Melanocortin 1 Receptor (MC1R) Gene Sequence Variation and Melanism in the Gray (Sciurus carolinensis), Fox (Sciurus niger), and Red (Sciurus vulgaris) Squirrel

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Sequence variations in the melanocortin 1 receptor (MC1R) gene are associated with melanism in many different species of mammals, birds, and reptiles. The gray squirrel (Sciurus carolinensis), found in the British Isles, was introduced from North America in the late 19th century. Melanism in the British gray squirrel is associated with a 24-bp deletion in the MC1R. To investigate the origin of this mutation, we sequenced the MC1R of 95 individuals including 44 melanic gray squirrels from both the British Isles and North America. Melanic gray squirrels of both populations had the same 24-bp deletion associated with melanism. Given the significant deletion associated with melanism in the gray squirrel, we sequenced the MC1R of both wild-type and melanic fox squirrels (Sciurus niger) (9 individuals) and red squirrels (Sciurus vulgaris) (39 individuals). Unlike the gray squirrel, no association between sequence variation in the MC1R and melanism was found in these 2 species. We conclude that the melanic gray squirrel found in the British Isles originated from one or more introductions of melanic gray squirrels from North America. We also conclude that variations in the MC1R are not associated with melanism in the fox and red squirrels.

Subject area: Molecular adaptation and selection

Key words: Melanism, Melanocortin 1 receptor, Sciurus carolinensis, Sciurus niger, Sciurus vulgaris, squirrel

Vertebrate pigmentation is the result of the coordinated action of many genes and cell types and more than 100 loci are thought to be involved (reviewed by Lin and Fisher 2007). Melanocytes, cells that synthesis pigment, produce 2 distinct forms of melain: eumelanin, which is a dark brown/black pigment, and pheomelanin, which is a pale red/yellow pigment. Hair color depends on the distribution and relative amounts of these 2 pigments, where darker hairs contain a greater proportion of eumelanin (Robbins et al. 1993).

The genetic basis of melanic (dark) phenotypes has been described in a number of mammals, birds, and reptiles including mice (Robbins et al. 1993), rock pocket mice (Nachman et al. 2003), pigs (Kijas et al. 1998), fox (Vage et al. 1997), cattle (Klungland et al. 1995; Theron et al. 2001), dogs (Everts et al. 2000), chicken (Takeuchi et al. 1996), bananquist (Theron et al. 2001), and wall lizards (Buades et al. 2013) (reviewed in Majerus and Mundy 2003). In the majority of reported cases, melanism is associated with variation in 1 gene, the melanocortin 1 receptor (MC1R) gene. This highly polymorphic gene encodes a 7-transmembrane G protein–coupled receptor, which is predominantly expressed in melanocytes. The MC1R plays a central role in regulating melanin production in these specialized cells. The MC1R is activated by its agonist, alpha melanocyte-stimulating hormone (α-MSH) (Donatien et al. 1992). When the MC1R is bound by α-MSH, intracellular levels of cyclic adenosine monophosphate (cAMP) are elevated through a G-protein signaling pathway and eumelanin is produced. However, if the MC1R is bound by its antagonist, agouti signaling protein (ASIP), α-MSH binding is blocked, cAMP levels are reduced.

The gray squirrel (Sciurus carolinensis) is a native of North America but has been repeatedly introduced to the British Isles where it has become a highly successful invasive species (Gurnell et al. 2004). These British gray squirrels have 3 distinct color morphs: wild-type gray, brown-black, and jet black. Brown-black and jet black morphs are both considered to be melanic (see Supplementary Materials online for images of all phenotypes). The first recorded sighting of a melanic gray squirrel in the British Isles was in Bedfordshire in 1912 (Middleton 1931). The range and population of these melanic squirrels have been increasing steadily over the last century and they can now be seen regularly in many areas across the south of England, particularly in the counties of Bedfordshire, Hertfordshire, and Cambridgeshire (McRobie 2012). We have previously reported that the genetic basis of melanism in these British gray squirrels is associated with a 24-bp deletion in the MC1R, where the gray phenotype is homozygous for a wild-type MC1R E+ allele, the jet black is homozygous for the MC1R Δ24 E B allele and the brown-black is heterozygous for the E+ and E B alleles (McRobie et al. 2009). Melanic gray squirrels are also common in North America where they live in mixed populations with gray squirrels. Following on from our previous work, here we investigate the origin of the melanic gray squirrel in Britain to establish if the 24-bp deletion is a new mutation here we investigate the origin of the melanic gray squirrel in Britain to establish if the 24-bp deletion is a new mutation or whether melanic gray squirrels have been introduced from North America.

Given the clear association between the 24-bp deletion and melanism in the British gray squirrel, we extended the study of variations in the MC1R to 2 other squirrel species with melanic variants, the fox squirrel (Sciurus niger) and the red squirrel (Sciurus vulgaris). All 3 squirrel species live in mixed populations where wild-type morphs interbreed freely with melanic morphs of the same species (Gurnell 1987). The fox squirrel is a native of North America and has a spectrum of color morphs ranging from grizzled russet-orange through various shades of gray to black (Moncrief et al. 2010). The red squirrel is native to Europe and also has a spectrum of color morphs ranging from russet-red, red-brown, brown, to black. Red squirrels can also be black with a gray dorsum and black with red flanks (Lapini L., personal communication). Given the previous finding of a mutation on the MC1R associated with melanism in the closely related gray squirrel and considering the widespread role of the MC1R in pigmentation, we chose this as the first candidate gene to investigate for the genetic basis of melanism in the fox and red squirrels. In this study, we sequenced the entire MC1R gene from both wild-type and melanic color morphs of the fox and red squirrels to test the hypothesis that MC1R variation is associated with melanism in these species.

Materials and Methods

Gray squirrel samples were obtained from Cumbria, West Yorkshire, Northamptonshire, and Cambridgeshire in Britain and from Massachusetts, Virginia, and British Columbia in North America. Of the 51 wild-type samples, 45 were from Britain and 6 from North America. Of the 44 melanic samples, 36 were from Britain and 8 were from North America. Forty-two of these melanic samples were brown-black and 2 were jet black. All melanic gray squirrel samples from Britain were obtained from Cambridgeshire, as melanic gray squirrels are absent from the other British locations tested. A total of 9 fox squirrel samples were obtained from Georgia, North America, 4 being wild type and 5 melanic. A total of 39 red squirrel samples were obtained, including 33 from Italy of which 10 were wild type and 23 melanic. All Italian samples were obtained from the Friuli-Venezia Giulia region in northeastern Italy; 30 from Udine, 1 from Pordenone, 1 from Gorizia, and 1 from Trento. A further 6 wild-type red squirrel samples were obtained from Inverness-shire and Cumbria in Britain.

Total genomic DNA was extracted from muscle tissue using Qiagen DNeasy extraction kits. The MC1R was amplified by polymerase chain reaction (PCR) in 2 stages: 90% of the gene, including the start codon at the beginning of the gene, was amplified using the primers MSHR4F (5’-TGC TTC CTG GAC AGG ACT ATG-3’) and MC1R11R (5’-TCG TGT CGT YGT YGT GRA GGA AC-3’). The rest of the gene, including the 3’ end, was amplified using MC1Rel (5’-AAC GCA CTG GAG GAC ACC ATC-3’) and MCIRer6 (5’-CTG GGC TTG AGA CCA GA-3’). A forward primer MC1RBHF (5’-CTG GTG AGG ACC TCT TCA CTG-3’) and reverse primer MC1RBRR (5’-CCA GCA GTA GAG AGG TG-3’) were used to sequence the MC1RΔ24 allele of the gray squirrel. All PCR reactions were carried out in duplicate on a DNA thermocycler (Techne touchgene gradient) in a total volume of 25 μL using approximately 25 ng template DNA, 1× TopTaq PCR buffer, 3 mM MgCl2, 0.2 mM deoxynucleotide triphosphates, 0.4 μM primers, and 1× TopTaq polymerase. The following PCR conditions were used: initial denaturation 94°C for 2 min, 30 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 1 min followed by a final extension at 72°C for 5 min. PCR products were purified and sent for sequencing at Source BioScience, Cambridge. Chromatograms were examined by eye to identify heterozygotes and sequences were aligned using ClustalW to obtain the full MC1R sequence of the gene for each sample. Sequences were compared with the MC1R sequences on GenBank to confirm that this was the MC1R gene. In fulfillment of data archiving guidelines (Baker 2013), we have deposited the primary data underlying these analyses with Dryad. Sequences have been deposited with GenBank.

Results

Our results showed that the melanog gray squirrels of North America and the British Isles had an identical 24-bp deletion in the MC1R. Figure 1 shows a schematic diagram of the MC1R protein with deleted amino acids indicated. Of the 44 melanic samples tested, the 42 brown-black samples were heterozygous and the 2 jet black samples were homozygous
for the MC1RΔ24 Eᵦ (melanic) deletion. Information about phenotypes, genotypes, and allele frequencies for all 3 species is summarized in Table 1.

Our results show no association between variation in the MC1R and melanism in either fox or red squirrel. The MC1R in the fox squirrel has 945 bp, giving a 314 amino acid receptor. Two alleles were detected with 3 nonsynonymous substitutions. The substitutions were as follows with the wild-type gray squirrel MC1R as reference. Allele 1: P15S and M167I (accession number KF052119). Allele 2: P30S (accession number KF052120). Positions of these amino acids are shown on the schematic diagram of the MC1R in Figure 1. No observable differences in phenotype were identified between these alleles.

Two alleles of the MC1R of the red squirrel were also identified. The first allele (accession number KF188571) has 942 bp giving a 314 amino acid receptor with the following substitutions compared with the wild-type gray squirrel MC1R: S10C, L21F, T105M, T108A, N114D, A158V, R233C, and A238V. The second allele (accession number KF188572) has 939 bp giving a 313 amino acid receptor with the same substitutions compared with the gray squirrel but also a single amino acid deletion, Y180del. Positions of these amino acids are shown on the schematic diagram of the MC1R in Figure 1. All 33 Italian red squirrel samples (both melanic and wild type) were homozygous for allele 1. Five of the 6 British samples of the red squirrel (all wild type) were homozygous for the allele 2 and the other was heterozygous.

Discussion

Our results strongly support the conclusion that the presence of melanic gray squirrels in Britain is the result of one or a few introductions from North America and not the result of a new mutation. Squirrels from both Britain and North America showed the same phenotypic and corresponding genotypic variation where wild-type squirrels were gray and homozygous for the wild-type $E^\pm$ allele, brown-black squirrels were heterozygous for the wild-type $E^+$ and MC1RΔ24

Figure 1. Schematic diagram of the MC1R showing amino acids of the wild-type gray squirrel (Sciurus carolinensis). Dark gray circles with white lettering indicate the 8 amino acids deleted in the MC1RΔ24 Eᵦ allele. Light gray circles and black circles indicate amino acids in the fox (Sciurus niger) and red (Sciurus vulgaris) squirrels, respectively, that differ from those of the gray squirrel. The circle with vertical stripes is the amino acid deleted in allele 2 of the red squirrel MC1R. Information for the predicted structure of the MC1R protein was obtained from Mundy (2005).
Table 1: Variations in amino acids of the MC1R between Sciurus carolinensis, Sciurus niger, and Sciurus vulgaris with MC1R E<sup>+</sup> (GenBank accession number EU604831.2) as the reference sequence.

| Species          | Amino acids | Allele frequency | Location | Phenotype/ genotype | wt | mel | wt | wt | wt | wt | wt | wt | wt | wt | mel | wt | mel |
|------------------|-------------|------------------|----------|--------------------|----|-----|----|----|----|----|----|----|----|-----|----|-----|
| S. carolinensis  | S P L P     | 105 108 114 158 167 180 233 238 |            |                   |    |     | 10 | 7  | 6  | 28 | 3  | 3  | 3  | 3  |
| S. niger         | — S — —     | —                | N1/N2    |                   |    |     | 15 |     | 1  | 1  | 3  | 3  | 1  | 2  |
| S. vulgaris      | C F — —     | M A D V C V V    | V1/V2    |                   | 67 | 10  | 23 |     | 1  | 1  | 1  | 1  |

Dashes indicate identity with the reference sequence and squares represent deleted amino acids. N1 and N2 refer to alleles 1 and 2 of S. niger and V1 and V2 refer to alleles 1 and 2 of S. vulgaris, respectively. Allele frequencies refer to the number of each allele observed in the samples tested. The numbers under loci refer to numbers of squirrels of each phenotype tested. Inv = Inverness-shire, Cu = Cumbria, Nor = Northamptonshire, WY = West Yorkshire, Cambs = Cambridgeshire, BC = British Columbia, MA = Massachusetts, VA = Virginia, GA = Georgia, wt = wild type, mel = melanic, bb = brown black, and jb = jet black.

In all of these cases, where there is a wide spectrum of color morphs, melanism is not associated with variations in this gene, but there are no such clear distinctions between phenotypes. Our results show no association between variation in the MC1R and melanism in either fox or red squirrel. In cases where melanism is associated with variations in other genes, the jet black squirrels were homozygous for the MC1R allele, and the jet black squirrels were homozygous for the MC1R allele.
as well as regulatory regions in elucidating the genetic and molecular basis of melanic phenotypes.

The genetics and molecular cell biology of pigmentation is clearly complex and it seems likely that melanism is polygenic in many cases, particularly where differences between phenotypes are relatively small as suggested by Wlasik and Nachman (2007). Given the wide spectrum of color morphs in the fox and red squirrels, and the complex phenotypes in the red squirrel where different body parts are differently colored, it seems possible that coloration is polygenic and may involve genes in the early stages of development, in the regulation of hormones or indeed the regulation of receptors. There are likely to be many more as-yet unidentified loci involved in pigmentation. The phenotypes observed in the fox and red squirrels may be associated with mutations in one or a few of these other candidate genes or in regulatory sequences of genes.

Overall our results confirm the polymorphic nature of the MC1R gene with even small sample sizes revealing substitutions and deletions. We conclude that the melanic gray squirrel found in the British Isles originated from one or more introductions of melanic gray squirrels from North America. We also conclude that variations in the MC1R are not associated with melanism in the fox and red squirrels. Future studies exploring other candidate genes or regulatory regions of genes, for example, ASIP, would no doubt provide insight into the genetics of melanism in the fox and red squirrels.

**Supplementary Material**


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**References**


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