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Citation

Mammal study, 41(3), 131-140

https://doi.org/10.3106/041.041.0303

Issue Date

2016-09

Type

article

File Information

Suzuki.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP
Variation in the coat-color-controlling genes, McI r and Asip, in the house mouse Mus musculus from Madagascar

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Abstract. Variability in the coat color of the house mouse, Mus musculus, provides an opportunity to study the evolution of phenotypes in this species. Here we associated genetic variations with coat color in seven mice from Madagascar that had identical M. m. gentilulus mitochondrial DNA sequences. The entire coding region of the 948-base pair (bp) coat-color-related gene, McI r, was shown to have no nonsynonymous changes. However, analyses of the two exon-1 promoter regions—termed 1A (317 bp) and 1B (499 bp)—from a second gene, Asip, which is also involved in the evolution of coat color, revealed two distinct haplotypes in each region. Associations between Asip promoter regions and dorsal color were ambiguous; however, two ventral color types—light and dark gray—were associated with the haplotypes of 1A, as determined by clustering analysis. Notably, the haplotype of the light gray animals was identical to the Asip A allele that is associated with white bellies.

Key words: Asip, coat color, Madagascar, McI r, Mus musculus gentilulus.

The house mouse, Mus musculus, often shows variations in coat color depending on the geographic location (Marshall 1998; Slábová and Frynta 2007). Moreover, this species often shows a polymorphic state for coat color within a local population. However, to date, the genetic systems underlying this variation have not been documented in field-caught wild mice. Trapping of small rodents at a zoo in Madagascar allowed our observations of considerable coat color variation in both the dorsal and ventral sides of the Mus musculus individuals. This provided an opportunity to assess the genetic systems underlying changes in coat color and to investigate the phylogenetic origins of the phenotype, further illustrating the ambiguous phylogenetic background of these mice introduced in Madagascar. Thus, studies such as this will shed light on the origins of the indigenous people of Madagascar (Hurles et al. 2005).

From previous sequence analyses of the mitochondrial control region, Madagascar mice were found to originate from Yemen, in the southern part of the Arabian Peninsula (Fig. 1; Duplantier et al. 2002). The mtDNA lineages constitute a “narrow” monophyletic group, which suggests a recent and probably single origin (Duplantier et al. 2002). The Yemen mtDNA lineage represents the fourth subspecies of M. m. gentilulus, which is distinct from the other three major subspecies groups (Prager et al. 1998; Suzuki et al. 2013): M. m. castaneus (CAS; southern Asia, Southeast Asia and southern China), M. m. domesticus (DOM; Eastern Europe), and M. m. musculus (MUS; northern Eurasia, excluding Eastern Europe). Currently, however, phylogenetic analyses are limited for Madagascar mice, particularly for nuclear genomes.

Numerous studies that have addressed coat color in wild populations have shown that a dimorphic state (indicative of the involvement of very few genes in color variation) can be used to identify the genes involved, as genetic variation may be linked to differential pigmentation pattern variations (e.g., Hoekstra et al. 2004; Fontanesi et al. 2006). In rodents, the melanocortin-1 receptor (McI r) and agouti signaling protein (Asip) are
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major determinants of red-yellow (pheomelanin) and black-brown (eumelanin) pigmentation (Tamate and Takeuchi 1981; Klungland et al. 1995; Suzuki 2013). Previous efforts to identify mutations related to phenotypic changes found a number of nonsynonymous mutations in the coding region (~954 bp in ordinal mammals but 948 bp in members of the genus *Mus*; Shimada et al. 2009) of the *Mc1r* gene that led to changes in coat color.

For the *Asip* gene, it is presumed that both the amino acid coding regions and their upstream promoter regions influence phenotypic changes in coat color. Changes in the coding region are thought to cause drastic changes in coat color, such as melanism, while changes in the promoter regions are expected to cause various degrees of color change, because the promoters regulate the expression level of the Asip protein, the antagonist/reverse agonist of the Mc1r receptor. In *M. musculus* there are two exon-1-associated promoter regions which have different functions. One promoter region located 100 kb upstream of the protein coding region harboring exons 2, 3, and 4 has two non-coding exons (exon 1), namely 1A and 1A’, and is responsible for ventral coat color (Vrielings et al. 1994). The other region also contains two non-coding exons (exon 1), 1B and 1C, located 30 kb upstream of the coding region, and is related to the agouti pattern of individual hairs with black and yellow bands (Vrielings et al. 1994).

Interestingly, these analyses of wild populations may provide useful phylogeographic information, not only indicating the genetic background of the phenotypic variation but also hinting at the genetic relationships among the geographic populations, in which sequence variation is used as a phylogenetic marker of coat color-related genes (e.g., Suzuki 2013; Kodama et al. 2015). In the case of black rats (*Rattus rattus* species complex), efforts to determine the gene responsible for the melanistic form have elucidated evolutionary genetic relationships and possible causative mutations (Kambe et al. 2011, 2012; Yasuda et al. 2014; Chingangbam et al. 2015).

To understand the involvement of the two candidate genes, *Asip* and *Mc1r*, we examined sequence variation in the promoters and coding regions of these genes, respectively, in specimens from Madagascar, where apparent color variations are present in both the dorsal and ventral sides. First, the origin of the population was determined by examining the mtDNA and nuclear *Mc1r* gene sequences. Next, a measure of coloration was performed using a spectrophotometer to determine the extent of variation among the individuals. A comparison of the sequence variations in *Mc1r* and *Asip* with the phenotypic variations in dorsal and ventral colors obtained from the qualitative measurements was performed, to determine the presence or absence of any association between the genotype and phenotype.

### Materials and methods

#### Specimens

Specimens of the house mouse (*M. musculus*; specimen 1–7), brown rat (*Rattus norvegicus*; field codes MG4, 5, 10, and 21), and black rat (*Rattus rattus* species complex; MG6–9) were collected from the Parc Botanique et Zoologique de Tsimbazaza, Madagascar, and nearby areas in 2015. Skull and flat skin specimens were prepared from the mice and the skull characters measured included the greatest skull length, condylobasal length, zygomatic breadth, and mandible length. Rats were included as the outgroups for phylogenetic analyses.

#### Measurement of coat color variation

We examined individual differences in coat color of the dorsal and ventral sides in all seven mice. A spectrophotometer (CM-700d, Konica Minolta Co., Osaka, Japan) with a 3-mm diameter window was used for all measurements under the Specular Component Included (SCI) option. A colorimetry model (CIE L*a*b*-model) was employed: (L*)-luminosity from black to white [0, 100], (a*)-component encoding the initial color from green to red [–60, 60], and (b*)-component encoding the initial color from blue to yellow [–60, 60]. Dorsal color was quantified using five independent measurements from the peripheral flat skin sections (at 2/3 of the vertical line). For the ventral section, five measurements were taken from various random locations. Cluster dendrograms were constructed from the average measurements of L*, a*, and b* by calculating pairwise distances in three-dimensional space. Hierarchical clustering algorithms were considered, and the hclust and farthest-neighbor methods in the R statistical package R3.2.1 (R Development Core Team 2015) were used. Based on this method, the maximum distance between two objects belonging to different clusters was taken into consideration.

#### Sequence analyses

Polymerase chain reaction (PCR) and direct sequencing of the mitochondrial control region (CR, ~800 bp) and *Cyb* (1,140 bp) were performed according to previously described methods (Suzuki et al. 2004, 2013;
Table 1. Seven specimens of the house mouse, *Mus musculus*, from Madagascar and their morphological and genetic characteristics

<table>
<thead>
<tr>
<th>Specimen code (Field code)</th>
<th>Skull morphology (mm)</th>
<th>Spectrophotometer measurements</th>
<th>Coat color types based on quantitative measurements</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSL</td>
<td>CBL</td>
<td>ZB</td>
<td>ML</td>
</tr>
<tr>
<td>No. 1 (MG-2)</td>
<td>20.01</td>
<td>17.35</td>
<td>10.65</td>
<td>10.55</td>
</tr>
<tr>
<td>No. 2 (MG-16)</td>
<td>20.01</td>
<td>17.35</td>
<td>10.65</td>
<td>10.75</td>
</tr>
<tr>
<td>No. 3 (MG-17)</td>
<td>20.55</td>
<td>17.55</td>
<td>10.90</td>
<td>10.25</td>
</tr>
<tr>
<td>No. 4 (MG-18)</td>
<td>21.00</td>
<td>18.55</td>
<td>10.20</td>
<td>11.20</td>
</tr>
<tr>
<td>No. 5 (MG-20)</td>
<td>19.55</td>
<td>15.55</td>
<td>10.60</td>
<td>10.60</td>
</tr>
<tr>
<td>No. 6 (MG-22)</td>
<td>20.70</td>
<td>17.35</td>
<td>10.90</td>
<td>11.00</td>
</tr>
<tr>
<td>No. 7 (MG-23)</td>
<td>20.35</td>
<td>17.50</td>
<td>10.80</td>
<td>10.85</td>
</tr>
</tbody>
</table>

The skull characters measured were the greatest skull length (GSL), condylobasal length (CBL), zygomatic breadth (ZB), and mandible length (ML). Coat color types were characterized by clustering analyses using the measurements of L*, a*, and b*, yielding two types on both dorsal and ventral sides. The color type of the ventral side in the specimen No. 5 was characterized as white based on naked eye observation (Fig. 3b) and light gray by spectrophotometry.

Yasuda et al. (2005). The entire coding region of the *McIr* gene (948 bp; 316 codons including a stop codon) was sequenced using the primer sets developed by Shimada et al. (2009). PCR was performed using the AmpliTaq Gold 360 DNA Polymerase Kit (Invitrogen, Carlsbad, CA, USA). The primers were 5'McIr-52 (5'-GCTCATACCACCTGGAGCTGCAGG-3') and 3'McIr+504 (5'-AAGAGGGTGCTGGAGACGATGC TGACC-3') for the upper section and 5'McIr+131 (5'-ATCCAGATGGCCTCT- TCCT-3') and 3'McIr+1025 (5'-CCCTTAGACAATGGAGACTCAGG-3') for the lower section. Following an initial heat activation step (95°C for 10 min), cycling conditions were 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C (30 cycles), followed by a

![Fig. 1. A map of the Madagascar collection sites. The predicted homeland areas of the five major mitochondrial lineages of *Mus musculus*, *M. m. castaneus* (CAS), *M. m. domesticus* (DOM), *M. m. gentilulus* (GEN), *M. m. musculus* (MUS), as well as mice from Nepal (NEP) are shown. The Madagascar mitochondrial lineage is thought to be derived from GEN mice introduced from Yemen (Duplantier et al. 2002).](image-url)
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**Fig. 2.** A schematic representation of the coat color-related gene, Asip (Vriel et al. 1994) (a). The two exon-1-associated promoter regions are indicated (1A-1A' and 1B-1C). The thick arrows indicate the inverted repeats, one of which carries the pseudo promoter, termed ps1A (Chen et al. 1996). The two amplified regions, Asip-1A (317 bp) and Asip-1B (499 bp), are indicated by the filled boxes. The chromosomal locations for the starting sites of exon 1A, 1B, and 2 are shown in parentheses and refer to the mouse genome sequence of chromosome 2 (strain C57BL/6J, NCBI build 37/mm9 http://genome.ucsc.edu). Two haplotypes recovered from the analyses of the sequenced regions are shown with the variable sites detected. An ML tree was constructed using the sequences of Asip-1A (317 bp) and three database sequences for the 1A and ps1A regions of allele A^a^, and the 1A region of allele a (Vriel et al. 1994) (b). Bootstrap support values (>50%) are shown at the nodes and are based on 1,000 resampling replicates.

The two exon-1-associated promoter regions of Asip were amplified using the primers Asip-1A_F (5'-TGGGTTCAGAAGCTGTCCCTGTG-3') and Asip-1A_R (5'-GGAGTTTAGACACATACTTGTTG-3') for the 1A region, and Asip-1B_F (5'-AGGAAAACCCAGAAGAGCGCCG-3') and Asip-1B_R (5'-TAAAGACCCAGAAGTGCCCTGG-3') for the 1B region (Fig. 2). Following an initial heat activation step (95°C for 10 min), 25 cycles of 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C were performed followed by a final 2 min extension at 72°C. PCR products were purified using a 20% polyethylene glycol–2.5 M NaCl precipitation method and sequenced directly (according to the manufacturer’s instructions) using the BigDye Terminator Cycle Sequencing Kit and ABI 3130. Sequence alignment was performed in MEGA5 (Tamura et al. 2011). Sequences with more than one polymorphic site were separated into alleles, mainly using the parsimony method (Karn et al. 2002). Nucleotide sequences were deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession numbers: LC146995–LC147023.

**Phylogenetic analyses**

Phylogenetic analyses were performed using the maximum likelihood (ML) method. The best-fit nucleotide-substitution model and other parameters were determined by AIC in MEGA5 software (Tamura et al. 2011). Boott-
strap support values were determined using 10,000 iterations, and a median-joining network was constructed using the Network program (ver. 4.6.1.2) (Bandelt et al. 1991).

Results

Morphological properties

The greatest length of skull (GSL) ranged from 19.55–21.00 mm (Table 1), with the relatively short skull lengths being comparable to those reported by Harrison (1970), who noted the short GSL of the South Arabian house mice compared with mice from other geographic locations, including Syria, Iran, and Afghanistan.

Coat color measurements

We examined coat color variations in both the dorsal and ventral sides in terms of the CIE L* a* b*-model color parameters L*, a*, and b* (Table 1) and created cluster dendrograms based on these measurements (Fig. 3). The dendrogram for the dorsal colors yielded two clusters, tentatively termed “light brown” (specimen code Nos. 2 and 4; dorsal color group D-I) and “dark brown” (Nos. 1, 3, 5, 6, and 7; dorsal color group D-II) (Fig. 3c). The
dendrogram for the ventral colors revealed two clusters, designated as “light gray” (Nos. 3, 5, and 6; ventral color group V–I) and “dark gray” (Nos. 1, 2, 4, and 7; ventral color group V–II) (Fig. 3d).

mtDNA sequence diversity

We determined the control region sequence in the seven mice from Madagascar. The sequences were aligned with those obtained from nucleotide databases of *M. m. gentilulus* from Yemen, and of *M. m. castaneus* (Prager et al. 1998). After removal of any gaps, the aligned sequences were 539 bp in length. Network analyses showed that our sequences from Madagascar, as well as those of the database sequences for mice from Yemen, were closely related with a small number of substitutions. These data indicate that our sequences belong to the same subspecies lineage of *M. m. gentilulus* (Fig. 4). The network revealed closer relationships among the Madagascar haplotypes than those from Yemen, supporting the idea of recent and limited introduction of mtDNA from the south Arabian Peninsula to Madagascar (Duplantier et al. 2002). Phylogenetic analysis of *Cytb* sequences from the Madagascar specimens and from others representing the four major mtDNA lineages, termed CAS, DOM, MUS, and Nepal (NEP), revealed the phylogenetic patterns of the five major lineages (Fig. 5). The phylogenetic data suggest a close relationship between DOM and NEP; however, the 2–3% sequence divergences indicate an ancient multifurcation of the major lineages. Using an evolutionary rate of 3% substitutions/site/million years (Suzuki et al. 2015), the multifurcation was estimated to have occurred between 300,000–500,000 years ago.

**M*clr* sequences

We examined the entire coding region of the coat color-related gene *M*clr (948 bp) and identified two variable sites that were synonymous substitutions (Table 1). A comparison of *M*clr sequences and those from databases representing the other three major subspecies groups of CAS, MUS, and DOM (Kodama et al. 2015) revealed that the *M*clr sequences of the Madagascar mice were positioned close to a cluster of sequences from CAS and DOM and distant from those from MUS. According to Kodama et al. (2015), the DOM group is represented by only a single haplotype of *M*clr, which is also shared by the CAS mice. The three Madagascar haplotypes comprised of the CAS/DOM haplotype and its satellite haplotypes differed by a single substitution.

**Asip sequences

Using the seven mice from Madagascar, two short *Asip* segments were identified and termed *Asip*-1A (317 bp) and *Asip*-1B (499 bp). These segments contained the pro-
Fig. 5. A ML tree for mitochondrial cytochrome b gene sequences (1,140 bp) of Madagascar mice and those from databases for the four major lineages of *M. m. castaneus* (CAS), *M. m. domesticus* (DOM), *M. m. musculus* (MUS), and Nepal (NEP). Rat species were used as the outgroup taxa. The Madagascar lineage was characterized as *M. m. gentilulus* (GEN) based on the phylogenetic analyses shown in Figure 3. Bootstrap support values (>50%) are shown at the nodes and were based on 1,000 resampling replicates.

The promoter regions, 1A and 1B, which were located 100 kb and 30 kb upstream of the coding regions (namely, exons 2, 3, and 4), respectively. There was considerable sequence variation, and two distinct haplotypes were present in both promoter regions 1A (1A-I, 1A-II) and 1B (1B-I, 1B-II) (Fig. 2, Table 1). Remarkably, the 1A haplotypes of 1A-I and 1A-II were associated with the ventral color groups V-I and V-II, respectively. The homozygous (1B-I/1B-I) mouse (No. 2) had a light brown (color group type D-I) dorsal side, while the four heterozygous 1B-I/1B-II mice were divided into light (D-I, No. 4) and dark brown (D-II, Nos. 3, 6, and 7) dorsal color types. Two additional homozygous (1B-II/1B-II) mice had dark brown fur on their dorsal sides (D-II).

We compared the two currently determined 1A sequences (317 bp) with the database sequences of spe-
cific 1A promoter region alleles of Asip (locus A), A" (white-bellied agouti), and a (nonagouti) (Vrieling et al. 1994; Chen et al. 1996). The pseudo promoter region (ps1A) of A" was also assessed. ML analyses indicated that the haplotype of Asip-1A-I, belonging to the lighter color type, was identical to the sequence of 1A from A", but differed substantially from the other sequences (Fig. 2b). The haplotype of Asip-1A-II was distinct from all sequences.

Discussion

Mus musculus often shows variation in coat color among populations and even within a population, some of which is due to human- and nonhuman-mediated secondary contact with genetically distinct subspecies groups. This may provide opportunities to evaluate the genetic systems responsible for the evolutionary changes in coat color, while also facilitating assessments of the evolutionary histories of mouse populations. In this study, we focused on the genetic systems of coat color variation seen in house mice of Madagascar, as well as the evolutionary origins of mouse lineages introduced by humans.

The morphological characteristics of skull size (Harrison 1970) and the appearance of a specific coat color type, “sandy” hair color (Thomas 1919), provide circumstantial evidence of the introduction of the South Arabian house mouse M. m. gentilulus in Madagascar. As hypothesized previously (Duplantier et al. 2002), the mtDNA sequence analyses provided evidence for the contribution of M. m. gentilulus from Yemen. Our Cytb sequence analyses also indicated that M. m. gentilulus was equally distinct from all other known major lineages of M. m. castaneus, M. m. domesticus, M. m. musculus, and mice from Nepal (Fig. 5). These data are consistent with previous studies of the CR marker (Suzuki et al. 2013) that estimated divergence at a half million years ago. Our analysis of nuclear gene McIr sequences revealed two single nucleotide polymorphisms (nucleotide positions 390 and 927 from the start site of exon 1) in mice from Madagascar (Table 1). The haplotypes were closely related to those of the South Asian subspecies group M. m. castaneus (Kodama et al. 2015), and one of the haplotypes is predominant in M. m. castaneus and M. m. domesticus (Kodama et al. 2015). Notably, both sequences of the two genic regions of Asip revealed two divergent haplotypes in the Madagascar mice (Fig. 2), implying a contribution of two distinct lineages. In summary, we characterized Madagascar mice based on morphological and molecular analyses, as well as previous data (Harrison 1970; Prager et al. 1998; Duplantier et al. 2002) to compare mouse populations from South Arabia to that of Madagascar. We also identified additional subspecies groups of M. musculus. Our future studies will validate whether the two divergent genetic properties of Asip implied multiple introgression of mice to Madagascar from Yemen and other regions, or an admixed state of South Arabian mice before arriving in Madagascar.

We addressed the genetic properties of coat color variation observed in Madagascar mice, which show considerable differences between the dorsal and ventral sides. The cluster analysis based on the measurements of L*, a*, and b* enabled us to categorize the coat color variations into two groups for the dorsal (light and dark brown; Fig. 3c) and ventral (light and dark gray; Fig. 3d) sides. Notably, irrespective of any apparent coat color variations in the seven mouse specimens examined in this study, there appeared to be no nonsynonymous changes in the coat color-related gene McIr, implying that genes other than McIr underlie coat color variation in Madagascar mice.

Accordingly, analyses of the two promoter regions suggested the involvement of another key gene, Asip, in the evolution of coat color. In Asip, sequences (300–500 bp) close to the two exon-1-associated promoter regions were significantly polymorphic among the seven specimens, with two distinct haplotypes found in each of the exon-1-associated promoter regions; exon 1A and exon 1B. As with the coat color patterns of the dorsal and ventral sides, there was no relationship between the patterns of the two promoter regions; namely, there appeared to be no linkage disequilibrium between the two intragenic regions, which are separated by ~70 kb, and this enabled analysis of any association between the two Asip haplotypes with coat color, separately.

Our results strongly suggest that allelic variation in the exon-1A-associated promoter region affects the ventral coloration phenotype (light gray, n = 3; dark gray, n = 4) (Table 1). This agrees with previous data indicating that the 1A promoter regulates ventral color, by modulating Asip expression (Vrieling et al. 1994). Interestingly, the haplotype Asip-1A-I that was associated with lighter coat color on the ventral side of Madagascar mice was identical to that of the Asip A" allele that is associated with white bellies (Vrieling et al. 1994; Chen et al. 1996). It is unclear how the inverted repeats lying 90–140 kb upstream of the coding region are responsible for changes
in ventral coat color (Fig. 2a, see Chen et al. 1996 for details). Thus, future studies will focus on such questions, as well as those related to the evolutionary histories leading to the geographic variations in Asip promoter regions.

Mice from the south Arabian Peninsula, an arid region, display a “sandy” color on their dorsal side (Thomas 1919); therefore, it is crucial to understand the genetic systems underlying this color variation. Our results did not identify a clear relationship between the 1B promoter region and dorsal color. The genotypes of the two mice with lighter dorsal color were different. One (No. 2) was homozygous for Asip-1B-I, while the other (No. 4) was heterozygous for Asip-1B-1/1B-II, whose genotype was shared by four mice with dark brown backs (Nos. 1, 2, 4, and 7). Thus, larger sample sizes should be used in the future to verify such findings.

In conclusion, our work using mtDNA sequences supports the previous notion that Madagascar mice originated from the south Arabian Peninsula. On the other hand, our sequence data suggest certain complex historical episodes associated with the genomic structure of Madagascar mice. In this study, we examined allelic association with coat color in both of the Asip promoter regions, which are ~70 kb apart, possibly resulting in the lack of linkage disequilibrium between these regions. The genetic analyses undertaken in this study could improve our knowledge of the genetic systems underlying variations in coat color of house mice, which display significant variation in natural populations of rodents.

Acknowledgments: We would like to thank Takamichi Jorgahara for assistance during capture efforts. We are grateful to Azusa Nakamoto, Mitsuru Sakaizumi, Jun J. Sato, and Toyoyuki Takada for their valuable comments. We extend our thanks to Satoko Mori who guided the quantitative analyses of coat color variation. This study was supported by Grants-in-Aid for Scientific Research (B) to HS (24405013) and to SO and SA (25304009) from the Japan Society for the Promotion of Science.

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*Received 29 March 2016. Accepted 26 May 2016.*

*Editor was Jun J. Sato.*