Title:

Melanocortin 1 receptor (MC1R) gene sequence variation and melanism in the grey (*Sciurus carolinensis*), fox (*Sciurus niger*) and red (*Sciurus vulgaris*) squirrel

Running title:

Melanism and the melanocortin-1 receptor in three species of sciuridae

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Abstract

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

Key Words

Melanism, Melanocortin-1 receptor, *Sciurus carolinensis, Sciurus niger, Sciurus vulgaris,* squirrel

Introduction

Vertebrate pigmentation is the result of the coordinated action of many genes and cell types and over 100 loci are thought to be involved (reviewed by Lin and Fisher 2007). Melanocytes, cells which synthesis pigment, produce two distinct forms of melanin: eumelanin, which is a dark brown/black pigment, and phaeomelanin, which is a paler red/yellow pigment. Hair colour depends on the distribution and relative amounts of these two 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), bananaquit (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 one gene, the melanocortin 1 receptor (MC1R) gene. This highly polymorphic gene encodes a seven-transmembrane G-protein coupled receptor which is predominantly expressed in melanocytes. The MC1R plays a central role in regulating melanin production in these specialised 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 cAMP are elevated through a G-protein signalling pathway and eumelanin is produced. However, if the MC1R is bound by its antagonist, agouti signalling protein (ASIP), α-MSH binding is

blocked, cAMP levels are reduced and phaeomelanin is produced (Abdel-Malek et al. 2001, Barsh et al. 2000, reviewed by Garcia-Borron et al. 2005 and Mountjoy et al. 1992).

The grey 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 grey squirrels have three distinct colour morphs; wildtype grey, brown-black and jet black. Brown-black and jet black morphs are both considered to be melanic. The first recorded sighting of a melanic grey 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 grey squirrels is associated with a 24 base pair (bp) deletion in the MC1R, where the grey phenotype is homozygous for a wildtype 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 grey squirrels are also common in North America where they live in mixed populations with grey squirrels. Following on from our previous work, here we investigate the origin of the melanic grey squirrel in Britain to establish if the 24 bp deletion is a new mutation which occurred since its introduction, or whether melanic grey squirrels were introduced from North America.

Given the clear association between the 24 bp deletion and melanism in the British grey squirrel, we extended the study of variations in the *MC1R* to two other squirrel species with melanic variants, the fox squirrel (Sciurus niger) and the red squirrel (Sciurus vulgaris). All three squirrel species live in mixed populations where wildtype 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 colour morphs ranging from grizzled russet-orange through various shades of grey to black (Moncrief et al. 2010). The red squirrel is native to Europe and also has a spectrum of colour morphs ranging from russet-red, red-brown, brown, to black. Red squirrels can also be black with a grey dorsum and black with red flanks (L. Lapini personal communication). Given the previous finding of a mutation on the *MC1R* associated with melanism in the closely related grey 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 of the fox and red squirrel for all colour morphs to test the hypothesis that MC1R variation is associated with melanism in these species.

Methods and Materials

Grey 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 wildtype 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. 42 of these melanic samples were brown-black and 2 were jet black. All melanic grey squirrel samples from Britain were obtained from

Cambridgeshire, as melanic grey squirrels are absent from the other British locations tested. A total of 9 fox squirrel samples were obtained from Georgia, North America, 4 being wildtype and 5 melanic. A total of 39 red squirrel samples were obtained, including 33 from Italy of which 10 were wildtype and 23 melanic. All Italian samples were obtained from the Friuli-Venezia Giulia region in north-eastern Italy: 30 from Udine, one from Pordenone, one from Gorizia and one from Trento. A further 6 wildtype 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 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 GRA GGA AC-3'). The rest of the gene, including the 3' end, was amplified using MC1Rdel (5'-AAC GCA CTG GAG ACG ACC ATC-3') and MC1Rer6 (5'-CTG GGC TTG AGA CCA GA-3'). A forward primer MC1RBF (5'-CTG GTG AGC ACC TTC CTA CTG-3') and reverse primer MC1RBR (5'-CCA GCA GTA GGA AGG TG-3') were used to sequence the *MC1RΔ24* allele of the grey 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, 1X TopTaq PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM primers, and 1X TopTaq polymerase. The following PCR conditions were used: initial denaturation 94°C for 2 minutes, 30 cycles of 94°C for 30 seconds, 59°C for 30 seconds and 72°C for one minute followed by a final extension at 72°C for 5 minutes. 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 to the *MC1R* sequences on GenBank to confirm that this was the *MC1R* gene.

Results

Our results showed that the melanic grey 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 two jet black samples were homozygous for the *MC1RΔ24* E^B (melanic) deletion.

Our results show no association between variation in the MC1R and melanism in either fox or red squirrels. The MC1R in the fox squirrel has 945 bp, giving a 314 amino acid receptor. Two alleles were detected with three non-synonymous substitutions. The substitutions were as follows with the wildtype grey 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 to the wildtype grey squirrel MC1R: S10C, L21F, T105M, T108A, N114D, A158V, R233C, A238V. The second allele (accession number KF188572) has 939 bp giving a 313 amino acid receptor with the same substitutions compared to the grey 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 wildtype) were homozygous for allele 1. Five of the six British samples of the red squirrel (all wildtype) were homozygous for the allele 2 and the other was heterozygous.

Discussion

Our results strongly support the conclusion that the presence of melanic grey 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 wildtype squirrels were grey and homozygous for the wildtype E^+ allele, brown-black squirrels were heterozygous for the wildtype E^+ and *MC1R-Δ24* E^B allele and the jet black squirrels were homozygous for the *MC1R-Δ24* E^B allele.

Our results show no association between variation in the MC1R and melanism in either fox or red squirrels. In cases where melanism is associated with variations in this gene, phenotypic differences are often discrete and relatively large as demonstrated in the grey squirrel (McRobie et al. 2009), bananaquit (Theron et al. 2001), chicken (Takeuchi et al. 1996), mice (Robbins et al. 1993), jaguars (Eizirik et al. 2003), and pigs (Kijas et al. 1998). In both the fox and red squirrel however, there

are no such clear distinctions between phenotypes and the colour differences are more subtle, presenting a continuous spectrum of colour variation. A similar spectrum of variation is also observed in the gopher (Wlasiuk and Nachman 2007), three mustelid lineages (Hosoda et al. 2005), Old World leaf warblers (MacDougall-Shackleton 2003) and the blue-crowned manakin (Cheviron et al. 2006). In all of these cases, where there is a wide spectrum of colour morphs, melanism is not associated with variations in the *M*C1R. These findings suggest that cases where melanism is graduated across a species, genes other than the MC1R may be responsible.

The MC1R gene is well characterised in a wide variety of species and the number of cases reporting the association of the MC1R to melanism indicates that this is a good candidate gene (Hoekstra 2006). However, it is likely that there is an ascertainment bias where positive results are more likely to be reported in this intronless gene which is relatively easy to sequence and analyse (Mundy 2005). Other key genes involved in pigmentation are more complex, for example ASIP which has three coding exons (Abdel-Malek et al. 2001) and is considerably harder to work with and therefore less likely to be reported. A number of other studies investigating the *MC1R* have highlighted the complex nature of the genetics of pigmentation. For example, large deletions in the first extracellular region of the receptor are associated with melanism in the jaguar (15 bp), jaguarundis (24 bp) (Eizirik et al. 2003) and grey squirrel (24 bp) (McRobie et al. 2009). In contrast, deletions almost identical to these are not associated with melanism in the wolverine (15 bp), stone marten (28 bp), four species of martens (Hosada et al. 2005) (45 bp) and the gopher (Wlasiuk and Nachman 2007) (21 bp). Further analysis of protein expression and function would be necessary to elucidate the effect of these deletions.

The study of melanism has provided many examples of convergent evolution. In some cases the same genes are associated with melanism in distantly related taxa and conversely different genes produce similar phenotypes in closely related taxa. Thus similar phenotypes can be the result of different underlying mechanisms (Gompel and Prud'homme 2009 and reviewed by Manceau et al. 2010). Rosenblum et al. (2010) demonstrated that independent mutations on the *MC1R* were associated with similar phenotypes in three species of white lizards (eastern fence lizard, little striped whiptail and lesser earless lizard). A number of studies have identified mutations in *cis*-regulatory elements involved in pigmentation (Gompel et al. 2005 and reviewed by Hoekstra 2006). These studies highlight the importance of investigating protein function 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 Wlasiuk and Nachman (2007). Given the wide spectrum of colour morphs in the fox and red squirrels, and the complex phenotypes in the red squirrel where different body parts are differently coloured, it seems possible that colouration 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 grey squirrel found in the British Isles originated from one or more introductions of melanic grey 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 squirrel.

Funding

This work was supported by Anglia Ruskin University.

Acknowledgements

We are very grateful to Luca Lapini from the Friuli Museum of Natural History, Udine, Italy for providing samples of red squirrel tissue from Italy, to Professor Buzz Hoagland from Westfield State College Department of Biology Massachussetts for samples of grey squirrels, to Dr Sheila Pankhurst of Anglia Ruskin University for samples of red squirrels, to Nicola Wain for experimental work and to Mark D'Arcy for IT support.

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Figure legend

Figure 1. Schematic diagram of the MC1R showing amino acids of the wildtype grey squirrel (*S. carolinensis*). Dark grey circles with white lettering indicate the eight amino acids deleted in the *MC1R-Δ24* E^B allele. Light grey circles and black circles indicate amino acids in the fox (*S. niger*) and red (*S. vulgaris*) squirrel respectively that differ from those of the grey 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).