Journal of Heredity 2007:98(6):555–566
doi:10.1093/jhered/esm072
Advance Access publication September 1, 2007

Four Independent Mutations in the Feline *Fibroblast Growth Factor 5* Gene Determine the Long-Haired Phenotype in Domestic Cats

James S. Kehler, Victor A. David, Alejandro A. Schäffer, Kristina Bajema, Eduardo Eizirik, David K. Ryugo, Steven S. Hannah, Stephen J. O'Brien, and Marilyn Menotti-Raymond

From the Laboratory of Genomic Diversity, National Cancer Institute-Frederick, Frederick, MD 21702 (Kehler, David, Bajema, O'Brien, and Menotti-Raymond); Center for Animal Transgenesis and Germ Cell Research, Room 102, Myrin Building, School of Veterinary Medicine, University of Pennsylvania, Kennett Square, PA 19348 (Kehler); Department of Health and Human Services, National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD 20894 (Schäffer); Centro de Biologia Genômica e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS 90619-900, Brazil (Eizirik); Department of Otolaryngology and Neuroscience, 720 Rutland Avenue, Johns Hopkins University, Baltimore, MD 21205 (Ryugo); and Nestlé-Purina PetCare Company, St. Louis, MO 63134 (Hannah).

Address correspondence to J. S. Kehler at the address above, or e-mail: jkehler@ncifcrf.gov.

Abstract

To determine the genetic regulation of "hair length" in the domestic cat, a whole-genome scan was performed in a multigenerational pedigree in which the "long-haired" phenotype was segregating. The 2 markers that demonstrated the greatest linkage to the long-haired trait (log of the odds ≥ 6) flanked an estimated 10-Mb region on cat chromosome B1 containing the *Fibroblast Growth Factor 5 (FGF5)* gene, a candidate gene implicated in regulating hair follicle growth cycle in other species. Sequence analyses of *FGF5* in 26 cat breeds and 2 pedigrees of nonbreed cats revealed 4 separate mutations predicted to disrupt the biological activity of the FGF5 protein. Pedigree analyses demonstrated that different combinations of paired mutant *FGF5* alleles segregated with the long-haired phenotype in an autosomal recessive manner. Association analyses of more than 380 genotyped breed and nonbreed cats were consistent with mutations in the *FGF5* gene causing the long-haired phenotype in an autosomal recessive manner. In combination, these genomic approaches demonstrated that *FGF5* is the major genetic determinant of hair length in the domestic cat.

The hair follicle provides a unique to mammalian model in which to study the complex genetic regulation between stem and stromal cells during self-renewal and terminal differentiation of a tissue. Genetic modulation of the hair follicle cycle can affect hair length, providing a rapid means for significant phenotypic change under either artificial or natural selection. The large variety of cat breeds with different hair textures and lengths provides a potential wealth of mammalian models with spontaneous mutations at unknown loci affecting hair follicle structure and function (Vella and Robinson 1999). Discovery and comparison of mutations in orthologous genes between mammals can provide additional understanding about the conserved domains that are required for protein function.

The initial molecular studies of the "long hair" locus were done in mice. Breeding experiments of spontaneously occurring "long-haired" Angora mice demonstrated that the *go* locus was the major determinant of "hair length" in the mouse and that the long-haired phenotype was inherited in an autosomal recessive manner (Dickie 1963; Pennycuik and Raphael 1984). Subsequent targeted mutation of the *fibroblast growth factor 5 (Fgf5)* gene and crossbreeding experiments between *Fgf5* knockout ($Fgf5^{neo}$) and Angora mice demonstrated that $Fgf5^{neo}$ and *go* represented null alleles of the same locus (Hébert et al. 1994).

FGF5 was originally identified as a human oncogene (Zhan et al. 1987), belonging to a family of 23 related FGF genes (For a review, see Katoh 2002; Katoh and Katoh

2005). FGF5 shares a β-trefoil superfold structure composed of 5 β-hairpin folds (that is required for receptor binding) and an N-terminal, signal peptide (that is required for paracrine secretion) with the prototypical members, FGF1 and 2 (Ornitz and Itoh 2001). During embryonic and fetal development of the mouse, *Fgf5* is first expressed in the extraembryonic ectoderm of the epiblast and then restricted to differentiating myotomes, skeletal muscles, and neurons (Haub and Goldfarb 1991; Hébert et al. 1991). The orthologous human FGF5 gene product is overexpressed in some mammary, prostatic, and renal carcinomas and can be presented on their major histocompatibility complex (MHC) class I receptors after intracellular processing, providing a potential antigenic target for cancer immunotherapy (Vigneron et al. 2004). However, experimental overexpression of FGF5 has been used to induce angiogenesis in the myocardium and to promote photoreceptor survival in animal models without inducing tumors in situ (Giordano et al. 1996; Green et al. 2001). In the adult mouse, FGF5 is normally expressed in neurons in most regions of the brain, in pancreatic β-islet cells involved in glucose-homeostasis, and in hair follicles in the skin (Haub et al. 1990; Gómez-Pinilla and Cotman 1993; Hart et al. 2000).

During the normal, cyclic hair growth of adult mice, expression of *Fgf5* is restricted to cells in the lower third of the outer root sheath (ORS) and in the inner root sheath at the base of the follicle during the anagen phase just prior to progression into the catagen phase (Hébert et al. 1994). Two major *Fgf5* isoforms have been described in the mouse (Suzuki et al. 2000); the full-length *Fgf5* mRNA results from transcription of all 3 exons, while splicing of exon 1 to an alternative acceptor site in exon 3 introduces a frameshift producing a shorter transcript (*Fgf5S*) that lacks exon 2 and most of exon 3. Both the FGF5 and FGF5S gene products bind primarily to the FGF receptors 1 and 2, Isoforms c (FGFR1c and 2c) (Ornitz et al. 1996) found on dermal papilla cells (DPCs) (Clements et al. 1993; Rosenquist and Martin 1996). However, the full-length FGF5 actively inhibits DPC stimulation of ORS cell proliferation and synthesis of hair fibers during anagen (Limat et al. 1993; Suzuki et al. 2000), thereby triggering catagen. In contrast, FGF5S antagonizes the inhibitory effects of FGF5, providing a degree of autoregulatory modulation (Ota et al. 2002). The overall effect of the absence of both isoforms in *Fgf5-null* mice is that the anagen stage is prolonged, resulting in longer hair (Hébert et al. 1994). However, even in these mice, the hair follicles do eventually proceed into the catagen phase, indicating the presence of additional signaling pathways (reviewed by Paus and Foitzik 2004). Comparative studies of *Fgf5* orthologs in other mammalian species exhibiting variation in hair length may reveal novel spontaneous mutations identifying key domains that influence protein function.

The inheritance of long hair has been documented as a recessive trait in other mammalian species including rabbits (Fraser 1953), dogs (Burns and Fraser 1963), and cats (Vella and Robinson 1999). While we were pursuing our study of the involvement of the *FGF5* locus in the control of hair length in cats, another group demonstrated that mutations in the canine *FGF5* gene explained the long-haired phenotype observed in multiple dog breeds (Housley and Venta 2006). Similar to dogs, the foundation of cat breed groups committed to the propagation of specific phenotypic traits resulting from spontaneous mutations has provided opportunities to study the genetic basis of several traits. Coat lengths and colors can be specified as part of a breed standard allowing for mutations carried by founders to become fixed within these closed breeding populations.

The large variety of breeds containing potential mutations affecting hair follicle structure and function from the hairless Sphynx to the kinked-haired Cornish, Devon, and Selkirk Rexes to the long-haired breeds such as Maine Coon, Persian, and Turkish Angora cats provides a wealth of animal models for understanding the complex gene regulation of the hair growth cycle. In this study, we have performed a genetic survey of "long-" and "short-haired" breeds, as well as genetic linkage and pedigree analyses of nonbreed cats to interrogate the influence of the *FGF5* locus on hair length in domestic cats.

Materials and Methods

Collection of DNA Samples and Phenotypic Data from Breed and Nonbreed Cats

Whole blood preserved with ethylenediaminetetraacetic acid (EDTA) or cheek swab samples were taken from cats for DNA extraction using a salt-precipitation/column-filtration kit (Qiagen, Valencia, CA). Samples were obtained from 119 unrelated cats with the permission of private owners, from 12 "short-haired" breeds (Abyssinian, American Shorthair, British Shorthair, Burmese, Chartreux, Cornish Rex, Devon Rex, Egyptian Mau, Havana, Ocicat, Russian Blue, and Siamese), 12 long-haired breeds (Angora, Balinese, Birman, Himalayan, Maine Coon, Norwegian Forest Cat, Persian, Ragdoll, Siberian, Somali, Turkish Angora, and Turkish Van), and 2 breeds (Manx and Scottish Fold) that currently maintain separate short- and long-haired registries within the Cat Fanciers Association (CFA). Private owners reported the phenotypes of their cats as either short- or long-haired in accordance with breed standards and provided photographs in most cases. All breed cats were assigned an anonymous registry (Fc) number, and phenotypic data were recorded in a database at the Laboratory of Genomic Diversity to preserve the anonymity of individual cats and their owners.

Samples were obtained from 261 related cats that were used for nutrition studies at the Nestlé-Purina PetCare Company in St. Louis, MO, and 50 related White Deaf cats that were used for hearing studies at Johns Hopkins University in Baltimore, MD, with the approval of their respective Internal Animal Care and Use Committees. Both colonies were established from random-bred, short- and long-haired cats obtained from commercial vendors and were maintained as closed colonies through planned matings. Because these cats were no longer being randomly mated or out or crossbred, they were defined in this study as "nonbreed" cats to distinguish them from the registered breed cats. As in breed cats, the hair length was

Table 1. Linkage analysis of the long-haired trait in the cat to *FGF5*

Marker	LOD to long haired[a]	θ[b]	Cat RH map position[c]	Cat chromosome number[d]	FCA start
FCA212	3.0	0.07	1188.1	B1	127, 108, 235
FCA074	3.3	0.09	1233.1	B1	133, 650, 025
FCA1144	4.5	0.07	ND	B1	135, 390, 194
FCA612	4.6	0.04	1276.7	B1	139, 688, 359
FCA824	9.5	0.03	1356.2	B1	156, 292, 359
FGF5[e]	(11.6)	(0.00)	ND	B1	158, 450, 684
FCA823	6.3	0.06	1476.2	B1	166, 387, 690

[a] LOD scores were calculated using 513 microsatellite markers typed on 135 potentially informative meioses with the allele associated with the long-haired phenotype assumed to have a frequency of 0.25. LOD scores were higher by at most 0.6 LOD units if an allele frequency of 0.50 was used.

[b] Estimates of the optimal recombination fractions differed at most by 0.01, depending upon the allele frequencies assumed.

[c] Assignment of microsatellite positions within a cat radiation hybrid (RH) map (Murphy et al. [2007]). ND indicates positions not determined in the cat RH map.

[d] Assignment of microsatellite positions to cat chromosome B1 contiguous sequences (contigs) in the 2× feline genome database using the Garfield Cat Genome Browser. *Felis catus* (FCA) start indicates sequences corresponding to the contig start sequences.

[e] Results of subsequent linkage analyses for the *FGF5* locus are presented in parentheses, after genotyping the 3 predicted mutations in the *FGF*5 CDS found within this pedigree.

sufficiently different to qualitatively phenotype nonbreed cats as either short or long haired. Photographs were used to confirm the reported phenotypes, but direct measurements of hair length were not taken. All nonbreed cats in this study were maintained in facilities inspected by the US Department of Agriculture, under conditions established by the American Association of Laboratory Animal Care in compliance with the federal Animal Welfare Act.

Whole-Genome Scan

The long-haired trait segregated in the Nestlé-Purina, multigenerational pedigree of nonbreed domestic cats (Eizirik et al. 2003). In conjunction with the development of a third generation, genetic linkage map, we genotyped 261 cats in this pedigree for 483 autosomal and 30 X-linked (513 total) microsatellite markers, using amplification conditions and analyses as previously described (Ishida et al. 2006).

Genetic Linkage Analysis

The long-haired phenotype was modeled as a binary trait with fully penetrant, autosomal recessive inheritance, and log of the odds (LOD) scores were computed using the Superlink software package (Fishelson and Geiger 2002, 2004). For the LOD scores shown in Table 1, the allele associated with the long-haired phenotype was assumed to have a frequency of 0.25 with all other markers of equal allele frequencies. If the trait allele frequency of 0.50 was used, peak LOD scores of all the linked markers were higher by at most 0.6 LOD units, and estimates of the optimal recombination fraction (θ) differed by at most 0.01.

PCR Amplification and DNA Sequence Analysis of *FGF5*

PCR primers were designed in the introns flanking the 3 exons of *FGF5* (Supplementary Figure A1 and Table A1). Primers flanking exon 1 were designed from domestic cat DNA sequence that was detected in the cat 2× whole genome by cross-species MegaBlast (Zhang et al. 2000) (http://www.ncbi.nlm.nih.gov/BLAST/tracemb.shtml). The forward primer for exon 2 was anchored in a region identical in both dog and cow, and the reverse primer was a consensus sequence between dog and cow and contained 2 degenerate oligonucleotide positions. The primers for exon 3 were derived from the dog DNA sequence from a region highly conserved between dog and human. Touchdown polymerase chain reaction (PCR) and DNA sequencing were performed as previously described (Guo et al. 2006; Ishida et al. 2006).

PCR–restriction fragment length polymorphism Genotyping Assay Development

Three fluorescence-based assays were developed to genotype the 4 *FGF5* mutations detected (Supplementary Table A2). Separate assays were developed to detect Mutation 1 (c.ins356T) and 2 (c.C>T406), and a third assay was designed which could detect both the adjacent Mutations 3 (c.del474T) and 4 (c.A>C475), as well as determine the phase of the 2 mutations. In all 3 assays, a fluorescent dye was incorporated into the PCR product (Boutin-Ganache et al. 2001) modified by attaching the −21M13F primer sequence (TGTAAAACGACGGCCAGT) to the 5′ end of one of the primers. PCR reactions and product detection were performed as previously described (Guo et al. 2006). For Mutation 1 (c.ins356T), PCR primers were designed flanking the mutation as the 2 alleles could be discriminated by their 1-bp size difference. For the other 2 assays, PCR–restriction fragment length polymorphism (RFLP) approaches were developed. Oligonucleotide substitutions were made in one of the primers such that a restriction site was generated that would recognize one of the alleles at each mutation. Additionally, the restriction enzyme recognition sequence and a pig-tail sequence (gtgtctt) were appended to the 5′ end of the primer adjacent to the

Table 2. DNA sequence analysis of *FGF5* in 50 breed cats with known hair length identifies 4 putative mutations associated with long hair

						Mutation 1	Mutation 2	Mutations 3, 4[a]				
		Polymorphism[b]	R51S	V61D	P65H	ins356T	R136X	del474T, T159P	N195N	Q223Q	L265L	
		Nucleotide position[c]	153	182	194	356	406	474, 475	585	669	795	
		Alleles[d]	G:T	T:A	C:A	−:+	C:**T**	T:**del474T**, A:**C**	C:T	G:A	G:C	Genotype[e]
Cat breed	Cat number	Hair length										
Abyssinian	Fc640	Short	G	T	C	–	C	A	C	A	G	N,N
Abyssinian	Fc653	Short	G	T	C	–	C	A	C	A	G	N,N
American Shorthair	Fc330	Short	G	TA	C	–	C	A	C	GA	CG	N,N
American Shorthair	Fc2387	Short	G	T	CA	–	C	A	C	A	C	N,N
British Shorthair	Fc2588	Short	G	A	C	–	C	A	C	A	G	N,N
British Shorthair	Fc2593	Short	G	T	A	–	C	**C**A	CT	G	G	N,4
British Shorthair	Fc2596	Short	G	TA	CA	–	C	A	CT	GA	G	N,N
Burmese	Fc305	Short	G	T	A	–	C	A	C	G	G	N,N
Burmese	Fc488	Short	G	T	A	–	C	A	C	G	G	N,N
Chartreux	Fc2505	Short	G	T	C	–	C	A	C	G	C	N,N
Chartreux	Fc2698	Short	G	T	A	–	C	A	C	GA	CG	N,N
Cornish rex	Fc2478	Short	G	T	C	–	C	A	C	G	C	N,N
Devon Rex	Fc1455	Short	G	T	CA	–	C	**C**A	C	G	C	N,4
Egyptian Mau	Fc523	Short	G	TA	C	–	C	A	C	GA	G	N,N
Egyptian Mau	Fc1671	Short	G	T	C	–	C	A	C	G	CG	N,N
Havana	Fc733	Short	T	T	C	–	C	A	C	G	C	N,N
Havana	Fc2591	Short	G	T	CA	–	C	A	C	GA	CG	N,N
Manx	Fc2813	Short	G	T	CA	–	C	**C**A	C	G	C	N,4
Ocicat	Fc2386	Short	G	TA	CA	–	C	A	C	GA	G	N,N
Ocicat	Fc2919	Short	G	T	A	–	C	A	C	G	G	N,N
Russian Blue	Fc1143	Short	G	A	C	–	C	A	C	A	G	N,N
Russian Blue	Fc2659	Short	G	TA	CA	–	C	A	CT	G	CG	N,N
Scottish Fold	Fc2536	Short	G	T	A	–	C	**C**A	C	GA	CG	N,4
Scottish Fold	Fc2843	Short	G	TA	C	–	C	**C**A	C	GA	CG	N,4
Siamese	Fc2735	Short	G	T	C	–	C	A	C	GA	G	N,N
Birman	Fc1916	Long	G	T	A	–	C	**C**	C	G	C	4,4
Birman	Fc2349	Long	G	T	A	–	C	**C**	C	G	C	4,4
Birman	Fc2569	Long	G	T	A	–	C	**C**	C	G	C	4,4
Maine Coon	Fc1231	Long	G	T	A	–	C	**del474T/C**	CT	G	CG	3,4
Maine Coon	Fc2325	Long	G	T	A	–	C	**C**	C	G	C	4,4
Maine Coon	Fc2586	Long	G	T	A	–	C	**del474T/C**	CT	G	CG	3,4
Maine Coon	Fc2608	Long	G	T	A	–	C	**del474T**	T	G	G	3,3
Norwegian Forest Cat	Fc1914	Long	G	A	C	–	**T**	A	C	A	G	2,2
Norwegian Forest Cat	Fc2086	Long	G	TA	CA	–	C**T**	**C**A	C	GA	CG	2,4
Norwegian Forest Cat	Fc2587	Long	G	A	C	–	**T**	A	C	A	G	2,2

Norwegian Forest Cat	Fc2598	Long	G	A	C	–	**T**	A	C	A	G	2,2
Persian	Fc1166	Long	G	T	A	–	C	**C**	C	G	C	4,4
Persian	Fc1931	Long	G	T	A	–	C	**C**	C	G	C	4,4
Persian	Fc2064	Long	G	T	A	–	C	**C**	C	G	C	4,4
Persian	Fc2276	Long	G	T	A	–	C	**C**	C	G	C	4,4
Ragdoll	Fc582	Long	G	T	C	**+**	C	A	CT	G	C	1,1
Ragdoll	Fc1621	Long	G	T	A	–	C	**del474T/C**	C	G	CG	3,4
Ragdoll	Fc2271	Long	G	T	CA	–+	C	**C**A	C	G	C	1,4
Ragdoll	Fc4013	Long	G	T	CA	–+	C	**C**A	C	G	C	1,4
Turkish Angora	Fc2521	Long	G	T	A	–	C	**C**	C	G	C	4,4
Turkish Angora	Fc2707	Long	G	T	A	–	C	**C**	C	G	C	4,4
Turkish Angora	Fc2742	Long	G	T	A	–	C	**C**	C	G	C	4,4
Turkish Van	Fc569	Long	G	T	A	–	C	**C**	C	G	C	4,4
Turkish Van	Fc2517	Long	G	T	A	–	C	**C**	C	G	C	4,4
Turkish Van	Fc2737	Long	G	T	A	–	C	**C**	C	G	C	4,4

[a] The haplotypes of the 2 adjacent mutations 3 and 4 were inferred from the DNA sequence.
del474T: del474T,A/**del474T**,A; A: No Del 474T,A/No Del 474T,A; **C**: No Del 474T,**C**/No Del 474T,**C**; **del474T/C**: No Del474T,**C/Del474T,A**; **C**A: No Del 474T,**C**/No Del 474T,A.

[b] Mutation 1 (ins356T) and Mutation 3 (del474T) result in the predicted truncation of FGF5 Isoform 1.

[c] Nucleotide position in CDS of predicted mRNA.

[d] Putative mutations are marked in bold. When only 1 base is shown, the cat is homozygous at that position. For ins356T, "+" and "–" refer to insertion and no insertion, respectively.

[e] Genotypes of both alleles are coded as follows: N, normal; 1–4, Mutations 1–4.

polymorphic site to introduce a positive control for enzyme digestion (Brownstein et al. 1996). Touchdown PCR cycling conditions were used to amplify Mutation 2 (c.C>T406). For the other 2 assays, the PCR conditions previously described were used (Menotti-Raymond et al. 1999) with the exception that the initial 93 °C denaturation cycle was extended to 10 min and the enzyme AmpliTaq Gold was substituted for AmpliTaq (Applied Biosystems, Foster City, CA). Fluorescent primer labeling, touchdown PCR amplification, and analysis on an Applied Biosystems Model 3100 DNA sequenher were performed as previously described (Guo et al. 2006). Prior to electrophoresis, digested products were purified by centrifugation through Multiscreen plates (Millipore, Bedford, MA) packed with Sephadex G-50 (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions.

Results

Whole-Genome Scan

We tested for genetic linkage between the long-haired trait and 513 microsatellite markers genotyped in the nonbreed, Nestlé-Purina pedigree. A portion of the Nestlé-Purina pedigree included 135 potentially informative meioses from mixed litters of 62 progeny produced from F1-by-F1 "short-haired" matings and 11 long-haired cats generated from F1 "short-haired" cats crossed with long-haired cats. The 6 microsatellites that demonstrated single-marker LOD scores at or above 3.0 were all found within contiguous sequences of the assembled cat genome and were assigned to an estimated 39.3-MB region of B1 through the Garfield Cat Genome Browser (http://lgd.abcc.ncifcrf.gov/cgi-bin/gbrowse/cat) (Pontius J, personal communication) (Table 1). All other markers not on B1 had peak single-marker LOD scores less than 1.75 (not shown). The 2 markers (FCA823 and FCA824) that demonstrated the highest LOD scores to the long-haired locus (6.3, $\theta = 0.06$ and 9.5, $\theta = 0.03$, respectively) flanked an estimated 10-Mb region of cat chromosome B1 containing the candidate gene, *FGF5* (Table 1). We next sequenced this candidate gene in related nonbreed cats in the Nestlé-Purina pedigree and in an independent research colony at Johns Hopkins University, as well as in unrelated long- and short-haired breed cats.

DNA Sequence Analyses of *FGF5* in 50 Short- and Long-Haired Breed Cats

To detect possible mutations in the predicted coding sequence (CDS) of the feline *FGF5* gene beginning at position 158, 481, 515 on chromosome B1 of the annotated feline genome (Pontius J, personal communication), primers were designed in flanking regions located in the 5′ and 3′ untranslated regions and in introns (Supplementary Table A1 and Figure A1) and used to amplify the 3 exons in 50 breed cats with known hair length (Table 2). The size of the assembled predicted CDS was 810 bp, and the inferred full-length, feline FGF5 protein (Isoform 1 translated from all 3 exons) was 270 amino acids (aas) exhibiting 91% residue identity with the human FGF5 protein (Figure 1A). The

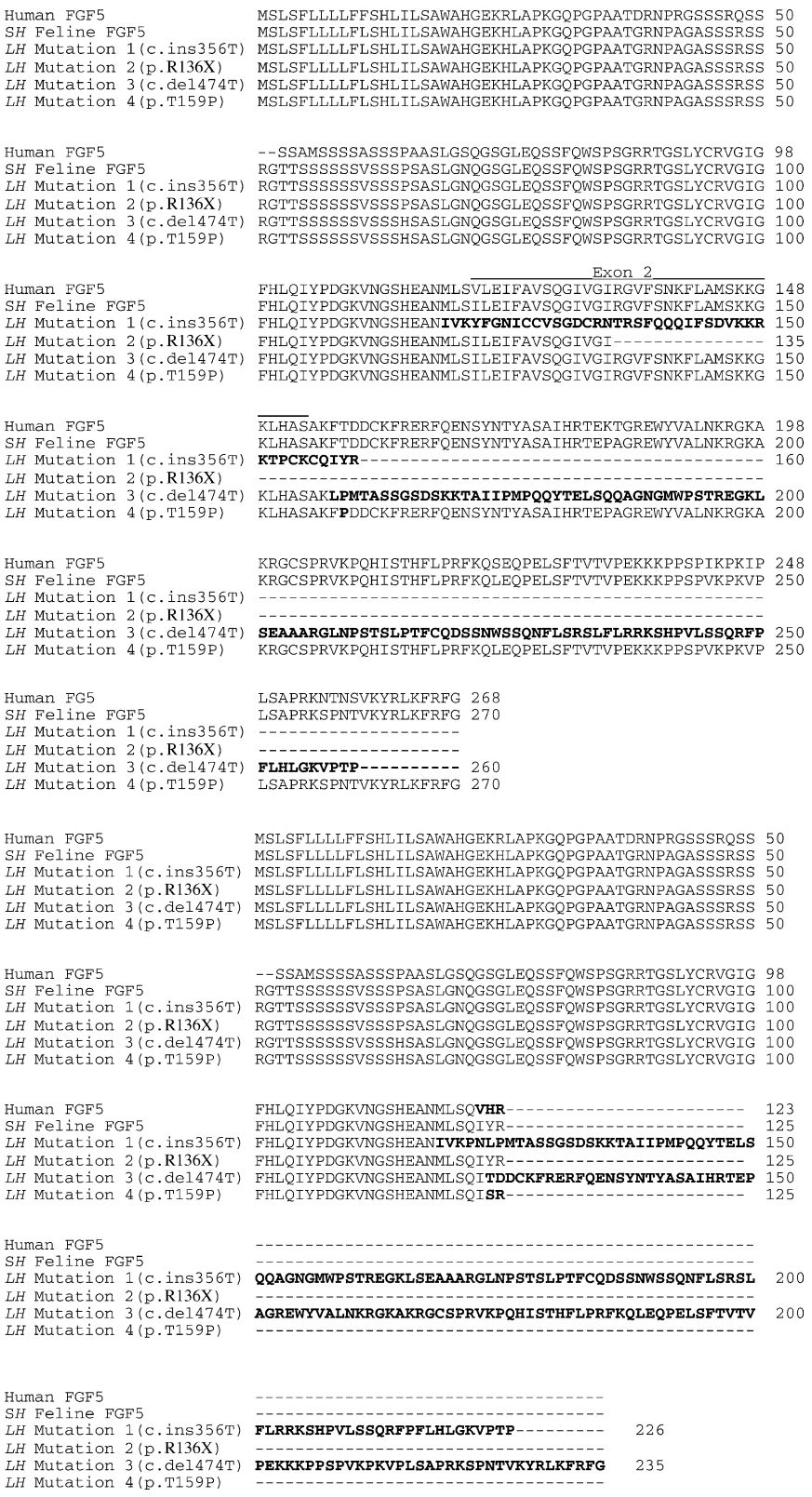

Figure 1. (**A**) Alignment of FGF5 Isoform 1 (full length form translated from 3 exons) for human, *short-haired* (*SH*) cats, and *long-haired* (*LH*) cats with 4 putative recessive mutations. Residue changes after putative mutations are marked in bold. (**B**) Alignment of FGF5 Isoform 2 (translated from exon 1 spliced to exon 3) for human, short-haired cats, and *long-haired* cats with 4 putative recessive mutations.

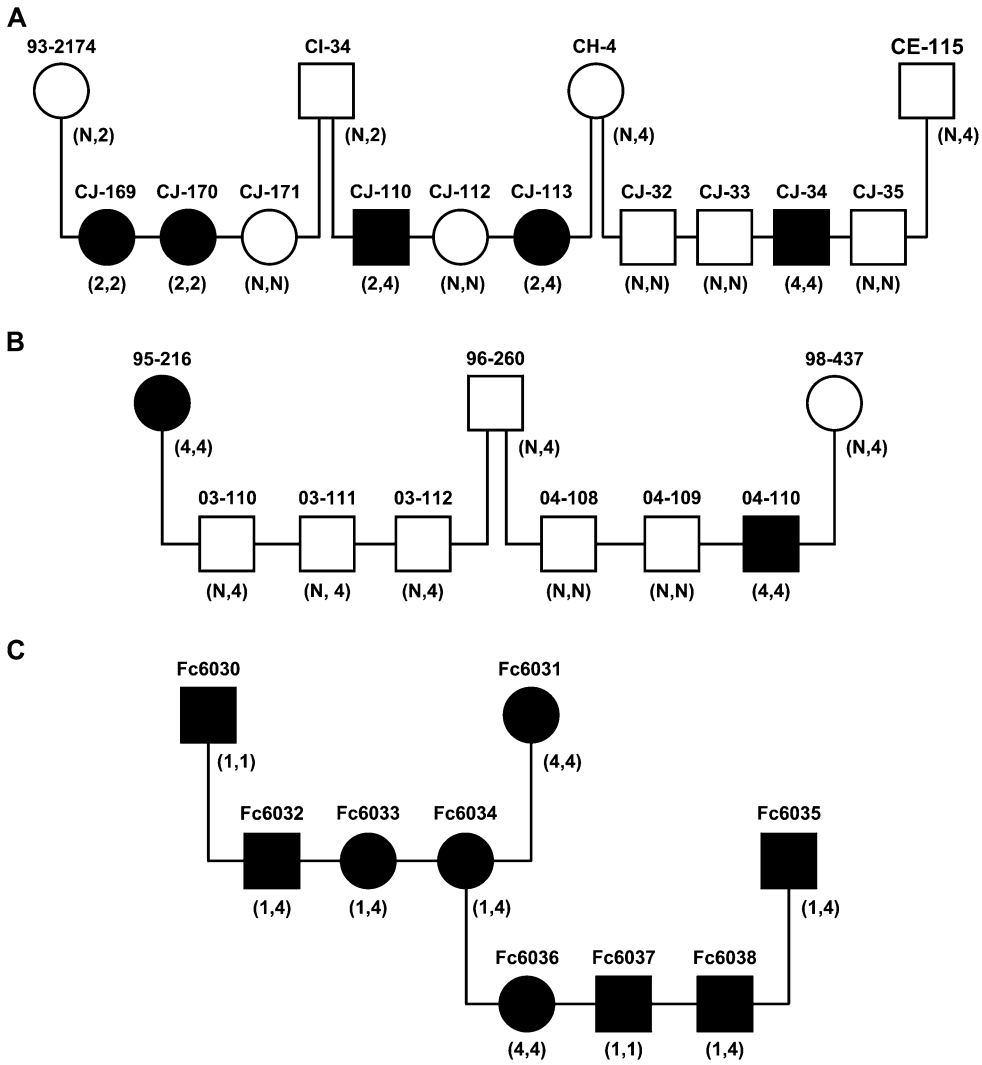

Figure 2. Three independent pedigrees demonstrating autosomal recessive inheritance of mutations1, 2, and 4 in *FGF5* with the long-haired trait. (**A**) Two-generation portion of the nonbreed Nestlé-Purina pedigree. (**B**) Two-generation portion of the nonbreed John Hopkins University pedigree. (**C**) Three-generation pedigree of long-haired Ragdoll cats. Square, male; circle, female. Open symbol, short haired; filled, long haired. The identification number of cats is written over top of individual symbols. Coded genotypes are listed in parentheses: N, no mutation; 2, Mutation 2 (c.C>T406); 3, Mutation 3 (c.del474T); 4, Mutation 4 (C.A>C475).

potential shorter feline FGF5S protein (Isoform 2 resulting from alternative splicing of exons 1 and 3) was predicted to be 125 aas in length and shared 86% residue identity with the human FGF5S (Figure 1B). A recent report confirmed that the transcripts for both predicted FGF5 isoforms are expressed in the skin of domestic cats (Drögemüller et al. 2007).

In our study, comparison of the feline *FGF5* CDS in 50 unrelated breed cats identified 10 single-nucleotide polymorphisms (SNPs) (Table 2). By examining the observed allele distributions between short- and long-haired cats (Table 2), 3 SNPs in exon 1 of *FGF5* producing nonsynonymous substitutions in both FGF5 isoforms and 3 SNPs encoding synonymous substitutions in exon 3 were all excluded as possible causative mutations for the long-haired phenotype. The 4 remaining independent changes detected in exons 1 and 3 appeared to be functionally significant (described below as Mutations 1–4). All 25 long-haired breed cats were either homozygous for 1 or compound heterozygous for 2 of these 4 predicted mutations, while none of the 25 short-haired cats carried 2 predicted mutant *FGF5* alleles.

Mutation 1: In 3 unrelated long-haired Ragdoll cats (Fc582, Fc2271, and Fc4013), an insertion of a thymine base was detected 356 bp (c.ins356T) downstream of the predicted translation initiation start site of *FGF5* CDS (Table 2). This frameshift mutation was predicted to introduce 3 nonsynonymous substitutions at the end of exon 1, 31 substitutions in exon 2, and a stop codon in exon 3, truncating the Mutation 1 FGF5 protein prematurely at 160 aas. In comparison, the size of the full-length, wild-type feline FGF5 protein was predicted

to be 270 aas in length (Figure 1A). The result of the c.ins356T mutation in the short *FGF5* mRNA (*FGF5S*), if spliced and translated correctly, would be to extend the predicted protein to 226 aas compared with 125 aas in the wild-type FGF5S (Figure 1B). One long-haired Ragdoll cat (Fc582) was found to be homozygous for the c.ins356T genotype, supporting the conclusion that the substantial changes caused by this frameshift mutation are likely to completely disrupt the biological activity of both Mutation 1 FGF5 isoforms. The other 2 Ragdoll cats (Fc2271 and Fc4013) were compound heterozygous for Mutation 1 and a second mutant allele (Mutation 4 discussed below). None of the 25 short-haired cats were found to carry the Mutation 1 allele in this survey.

Mutation 2: Among all the sequenced breed cats, only Norwegian Forest Cats possessed a potential nonsense mutation introduced in exon 2 by the substitution of a cytosine to a thymine base at nucleotide position 406 (c.C>T406). While the Mutation 2 *FGF5S* mRNA lacking exon 2 should be translated normally, the full-length Mutation 2 FGF5 protein was predicted to contain a change at aa position 135 from an arginine to a premature stop codon (p.R136X) (Figure 1A). Three long-haired Norwegian Forest Cats (Fc1914, Fc2587, and Fc2598) were homozygous for Mutation 2 in the *FGF5* gene, suggesting a loss of biological activity. One Norwegian Forest Cat (Fc2086) was compound heterozygous for Mutations 2 and 4 (discussed below) (Table 2).

Mutation 3: A deletion of a thymine base at nucleotide position 474 (c.del474T) was found to introduce a frameshift mutation near the beginning of exon 3 in 1 Ragdoll (Fc1621) and 3 Maine Coon cats (Fc1231, Fc2586, Fc2608) (Table 2). The predicted Mutation 3 FGF5 protein should contain extensive nonsynonymous substitutions starting at aa position 158 and truncate prematurely at 260 residues (Figure 1A). Whereas the predicted Mutation 3 FGF5S should have extensive changes starting at aa position 144 and extended to 235 aas in comparison to 125 aas for the wild-type FGF5S (Figure 1B). As a result of Mutation 3, both isoforms were unlikely to maintain their normal biological activity. One Maine Coon cat (Fc2608) was found to be homozygous for Mutation 3, while the other 3 long-haired cats were compound heterozygous for Mutations 3 and 4. Recombination spanning the single-nucleotide position between Mutations 3 and 4 was not detected by phase haplotype analyses in any long-haired cats (Table 2). None of the 25 short-haired breed cats genotyped carried the Mutation 3 allele.

Mutation 4: A change from an adenine to a cytosine base was detected at nucleotide position 475 (c.A>C475) in exon 3 in at least one individual from all the long-haired cat breeds sequenced (Table 2). This change was predicted to result in the missense substitution of a single threonine with a proline residue at aa position 159 (p.T159P) in FGF5 (Figure 1A) and the replacement of a tyrosine with a serine residue at the penultimate position in the potential FGF5S isoform (Figure 1B). The majority of long-haired cats sequenced in this initial survey were homozygous for Mutation 4 (Table 2). While 5 breed cats from short-haired registries (British Shorthair [Fc2593], Devon Rex [Fc1455], Manx [Fc2813], and Scottish Fold cats [Fc2536 and FC2843]) were heterozygous carriers of this allele, no short-haired cats were found to be homozygous for Mutation 4 in the *FGF5* gene.

Table 3. *FGF5* allele frequencies among 116 cats from 26 registered breeds

CFA registered breeds	Number of cats	Mutation number 1	2	3	4	None	Coat length1
Long haired							
Angora	2				1.00		Long
Balinese	2				1.00		Long
Birman	6				1.00		Long
Himalayan	4				1.00		Long
Maine Coon	4			0.50	0.50		Long
Norwegian Forest Cat	4		0.88		0.12		Long
Persian	7				1.00		Long
Ragdoll	13	0.27		0.23	0.50		Long
Siberian	3				1.00		Long
Somali	6				1.00		Long
Turkish Angora	4				1.00		Long
Turkish Van	5				1.00		Long
Long or short haired							
Manx[a]	1				1.00	0.00	Long
	2				0.50[d]	0.50	Short
Scottish Fold[a,b]	1				1.00	0.00	Long
	3				0.33[d]	0.66	short
Short haired							
Abyssinian	8					1.00	Short
American Shorthair	7					1.00	Short
British Shorthair[c]	5				0.20[d]	0.80	Short
Burmese	2					1.00	Short
Chartreux	2					1.00	Short
Cornish Rex	1					1.00	Short
Devon Rex[b,c]	3				0.33[d]	0.67	Short
Egyptian Mau	10					1.00	Short
Havana	2					1.00	Short
Ocicat	2					1.00	Short
Russian Blue	3					1.00	Short
Siamese	4					1.00	Short

[a] Breeds with separate short- and long-haired CFA registries.

[b] Breeds that the CFA allows outcrossing with British Shorthair cats.

[c] Breeds with histories of outcrossing with Persian cats.

[d] Mutation 4 alleles were only present in the heterozygous state in short-haired cats.

DNA Sequence Analyses of *FGF5* in Additional Short- and Long-Haired Breed Cats

To assess the frequency of normal and mutant *FGF5* alleles in cat populations of 26 breeds, we genotyped 66 additional breed cats (29 short-haired and 37 long-haired) using RFLP assays designed to detect Mutations 1–4 (Supplementary Table A2) and then sequenced the entire *FGF5* CDS to detect any other potential mutations. Their genotypes were combined with those of the original 50 cats (Table 2) to estimate the allele frequencies in the 21 original breeds and 5 additional long-haired breeds (Angora, Balinese, Himalayan, Siberian, and Somali) (Table 3). Mutations 1 and 2 remained

unique to the Ragdoll and Norwegian Forest Cat breeds, respectively, whereas Mutation 3 continued to be detected only in Maine Coon and Ragdoll cats. Mutation 4 was the most prevalent mutant *FGF5* allele in this study. It was detected in all 14 long-haired breeds sampled and was the only mutation detected in 11 of these breeds (Table 3). No additional mutations were detected in the *FGF5* CDS in these additional cats. Most importantly, no short-haired breed cats ($n = 54$) were homozygous for any of the 4 *FGF5* mutant alleles, whereas all long-haired breed cats ($n = 62$) were either homozygous or compound heterozygous for 2 separate mutant alleles. Only 3 of the 6 possible compound heterozygous allelic combinations (1/4, 3/4, 2/4) were observed in breed cats in this study (Table 3). The results of this extended breed analysis spurred us to genotype and test whether the inheritance of the 4 mutations detected in the *FGF5* gene segregated in pedigrees of nonbreed cats with the long-haired phenotype in an autosomal recessive manner as previously reported (Vella and Robinson 1999).

FGF5 Sequence and Linkage Analyses in Nonbreed Cats

To test for Mendelian segregation of the 4 mutations identified in *FGF5* with the long-haired trait, all 3 exons were successfully sequenced in 225 of the 261 cats in the Nestlé-Purina pedigree and 40 of 50 cats in a second, nonbreed pedigree from Johns Hopkins University. Of the 211 short-haired cats genotyped in the Nestlé-Purina pedigree, all were either homozygous for the wild-type *FGF5* allele or heterozygous for Mutations 2, 3, or 4 (Supplementary Table A3). Whereas all the 14 long-haired cats were either homozygous for Mutations 2 or 4 or compound heterozygous for paired combinations of Mutations 2, 3, or 4. When the genotypes of the 225 individuals in the Nestlé-Purina pedigree *FGF5* were coded for the 1 wild-type and 4 mutant alleles (Supplementary Table A3) and included in additional linkage analyses, linkage between the long-haired phenotype and *FGF5* locus was established with peak LOD scores of 11.6 at $\theta = 0.00$ (Table 1). Similarly, all the 9 genotyped long-haired cats in the Johns Hopkins University pedigree were either homozygous for Mutation 4 or compound heterozygous for Mutations 3 and 4, whereas all the 31 short-haired cats were either heterozygous carriers of Mutations 3 or 4 or homozygous for the wild-type *FGF5* allele (Supplementary Table A3). As in the breed survey, the concordant phenotype and genotype data for the total of 242 short-haired and 23 long-haired, nonbreed cats were consistent with mutations in the *FGF5* gene segregating with the long-haired phenotype in an autosomal recessive manner.

Inheritance of the Long-Haired Phenotype Segregates with Mutations in *FGF5*

An informative portion of the Nestlé-Purina pedigree clearly demonstrated autosomal recessive inheritance of both Mutations 2 and 4 in the *FGF5* locus segregating with the long-haired phenotype (Figure 2A). Multiple crosses of short-haired cats that were heterozygous for either *FGF5* Mutations 2 or 4 produced mixed litters with all the long-haired kittens possessing either 2/2, 2/4, or 4/4 genotypes. Likewise, an informative portion of the Johns Hopkins University pedigree also demonstrated Mendelian inheritance of Mutation 4 segregating with the long-haired phenotype in an autosomal recessive manner (Figure 2B). Although not exclusive of autosomal dominance, a 3-generation pedigree of 9 long-haired Ragdoll cats produced long-haired offspring with all 3 possible combinations of Mutations 1 and 4 consistent with autosomal recessive inheritance (Figure 2C). In combination, the pedigree analyses all support an autosomal recessive mode of inheritance of multiple mutant alleles in the *FGF5* locus segregating with the long-haired phenotype in domestic cats.

Discussion

A genomic approach was effective at testing the hypothesis that the *FGF5* locus is the major determinant of hair length in the cat. A genome wide scan of the Nestlé-Purina pedigree identified a region on cat chromosome B1 containing a likely candidate gene, *FGF5*, controlling the long-haired trait in the domestic cat. When individuals were genotyped for changes within the CDS and the *FGF5* "marker" was included in subsequent linkage analyses, a peak LOD score of 11.6 (θ of 0.00) was obtained. Sequence analyses of the feline *FGF5* gene in our survey of 12 short-haired, 12 long-haired, and 2 breeds with separate short- and long-haired registries revealed 4 separate mutations that were predicted to disrupt the biological activity of the FGF5 protein. Association analyses of all breed and nonbreed cats demonstrated uniformly that a combined total of 85 genotyped, long-haired cats were either homozygous for a single or compound heterozygous for 2 of 4 *FGF5* mutant alleles, while all 296 short-haired cats were either heterozygous or homozygous for the wild-type allele (Table 3 and Supplementary Table A3). Pedigree analyses of 2 independent, nonbreed colonies and 1 family of Ragdoll cats demonstrated that multiple combinations of mutations in the *FGF5* gene segregated with the long-haired phenotype in an autosomal recessive manner (Figure 2A–C). All these genetic analyses support our conclusion that the *FGF5* gene is the major determinant of hair length in the domestic cat.

While this study was under review, another group published their interpretation of SNPs within the feline *FGF5* gene associated with hair length (Drögemüller et al. 2007). They concluded that the c.194C>A (p.P65H) SNP in exon 1 was a predicted mutation for the long-haired trait, although it was clearly in linkage disequilibrium in their pedigree analyses with the c.475A>C SNP in exon 3 (Mutation 4 in this study). They discounted this change as a causative mutation due to the detection of 1 out of 50 crossbred short-haired cats that was homozygous for c.475A>C. Reevaluating the genotype and phenotype of this animal is clearly critical in light of our results. None of the 296 short-haired cats that we genotyped in our study were homozygous for the c.475 A>C change (the most common mutation found in most long-haired cats in our

study), whereas 6 short-haired cats were homozygous for the c.194C>A SNP (Table 2). Similarly, a second SNP in exon 1 c.182T>A was detected within 5 Norwegian Forest Cats and concluded to be a causative mutation in their study, but it was likely in linkage disequilibrium with the c.406C>T nonsense mutation in exon 2 (Mutation 2 found in Norwegian Forest Cats in our study). The authors did not detect this c.406>T change because they did not sequence exon 2 in the feline *FGF5* gene. However, we detected 2 unrelated short-haired, registered breed cats (Fc1143 and Fc2588) that were homozygous for the c.182T>A SNP (Table 2) and ruled out this change as a causative mutation for long hair. In addition, the other study did not detect the rare c.ins356T frameshift mutation in exon 1 (Mutation 1 found exclusively in Ragdoll cats in this study). Importantly, all 85 of the long-haired cats in our study were either homozygous or compound heterozygous for 2 of the 4 independent, predicted mutations, whereas none of the 296 short-haired cats were homozygous for any of these 4 predicted mutations in the *FGF5* gene.

Discovery of spontaneous mutations in *FGF5* orthologs of other domestic and wild species may implicate key functional protein domains. As in the Angora mice that lack all transcription of the *Fgf5* gene, long-haired dogs with a single *FGF5* mutation and long-haired cats with multiple combinations of *FGF5* mutations show no additional gross phenotypic changes. A SNP detected in exon 1 of the canine *FGF5* gene in long-haired dogs (Housley and Venta 2006) was predicted to substitute a phenylalanine for a conserved cysteine residue at aa position 95 within the β-1 loop required for the formation of hydrogen bonds with FGFRs (Yeh et al. 2003). Whereas the profound predicted changes caused by Mutations 1, 2, and 3 in the feline FGF5 protein preclude further functional interpretation, the feline Mutation 4 (T159P) FGF5 protein implicates an additional key domain that is likely to be required for FGF5 inhibitory activity. The threonine residue at aa position 159 falls within β-chain 7 of FGF5 (Katoh and Katoh 2005) and is a part of a conserved motif found in FGF9, 14, and 20 orthologs from multiple mammalian species (Supplementary Figure A2). Substitution with a proline residue may change the orientation of the following β-chains 8 and 9 and interfere with FGF5′s ability to bind to its cognate receptors (Mohammadi et al. 2005, Zhang et al. 2006). Although we have not directly assessed the biological activity of the feline wild-type and 4 mutant FGF5 proteins, singly or in combination, the observation of multiple long-haired cats that were homozygous for each of the 4 *FGF5* mutant genotypes supports the conclusion that each allele is dysfunctional. By analogy to FGF5 function in the mouse, all 4 feline mutant FGF5 proteins likely lose their inhibitory activity on DPC proliferation, prolonging the anagen stage and extending hair growth in long-haired domestic cats.

The 4 mutations detected in the feline *FGF5* locus may have arisen independently in geographically isolated, free-ranging populations of domestic cats and increased in frequency through natural selection in cold climates where the long-haired trait might be advantageous. Subsequent artificial selection for this recessive phenotype by breeders may have fixed the alleles carried by founders within specific long-haired breeds. While reconstructing the origins of the *FGF5* mutations within the context of breeds is largely conjecture due to their limited historical documentation, Mutation 4 may represent the oldest mutant allele. It was present in all 14 and was the only mutant allele in 11 long-haired breed registries sampled in this survey, including the Turkish Angora with a documented history older than 400 years (Fogle 2001).

In contrast Mutations 1, 2, and 3 were limited to 3 breeds with documented histories less than 200 years old (Vella and Robinson 1999). In this study, Mutations 1 and 2 were found to be unique to the Ragdoll and Norwegian Forest Cat breeds, respectively. Although Mutation 3 was found in both Maine Coon and Ragdoll cats, the more recently established Ragdoll breed (1960) may have obtained this allele by interbreeding with Maine Coon cats (first shown in 1860) (Fogle 2001). The inadvertent introduction of heritable congenital cardiomyopathy reported in Maine Coon cats (Kittleson et al. 1999) into some lines of Ragdolls supports these reports of prior crossbreeding with Maine Coon cats (Traas A, personal communication). The distribution of mutant alleles detected in the breeds sampled in this study supports the hypothesis that Mutations 1, 2, and 3 arose independently in nonbreed cats that were used as founders of Ragdoll cats, Norwegian Forest Cats, and Maine Coon cats, respectively, whereas Mutation 4 was most likely also present in these early founders or subsequently introduced through crosses with other long-haired breeds such as Persian cats.

The persistence of heterozygous carriers of *FGF5* Mutation 4 among 4 short-haired breed registries (Table 2) may also derive from this practice of outcrossing or from the original founders of these breeds originally established in the British Isles. Although long hair is now a disqualification for registry as a British Shorthair cat with the CFA, British Shorthair cats were occasionally crossed with Persian cats beginning after World War II, up until 1978 (Fogle 2001). Despite selection against the long-haired phenotype by breeders, the Mutation 4 allele was still present in the American population of British Shorthair cats sampled in this study. The CFA currently permits both Devon Rex and Scottish Fold cats to be outcrossed with British Shorthair cats, a possible source of Mutation 4 in these breeds. In addition, the founders of the Scottish Fold and Manx breeds who maintain separate registries based on hair length included long-haired individuals (Fogle 2001; Stephens 2001). In support of this historical reconstruction, the long-haired Manx and Scottish Fold cats that we sequenced were homozygous for Mutation 4 (Table 3). Although not detected in our study, it would not be unexpected to find carriers of Mutation 4 in extended populations of short-haired breeds such as the Siamese from which the long-haired Balinese breed was derived and recognized by the CFA during the 1960s (Vella and Robinson 1999).

Although this study included 62 unrelated individuals from 14 long-haired breed registries and 23 long-haired nonbreed cats from the Johns Hopkins University and

Nestle-Purina pedigrees, it is possible that additional mutations in the feline *FGF5* gene may be present in unsampled long-haired breed and nonbreed cats. In addition, the reported quantitative and qualitative differences between the coats of long-haired cat breeds indicate that other independent loci may modify the major influence of *FGF5* on hair length in the domestic cat (Vella and Robinson 1999). Finally, this study raises the question as to whether wild felid species with long hair possess *FGF5* mutations, providing an avenue to test whether free-ranging populations have undergone natural selection for genes that could affect coat length and their current phylogeographic distribution.

Funding

National Institutes of Health/National Center for Research Resources KO1/Special Emphasis Research Career Award (RR019677-01) to J.S.K; National Institutes of Health/ National Institutes on Deafness and Other Communication Disorders (DC00232) to D.K.R.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

Acknowledgments

J.S.K. and V.A.D. contributed equally to this work. The authors would like to thank Bethany Buzzell and David Wells for technical assistance and Dr Guo Kei Pei and Lisa Maslan for operating the automated DNA sequencers. We thank the many cat breeders who have contributed in the past to our population genetic database of cat breeds at the Laboratory of Genomic Diversity through cooperation with the CFA and the International Cat Association. We would also like to thank Dr Yasuko Ishida, Dr Solveig Pflueger, and Dr Anne Traas for their useful information about cat breeds and Dr Joan Pontius about the annotation of the cat genome. This research was supported in part by intramural research programs at the National Institutes of Health, National Cancer Institute, and National Library of Medicine. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services or mention of trade names, commercial products, or organizations imply endorsement by the US government.

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Received March 19, 2007
Accepted July 11, 2007

Corresponding Editor: Leif Andersson