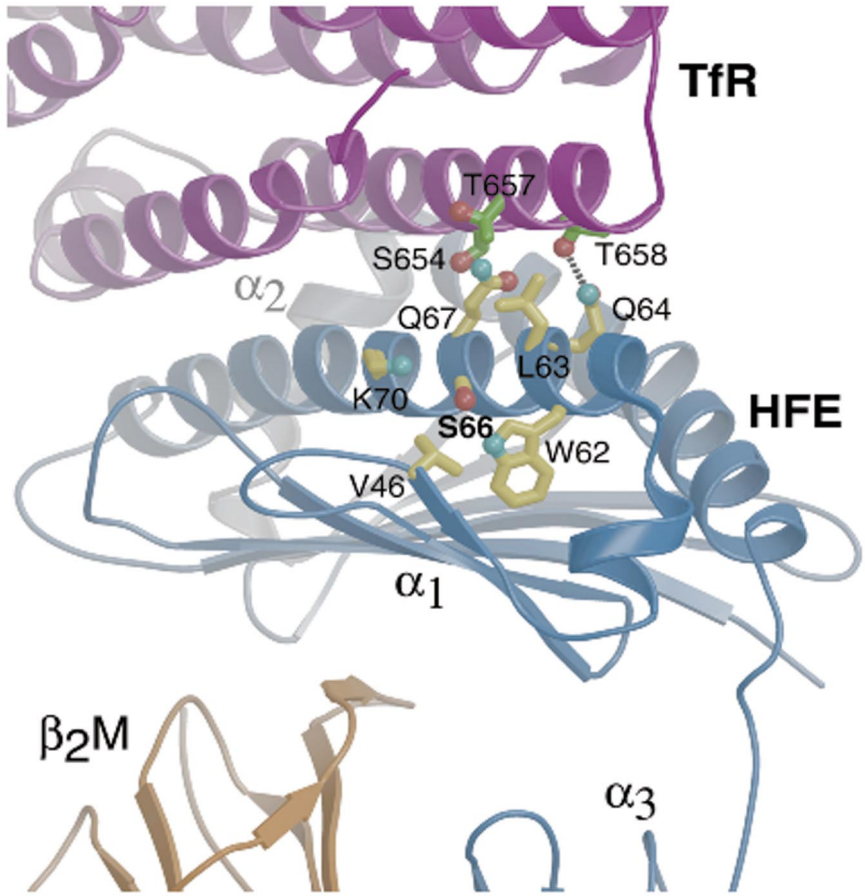

FIG. 2. Location of the serine-88 to threonine mutation of black African rhinoceros HFE from the analysis of the crystal structure of the complex between human HFE (blue) and TfR (magenta) [PDB access code 1de4 (22)]. Residue numbers are from the atomic coordinates numbered according to the processed protein; hence, S88, the mutation found in the black African rhinoceros, is designated S66, and the numbers of the other amino acids are also 22 less than those in the processed protein as shown in the text and tables.

the black African rhinoceros evolved. The change is not present in the other browsing species tested, the Sumatran rhinoceros, and it is likely that in that species, and in the black African rhinoceros as well, other proteins may play a role in upregulating iron absorption.

APPENDIX

Nucleotide Differences in Intervening Sequences between Rhinoceros Species

				Browsers		Grazers	
Intron location		Mutation	Genomic No.	African Black	Sumatran	African White	Indian
Upstream of the ATG							
	−85 to −1	a/g	−82	a/a	g/g	a/a	g/g
		c/t	−56	c/c	t/t	c/c	c/c
		a/g	−32	g/g	a/a	g/g	a/a
Intron 1, 5′	77 to 166						
		t/c	84	t/t	c/c	t/t	c/c
		c/a	108	c/c	c/c	c/c	c/a
		c/t	147	c/c	c/c	c/c	t/t

APPENDIX—*Continued*

Intron location		Mutation	Genomic No.	Browsers		Grazers	
				African Black	Sumatran	African White	Indian
		c/t	149	c/c	t/t	c/c	c/c
Intron 1, 3′	3135 to 3192	t/c	3139	t/t	t/t	t/t	c/c
		c del	3150–3153	ccc/ccc	cccc/cccc	cccc/cccc	cccc/cccc
		gcctcc insert	After 3139	No insert	gcctcc	No insert	No insert
		c/t	3154	c/t	c/c	c/c	t/t
		c/g	3155	g/g	c/c	c/c	c/c
		g/a	3166	g/g	g/g	g/g	a/a
		c/t	3187	c/c	c/c	c/c	t/t
Intron 2	3457 to 3649	c/a	3465	c/c	a/a	c/c	a/a
		g/a	3469	g/g	g/g	a/a	g/g
		t/c	3479	t/t	c/c	t/t	c/c
		g/a	3486	g/g	a/a	g/g	a/a
		a/g	3513	a/a	a/a	g/g	a/a
		g/a	3525	g/g	a/a	g/g	g/g
		a/g	3539	a/a	g/g	g/g	g/g
		g/t	3558	g/g	t/t	g/g	g/g
		a/g	3561	a/a	a/a	a/a	g/g
		c/t	3595	c/c	c/c	c/c	t/t
		c/t	3617	c/c	c/c	t/t	c/c
		a/c	3618	a/a	c/c	c/c	c/c
Intron 3, 5′	3936 to 4100	t/g	3953	t/t	t/t	t/t	g/g
		c/t	3964	c/c	t/t	c/c	c/c
		g insert	After 3970	None	g/g	None	g/g
		g/t	3993	g/g	g/g	g/g	t/t
		g/a	3999	g/g	a/a	g/g	g/g
		acc del	4029–4031	acc	acc del	acc del	acc del
		c/t	4075	c/c	c/c	t/t	c/c
		g/c	4086	g/g	g/g	g/g	c/c
		t/c	4095	t/t	c/c	c/c	c/c
Intron 3, 3′	4730 to 4779	c/t	4771	c/c	c/c	c/c	t/t
Intron 4	5056 to 5186	t/c	5090	t/t	t/t	t/t	c/c
		a/g	5100	a/a	a/a	a/a	g/g
		c/t	5114	c/c	t/t	c/c	c/c
		t/a	5147	t/t	a/a	t/t	t/t
		t/c	5150	t/t	c/c	c/c	c/c
		g/a	5154	g/g	g/g	g/g	a/a
Intron 5, 5′	5301 to 5400	a/g	5311	a/a	g/g	a/a	a/a
		t/c	5336	t/t	c/c	t/t	c/c
		t/c	5360	t/t	c/c	c/c	c/c
		t/a	5362	t/t	a/a	t/t	t/t
		t/c	5364	t/t	c/c	c/c	c/c
		c/g	5367	c/c	g/g	c/c	g/g
		a/g	5388	a/a	g/g	a/a	a/a
		a/g	5393	a/a	g/g	g/g	g/g
Intron 5, 3′	6217 to 6300	g/a	6231	g/g	g/g	a/a	g/g
		a/g	6238	a/a	g/g	a/a	a/a
		g/c	6239	g/g	g/g	g/g	c/c
		c/a	6253	c/c	a/a	a/a	a/a
		g/a	6271	g/g	g/g	g/g	a/a
Intron 6	6342 to 6529	c/a	6342	c/c	a/a	c/c	a/a
		g/c	6357	g/g	c/c	c/c	c/c
		t/c	6404	t/t	t/t	t/t	g/g
		c/t	6406	c/c	t/t	c/c	t/t
		a/g	6410	a/a	g/g	a/a	g/g

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APPENDIX—*Continued*

Intron location	Mutation	Genomic No.	Browsers		Grazers	
			African Black	Sumatran	African White	Indian
	t/c	6416	t/t	c/c	t/t	c/c
	g del	6471	g/g	g/g	g/g	g del
	c/t	6505	c/c	t/t	c/c	t/t
	c/t/g	6525	c/c	t/t	g/g	g/g

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