Identification of a novel missense mutation in the fibroblast growth factor 5 gene associated with longhair in the Maine Coon Cat

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Abstract
Hair length can be a highly variable trait within the Felis catus species, varying between and within different cat breeds. Previous research has demonstrated this variability is due to recessive mutations within the fibroblast growth factor 5 (FGF5) gene. Following a genetic screen, four longhaired Maine Coons were identified that had only one copy of a known FGF5 mutation. We performed DNA sequencing on samples from two of these Maine Coons and identified a missense mutation in FGF5 c.577G > A p.Ala193Thr. Genetic screening via restriction digest was then performed on samples from the other two Maine Coons and an additional 273 cats of various breeds. This screening found that only the two additional Maine Coons were heterozygous for the novel variant. Furthermore, the novel variant was not identified after in silico analysis of 68 whole genome cat sequences from various breeds, demonstrating that this novel mutation is most likely a breed-specific variant for the Maine Coon, contributing to the longhair phenotype in about 3% of these cats.

Introduction
The fibroblast growth factor 5 (FGF5) protein encoded by the FGF5 gene mediates the mammalian hair follicle cycles through the periods of growth, involution, and rest, resulting in various hair lengths between and among species due to variants within the FGF5 gene (Housley and Venta, 2006). Demonstrated in both dogs (Dierks et al 2013) and cats (Drögemüller et al 2007; Kehler et al 2007), longhair is recessive to shorthair and requires homozygosity or compound heterozygosity of variants in FGF5 for longhair to be expressed.

Specifically in cats, the impact of FGF5 on hair length has been demonstrated within different breeds, showing how variants of FGF5 cause longhair (Drögemüller et al 2007). Mutation 1 (M1), is an insertion of a thymine base (c.ins356T) originally found in the Ragdoll breed; Mutation 2 (M2), is a cytosine to thymine conversion (c.406C > T) originally found in the Norwegian Forest Cat breed; Mutation 4 (M4), is an adenine to cytosine conversion (c.475A > C) found in most longhair cat breeds (Drögemüller et al 2007; Kehler et al 2007). All four variants can be found in a homozygous state or a compound heterozygous state involving multiple variants (Kehler et al 2007).

Two additional variants in FGF5 were identified by Drögemüller et al. (2007) as potentially causative for the longhair phenotype. However, Kehler et al. (2007) found multiple shorthaired cats homozygous for these two mutations and excluded these variants as causative. Therefore, these variants are not included in the genetic screen used in this study. Both groups found the M4 variant, but it is worth mentioning that Drögemüller et al. (2007) found a single shorthaired crossbred cat that was homozygous for M4. In contrast, Kehler et al. (2007) found that M4 was the most common mutation among longhaired cats and was not found in a homozygous state in any shorthaired cats in their large study.

We have a developed a genetic screen for cats that tests 85 different variants causing diseases and traits. Among the trait tests are the four known variants in FGF5 for longhair (M1-M4). Genetic screening of more than 950 cats identified four Maine Coons with only one of the known variants in FGF5, yet exhibiting longhair. According to the Cat
Fanciers’ Association breed standards, Maine Coon cats should be longhaired with an overall shaggy appearance (https://cfa.org/maine-coon-cat/maine-coon-cat-breed-standard/). Sequencing of FGF5 in samples from two of these Maine Coons that had only one of the four known FGF5 variants, with additional screening in 144 Maine Coon cats and confirmation in relatives, led to the identification of a fifth, novel variant, Mutation 5, (M5) associated with long-hair in the Maine Coon.

Materials and methods

Study cohort

All cats in the study were privately owned and samples were collected by the cats’ owners. PERFORMAgene PG-100 (DNA Genotek, Ontario, Canada) buccal swabs were used to collect cheek cells from cats. DNA was extracted using the KingFisher Flex Purification System (ThermoFisher Scientific, MA, USA) as specified by the manufacturer. DNA concentrations were quantified with a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific), and samples over 40 ng/μL were diluted in DNA suspension buffer to 20 ng/μL. Samples were genotyped with the CatScan (Genetic Veterinary Sciences, Inc., Spokane, WA, USA) genetic screening panel using an Agena Biosciences MassArray with the iPLEX Gold PCR kit and SpectroCHIP kit-CPM (Agena Biosciences, San Diego, CA, USA) with methods as previously described (Adams et al. 2003; Maksymowych et al. 2003). Among the over 950 cats originally screened, four Maine Coon cats that were identified to be heterozygous for only one of the known mutations and 273 additional cats of various breeds were selected for further study (Table 1). Maine Coons that were found to only have one copy of the known variants were confirmed to be longhaired via personal communication with the owners and pictures when possible. Owners were also contacted for related cat samples, preferably tom and queen or full siblings when available, to determine the mode of inheritance.

Sequencing

Sequencing was performed on samples from two of the Maine Coon cats that were heterozygous for a known variant using the ThermoFisher SeqStudio Genetic Analyzer. Four sets of primers were designed with M13 adapters to cover the exons and intronic boundaries of FGF5 (See Table 2). PCR was prepared using the BigDye Terminator v3.1 Sequencing Kit (ThermoFisher Scientific) and purified using the BigDye X Terminator Purification Kit (ThermoFisher Scientific) then run on the SeqStudio Genetic Analyzer using the Medium-Seq Sequencing Run Module. Sequence runs were analyzed manually using Chromas (Technelysium, Brisbane, Australia) and after the variant was identified, the sequence was BLAfed to the ICGSC Felis_Catus_9.0 Assembly (UCSC Genome Browser) to confirm the variant was not a known benign polymorphism.

Variant screening

Using the primer pair FGF5_5 (Table 2), a screening assay was developed via restriction enzyme digestion. PCR was set up using the ZymoTaq PreMix (Zymo Research, Irvine, CA, USA) and amplified with standard PCR conditions (40 cycles with the annealing temperature of 60°C). The restriction enzyme (Sau96I) was selected using NEBcutter V2.0 and sourced from New England Biolabs (NEB, Ipswich, MA, USA). Sau96I (G^GNCC) was used to digest the samples using standard conditions set by NEB. The resulting digested product was diluted 2:15 with PCR certified water and run on a 48 Well 2% Agarose E-Gel (ThermoFisher Scientific). E-Gels were visualized on the Analytik Jena UVP Transilluminator (Jena, Germany) and scored manually based on digested band sizes and intensity. Samples from 275 cats were screened with this restriction digest assay, including the two remaining Maine Coons that were heterozygous for only one of the known variants in FGF5.

In Silico analysis

The genomes of 68 previously sequenced cats of various breeds (North Carolina State College of Veterinary Medicine) were compared via sequence alignment to the FGF5 DNA variant sequences obtained from the two sequenced cats. The in silico analysis evaluated the presence of the novel variant and did not evaluate the presence of previously known variants (M1–M4).

Results

To date, more than 950 cats have been tested using the screening panel in our laboratory. Of these, four Maine Coons, which are typically longhaired cats, were identified to be heterozygous for only one of the known variants in the FGF5 gene. The samples from two of these cats were used for sequencing of FGF5. An additional 275 cats of various breeds, including 144 Maine Coons, which included the two remaining Main Coons identified as heterozygous with only one known variant, were screened for the presence of the novel variant via restriction enzyme digestion on a 2% E-Gel and 68 cats were screened via in silico sequence alignment for a total of 345 different cats included in this portion of the study (Table 1). Samples represented 33 different breeds of cats of varying hair lengths: 3 hairless breeds, 15
shorthaired breeds, 7 longhaired breeds, and 8 breeds that can have either short or longhair.

### Table 1

| Hair length     | Breed                  | Mutations |
|-----------------|------------------------|-----------|
|                 |                        | M1        | M2 | M3 | M4 | M5 | Total cats tested |
| Hairless        | Bambino                | –         | –  | –  | –  | –  | 1               |
|                 | Peterbald              | –         | –  | –  | –  | –  | 1               |
|                 | Sphynx                 | –         | –  | 2  | –  | –  | 11              |
| Longhair        | Birman                 | –         | –  | 2  | –  | –  | 1               |
|                 | British Longhair       | 1         | 1  | 9  | –  | –  | 5               |
|                 | **Maine Coon**         | 1         | 96 | 191| 4  | 146|                 |
|                 | Norwegian Forest Cat   | –         | 3  | –  | 1  | –  | 6               |
|                 | Persian                | –         | 1  | 11 | –  | –  | 14              |
|                 | Ragdoll                | 9         | 11 | 26 | –  | –  | 23              |
|                 | Siberian               | –         | 2  | 10 | –  | –  | 6               |
| Shorthair       | Abyssinian             | –         | –  | –  | –  | –  | 3               |
|                 | British Shorthair      | –         | 1  | –  | –  | –  | 5               |
|                 | Burmese                | –         | –  | –  | –  | –  | 2               |
|                 | Chausie                | –         | –  | 2  | –  | –  | 5               |
|                 | Cornish Rex            | –         | –  | 1  | –  | –  | 5               |
|                 | Egyptian Mau           | –         | –  | –  | –  | –  | 2               |
|                 | Exotic Shorthair       | –         | –  | 2  | –  | –  | 3               |
|                 | Highlander             | –         | –  | –  | –  | –  | 1               |
|                 | Jungle Cat             | –         | –  | –  | –  | –  | 1               |
|                 | Oriental               | –         | –  | 1  | –  | –  | 1               |
|                 | Oriental Shorthair     | –         | –  | –  | –  | –  | 2               |
|                 | Russian Blue           | –         | –  | –  | –  | –  | 1               |
|                 | Savannah               | –         | –  | 1  | –  | –  | 4               |
|                 | Thai                   | –         | –  | 1  | –  | –  | 1               |
|                 | Tonkinese              | –         | –  | –  | –  | –  | 5               |
| Short/Longhair  | Bengal                 | –         | –  | 1  | –  | –  | 6               |
|                 | Devon Rex              | –         | 5  | 6  | –  | –  | 14              |
|                 | Domestic Cat           | –         | –  | 1  | –  | –  | 45              |
|                 | Pixie–Bob              | –         | –  | 1  | –  | –  | 1               |
|                 | Scottish Fold          | –         | 1  | –  | 11 | –  | 10              |
|                 | Scottish Straight      | –         | –  | 7  | –  | –  | 8               |
|                 | Selkirk Rex            | –         | –  | 3  | –  | –  | 2               |
|                 | Siamese                | –         | –  | 1  | –  | –  | 4               |
| Total           |                        | 10        | 8  | 113| 292| 4  | 345             |

### Table 2

| Primer set name | Forward                     | Reverse                        |
|-----------------|-----------------------------|--------------------------------|
| FGF5_1          | 5'-AATGAACACTTGACTGCTAGGC-3' | 5'-AGTCCCTCTAAGCAAATTTGCCC-3'  |
| FGF5_6          | 5'-AGTTTCTGTGATTACAGCCCC-3'  | 5'-AGTGAGCTGAGGTTGATCC-3'      |
| FGF5_5          | 5'-GAGGAAGTTTCTGCCGTAGTG-3'  | 5'-AAGTGGGTAGAGATGTGCTGG-3'    |
| FGF5_9          | 5'-TTCCATCTGAGATCTACCAG-3'   | 5'-TAGATGCACTTCCACCCAACC-3'    |

Sequence

We used samples from two Maine Coon cats heterozygous for one known mutation and a control cat that is a compound heterozygote for M3 and M4 to search for additional variants within the FGF5 gene. Based on a recessive mode
of inheritance, it was hypothesized that cats with longhair having only one copy of a known mutation were likely compound heterozygotes for a different variant. Through sequencing, the known variants were confirmed in each cat. Furthermore, a single, novel missense variant was identified c.577G > A in the FGF5 sequencing (Fig. 1). The sequence data were submitted to NCBI GenBank with the accession number MZ311544. Figure 2 shows the location of this novel M5 variant as compared to the four previously known variants. This variant causes an amino acid substitution of alanine to threonine at amino acid position 193 (p.Ala193Thr). PolyPhen predicts this mutation to be deleterious with a score of 1 (PolyPhen). The amino acid sequence was also compared against other species, which showed that the region is highly conserved among various species (Fig. 3). No other variants were identified.

### Genetic screen

A PCR assay was developed to screen cats for this mutation to correlate its occurrence with longhair and to determine if the variant was associated with any of the known longhair mutations, as longhair mutations were already known in each of the cats. The screening was performed using a Sau96I restriction digest, with the wildtype sequence resulting in a 350 bp and 400 bp digested product and M5 sequence resulting in a 600 bp (uncut) product. Cats with an M5 allele and one other known variant, showed the 350 bp, 400 bp and 600 bp products (Fig. 4). Samples from all 277 cats were screened using this assay. No shorthair or hairless cats were found to have this novel variant and no longhaired cats with two known mutations were found to have this variant. Two additional Maine Coons, who were not used for the sequencing, were found to be heterozygous for M5, for a
Of the 68 additional cat sequences from various breeds analyzed in silico for the M5 variant, none were found to have this newly identified variant. No Maine Coons were among those sequences analyzed.

**Inheritance**

After finding the variant in four unrelated Maine Coons, the owners were contacted and we requested buccal swabs from related cats, preferably parents or offspring. We received samples from three offspring of one Maine Coon who has the M5 variant (Fig. 5a) and received samples from the queen of a second Maine Coon with M5 (Fig. 5b). Each related Maine Coon was tested for M5 using the PCR screening assay and tested for the four known variants using our genetic screening panel. All 3 offspring (Fig. 5a) and the queen in the second pedigree (Fig. 5b) were found to have the M5 variant, consistent with a recessive mode of inheritance.
Discussion

FGF5 is a known regulator of mammalian hair growth (reviewed in: Housley and Venta, 2006). Recessive mutations in mice contribute to the angora phenotype (Hébert et al. 1994), with variants in FGF5 contributing to long-hair in dogs (Dierks et al. 2013) and other mammals (Legrand et al. 2014; Yoshizawa et al. 2015; Daverio et al. 2017; Yu et al. 2018; Zhang et al. 2019). Interestingly, in humans, molecular characterization of trichomegaly in consanguineous families identified recessive variants in FGF5 (Higgins et al. 2014).

The domestic cat is defined by a number of different breeds based on morphological appearance, including the presence of short hair or long-haired varieties (Alhaddad et al. 2021). Selective breeding for desired characteristics will fix certain phenotypes within a breed. Previously, four variants were described in the FGF5 gene that contribute to longhair in cats (Drögemüller et al. 2007; Kehler et al. 2007). We describe here a novel recessive allele in the FGF5 gene associated with longhair in the Maine Coon, following previous nomenclature, called Mutation 5, M5. This c.577G > A results in an alanine to threonine substitution and is found in cats with known M3 or M4 FGF5 alleles in this breed as compound heterozygotes with phenotypically longhair. The alanine at position 193 has been found to be highly conserved among various mammals and is assumed to be important to the protein function. We assume that it is possible to be homozygous for M5, like the other known mutations, but this was not found in this sample group. No shorthair cats or longhair cats with two of the known mutations were found to have M5. All Maine Coon cats with the M5 variant and one other variant were longhaired, demonstrating high to complete penetrance. No cats outside of Maine Coons were found to have the M5 variant, indicating it may be a breed-specific mutation, arising after selective breeding. M5 was found in unrelated cats from different areas of the United States and Canada, showing that although it is specific to Maine Coons, it is likely not a private mutation. However, genetic screening did not identify any additional carriers in Maine Coons, indicating a possible allele frequency of this mutation within the breed of about 1.4%.

The variety of mutations within FGF5 in domestic animals is an excellent example of allelic heterogeneity. Here, we describe a novel variant within the FGF5 gene of Maine Coon cats causing the expression of longhair. With the addition of M5, there are five known FGF5 longhair causing variants within the Felis catus species. Adding this variant to tests offered by genetic testing facilities will provide additional information to breeders who use genetic results to control for certain diseases and predict the possible phenotypic outcomes for their litters.

Author contributions GDS and HF-S contributed to the study conceptualization and design. Any additional sample recruitment was arranged by LGS. All experiments were performed by GDS. Results were analyzed by GDS and HF-S. GDS produced the figures. In silico analysis was done by KM. GDS wrote the first draft of the manuscript. LGS and BCB provided critical review of the manuscript. GDS and BCB submitted the variant to GenBank. All authors commented on and approved the final manuscript.

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Declarations

Conflict of interest LGS and GDS are owners of Genetic Veterinary Sciences, Inc, DBA Paw Print Genetics which provides genetic testing on a fee-for-service basis including the CatScan genetic screen for cats. The remaining authors have no conflicts of interest to declare.

Availability of data and material All relevant data generated in this study are included in this published article.

Code availability Not applicable.

Ethics approval Not applicable.

Consent to participate All feline samples included in this study were obtained through consent of the individual owners or were obtained from otherwise discarded DNA samples after clinical testing at Paw Print Genetics.

Consent for publication Not applicable.

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