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小家鼠與黑腹絨鼠毛色基因之演化遺傳分析

Evolutionary genetic analysis of coat color genes in the house mouse (*Mus musculus*) and the Pére David's vole (*Eothenomys melanogaster*)

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中文摘要

遺傳多樣性爲生物多樣性研究之重要一環，實驗動物-小家鼠(*Mus musculus*)及其近親鼠類的毛色基因，因毛色變化多端，爲研究遺傳多樣性的良好材料。

我們使用 Munsell 土壤比色卡描述亞洲小家鼠的毛色變異，發現符合 Gloger's rule：恆溫動物在潮溼環境的個體毛色偏黑，在乾燥的棲地毛色偏淡。小家鼠的背部毛色從黃色、褐色到黑色間變化，腹部毛色從白色至黑色間變化。背部毛色的變異較腹部毛色小，且在野外族群發現的毛色種類，遠不如實驗室純品系老鼠的各式毛色變化。小家鼠背部毛色的明暗和雨量有顯著相關，植被茂密且潮溼的環境(環境背景色較深)，背部毛色偏黑，在植被稀疏且乾燥的環境(環境背景色較淡)，背部毛色偏淡，暗示背部毛色可能是受保護色的天擇作用。

分析小家鼠 *Agouti* 基因，發現在 intron 1 的位置上有 endogenous retroviruses (ERVs)和 long terminal repeats (LTRs)的插入，插入的比率不低，同時插入的位置非常保守。沒有找到插入的突變和毛色或體重的關聯性，暗示插入的突變對個體沒有不利的影響，因此在野生族群中能維持如此比率的突變基因型。

另一個基因 melanocortin 1 receptor (*Mc1r*)亦被發現會影響許多野生動物的毛色，然而有關它的調節區研究卻很少。我們首先取得黑腹絨鼠 *Mc1r* 基因上游的序列，結果顯示無論是調節區或編碼區的序列變異，均與毛色變異無關，表示黑腹絨鼠的毛色應是受其它基因的作用影響。

關鍵字：黑色素皮質素受體 1 基因、鼠灰色基因、毛色、格洛格氏法則、5 端快速基因放大技術、小家鼠、黑腹絨鼠

英文摘要

The genetic diversity is an important research area for biodiversity. The candidate genes for highly varied coat color of the laboratory mouse (house mouse, *Mus musculus*) and other rodents are promising subjects for the research of genetic diversity in wild populations.

We described the coat color variation of house mice in Asia by using Munsell Soil Color Charts and found it is consistent with Gloger's rule, *i.e.*, individuals of endothermic animals are darker in humid habitats than those in drier habitats. Dorsal coat color ranged from yellow through brown to black, whereas ventral coat color ranged from white to black. Dorsal coat color varied less than the ventral color. In our samples, the variation in coat color in natural populations was far less than that has been observed in the laboratory. We found a significant correlation between the lightness variable of dorsal coat color and precipitation. Mice with dark coat color were observed in more humid and closed habitats (darker background color), and pale coat color in drier, more open habitats (lighter background color). This result might imply the role of concealment as a selective force affecting dorsal coat color in house mice.

We screened insertional mutations in intron 1 of a coat color candidate gene – *Agouti*, in wild mouse populations. These insertions were found to be caused by transposition of endogenous retroviruses (ERVs). The frequency of retrotransposition in *Agouti*'s intron 1 is not low and the insertion site is very conserved. No association between the retrotransposition and the variation of coat color or body weight was found. The insertional mutations may be non-deleterious alleles, therefore maintaining a certain frequency in wild populations.

On the other hand, the molecular genetic changes associated with adaptive morphologies remain an interesting puzzle in evolutionary biology. Previous studies have shown that mutations in the coding regions of another coat color candidate gene, melanocortin 1 receptor (*Mc1r*) underlies coat color variation in a wide range of animal species. However, the effects of regulatory regions of *Mc1r* on coat color variation still remains unclear. In this study, we obtained the upstream sequences of the *Mc1r* gene from Pére David's vole (*Eothenomys melanogaster*). No association was found between the coat color variation and the polymorphisms in either regulatory or coding

sequences. This implies that there may be other genes, acting alone or in concert with *Mc1r*, underlying coat color variation in Pére David's vole.

Keywords: *Mc1r*, *Agouti*, coat color, Gloger's rule, 5'RACE, *Mus musculus*, *Eothenomys melanogaster*

目　　錄

第一章、文獻回顧

1.1 適應性演化表型與基因型的相關性

Stearns and Hoekstra(2000)認爲隨著科技與知識的進步，演化生物學於 21 世紀需挑戰的兩大謎團爲：爲何有些特徵在不同親緣物種(lineage)間非常穩定，如陸域脊椎動物的四足特徵；相對的，某些特徵卻變化很大，如動物的毛色，即使在特定族群內亦有變化。他們認爲上述兩個問題的解答，有待特徵的表型與基因型關係(phenotype-genotype relationship)獲得澄清，方能得到明確的答案。適應性特徵之表型與基因型關係的釐清，在演化生物學具有很重要的意義，因爲天擇作用在表型上，它挑選具特定表型的個體，然而，天擇選汰下存活的個體，遺傳給子代的不是表型，而是特定的基因型。因此，我們惟有清楚了解表型與基因型之間的落差，方能理解適應性演化發生的遺傳機制，除了知道天擇作用在哪些基因上，並能檢驗目前已被提出的一些適應性演化假說，即(一)主要由多基因造成、(二)主要爲顯性對偶基因造成、(三)主要由基因調節區(regulatory region)造成(Steiner, Weber & Hoekstra, 2007; Wlasiuk & Nachman, 2007)。

本研究的主旨爲探討一個受選汰作用的特徵(適應性表型)，其與基因型之間的關聯性。這類型的研究，一開始出現在探討人爲選汰(artificial selection)特徵，如老鼠對滅鼠靈(warfarin)的抗藥性基因(Kohn, Pelz & Wayne, 2000; Kohn, Pelz & Wayne, 2003)，另外像對重金屬、殺蟲劑、抗生素等的抗藥性研究亦屬此類(Wootton et al., 2002)。關於天擇選汰特徵的探討，從 Nachman, et al. (2003)於 PNAS 上發表第一篇天擇選汰(natural selection)特徵的表型-基因型相關性研究，爲這類研究跨出了第一步。他們發現口袋鼠 (*Chaetodipus intermedius*)毛色變化是受天擇作用的結果，藉由表型-基因的相關研究，證實毛色變化是 *Mc1r* 基因上的點突變(point mutation)造成的結果，且連鎖不平衡(Linkage disequilibrium)分析亦發現 *Mc1r* 有受天擇影響的證據。在他們之後，Mundy et al. (2004)研究鳥類、以及 Rosenblum et al. (2004)研究爬蟲類，都發現 *Mc1r* 基因的點突變，能解釋受性擇或天擇影響的適應性羽色或體色變化，且一致發現天擇作用在少數的毛色基因上，而適應性 allele 皆爲顯性的 coding mutation。其中，Mundy et al. (2004)研究的是連續性羽色變化，非類別特徵(如毛色變化只有黑化與非黑化兩種類型)，同時發現

兩種不同鳥類(雪雁與賊鷗)，有相同的適應性突變，爲平行演化的結果，爲這個領域的研究，又往前跨了一步。之後，Rompler et al. (2006)利用相同的方法，發現 *Mc1r* 基因影響猛瑪象的毛色，爲這方面的研究，在古生物學上，開拓一個全新的視野。

上述的研究，皆僅針對一個基因和一個適應性表型做研究(圖 1)。直到 Hoekstra et al. (2006)研究 beach mouse (*Peromyscus polionotus*)的毛色變異，發現 *Mc1r* 基因同時會影響老鼠身上七個部位的毛色變異(鬍鬚、吻、頰、眉、耳、腹、腳踝)，算是初步開始了一個基因對多個性狀的研究。另一方面，他們進一步的研究發現，*Mc1r* 和 *Agouti* 基因，同時會影響 beach mouse 的毛色(Steiner et al., 2007)，而且兩個基因有交互作用的影響(gene-gene interaction)，算是開啓了多個基因對一個性狀研究的序幕。未來，適應性表型之遺傳機制的研究，會不斷的深化，慢慢往多基因對多性狀的方向邁進，最終期望能了解整個 genome 對個體所有表型的影響，解開特徵演化的謎團。

最後值得一提的是，在適應性表型與基因型相關性的研究領域中，有一些研究的結果呈現不顯著或無關聯。包括 *Mc1r* 基因(Kerns et al., 2003; MacDougall-Shackleton, Blanchard & Gibbs, 2003; Mundy & Kelly, 2003; Rosenblum et al., 2004)、*Agouti* 基因(Kerns et al., 2003)和 *TYRP1* 基因(Guibert et al., 2004)，皆有例子顯示毛色基因的基因型(genotype)和毛色(phenotype)間沒有關聯。然而上述的結果未必表示毛色的變化不受這些基因的影響。若以 *Mc1r* 基因爲例，有可能 *Mc1r* 造成毛色變異的序列位置尚未被分析，也可能毛色的變化是基因-基因交互作用的結果。克服的方法需要分析候選基因的更多區域，或是分析更多的基因。另一方面，從口袋鼠(Hoekstra & Nachman, 2003; Nachman, 2005)的研究得知，某些族群的毛色變化是由 *Mc1r* 決定，其它族群則由另外的基因突變產生。所以也可能是因爲所研究的族群剛好不受 *Mc1r* 突變影響，因此偵測不出有相關性，克服的方法需研究更廣泛的族群做取樣分析。

1.2 動物毛色的適應性演化

Stearns and Hoekstra(2000)提出的兩大謎團中，動物的毛色屬於變化很大特徵的典型代表。毛色對動物而言，扮演非常重要的角色。它的功能包括形成保護色

(Ortolani, 1999; Stoner, Bininda-Emonds & Caro, 2003a)、警戒色、具有擬態、隔離紫外線(photoprotection)等作用、且具有調整體溫的生理功能(Stoner et al., 2003a)，亦可能有與其它個體溝通的作用 (Ortolani, 1999; Stoner et al., 2003a; Stoner, Caro & Graham, 2003b)，或者在求偶上具有特別的意義。因爲上述的諸多原因，動物的毛色變化，往往被視爲天擇或性擇的產物(Burtt, 1981; Cloudsley-Thompson, 1999; Caro, 2005)。

最近一些有關食肉目(Ortolani, 1999)、偶蹄目(Stoner et al., 2003b)，和兔形目(Stoner et al., 2003a)的毛色變化研究，皆顯示毛色爲適應性演化的結果。而在齧齒目 oldfield mice 的研究 (Sumner, 1926; Kaufman, 1974; Belk & Smith, 1996)，亦發現毛色是鼠類的重要保護色。因此本實驗以毛色爲研究適應性演化的特徵，是很好的研究材料。

1.3 動物色素形成機制

哺乳類的毛色，是由黑色素(melanin)的數量與分佈情形所決定。哺乳類的黑色素可分爲二大類：真黑色素(eumelanin)與僞黑色素(pheomelanin)，二者皆由黑色素細胞(melanocyte)在毛球(hair bulb)產生。真黑色素爲黑色或褐色，僞黑色素爲黃色或紅色，二種黑色素的數量、密度、大小，以及混合比例會形成哺乳類的各種毛色，包括黑色、褐色、黃色、灰色，以及白色等。二種黑色素，主要都是由左旋酪氨酸(*L*-tyrosine)轉變而成(圖 2)，黑色素體表面有 active tyrosine transporter 負責運輸此原料。不過左旋苯丙氨酸(*L*-phenylalanine)亦可能成爲黑色素的原料，它可以先轉變成左旋酪氨酸，再進一步合成黑色素。真黑色素造成毛髮成黑色到褐色的變化，它的結構較緊密，不溶於大多數的溶劑中，隔絕紫外線能力較強(DHI-eumelanin > DHICA-eumelanin)，但合成過程中產生的毒性亦較高(DHI-eumelanin > DHICA-eumelanin)。反之僞黑色素使毛髮形成黃色到紅褐色的變化，易溶於鹼性溶液中，隔絕紫外線的能力較弱，但毒性較低。黑色素中，DHICA-eumelanin 爲毒性與隔絕紫外線功能的折衷選擇，亦是哺乳類動物最主要合成的黑色素種類(Hearing, 2000)。

真黑色素的生成機制目前較爲清楚，它經由 tyrosinase 將左旋酪氨酸氫氧化(hydroxylation)成 *L*-DOPA (*L*-dihydroxyphenylalanine)；接著 *L*-DOPA 再被

tyrosinase 脫氫(dehydrogenation)成 dopaquinone，其中 Mn^{2+}、Cu^{2+}等金屬離子可加快 DOPA 氧化的速度；dopaquinone 轉變成 leukodopachrome 後，經歷一系列氧化還原反應(oxidation/reduction reactions)，分別產生 DHI (5,6-dihydroxyindole)和 DHICA (5,6-dihydroxy-indole-2-carboxylic acid)。它們分別受到 tyrosinase 和 Tyrp1 (tyrosinase-related protein 1)的催化，形成 DHI-eumelanin 和 DHICA-eumelanin。DHI-eumelanin 爲黑色化合物，分子量較高，較不易溶解；DHICA-eumelanin 爲褐色化合物，分子量較低，且較易溶解。二者以各種比例混合成真黑色素，故真黑色素其實是一種異質聚合物(heterogeneous polymers)。

另一方面，僞黑色素的形成機制雖然較不清楚，但亦是先經由 tyrosinase 將左旋酪氨酸氫氧化成 *L*-DOPA；接著 *L*-DOPA 再被 tyrosinase 脫氫成 dopaquinone，故 dopaquinone 爲真黑色素和僞黑色素的共同前驅物(precursor)。之後，dopaquinone 和 cysteine 或 glutathione 聚合，分別形成 cysteinyldopa 以及 glutathionyldopa (主要爲 cysteinyldopa，因爲 melanosome 只有 cysteine 的 active membrane transporter，但沒有 glutathione 的 active membrane transporter，經過一系列反應合成僞黑色素。其中，黑色素細胞內 cysteine 和 glutathione 濃度，對形成哪種黑色素具有決定性影響。當濃度高時，它們會和 dopaquinone 結合而形成 cysteinyldopa 和 glutathionyldopa，進一步成爲僞黑色素；相對的，濃度低時，dopaquinone 會轉變成 dopachrome，進而形成真黑色素。

黑色素僅分佈於黑色素細胞的黑色素體(melanosome)內。黑色素體分爲二類：真黑色素體(eumelanosome)和僞黑素體(pheomelanosome)，前者爲楕圓形，內部成纖維狀(fibrillar matrix)；後者形狀較多變，但大致成圓形，內部成氣泡狀(vesiculoglobular matrix)。兩者可以共存於一個黑色素細胞中。真黑色素體的形成包含 4 個步驟(Slominski et al., 2004)：第一、早期的 matrix 開始聚合；第二、matrix 聚合完成，但是黑色素尚未成形；第三、黑色素生成；第四、黑色素體完全黑化(melanized)。環境 pH 值對黑色素生成具有決定性的影響，黑色素體具有質子幫浦(proton pump)可調控內部的 pH 值，當黑色素開始生成時，環境成酸性，促使 tyrosinase 將左旋酪氨酸水解成 *L*-DOPA。此時爲黑化(melanogenesis)的速率決定步驟(rate-limiting step)，因爲之後的步驟可在正常生理 pH 值環境中自發反應。隨後，黑色素體內部轉變成中性或鹼性環境，以利黑色素的合成。另一方面，僞

黑色素體的形成機制較不清楚，不過它在第二步驟時已有僞黑色素成形，表示 phenomelanogenesis 過程中的 tyrosinase，較 eumelanogenesis 還早活化。

黑色素細胞爲特化的樹突細胞(dendritic cells)，起源於外胚層的神經脊(neural crest)。胚胎發育過程中，黑色素細胞會受基因調控而遷移到特定的位置，黑色素細胞在表皮的分佈並不均勻，因此形成動物身上的各種花紋。黑色素細胞會受許多因子影響，決定黑色素的生成與種類(Hearing & Tsukamoto, 1991; Hearing, 2000)。舉例說明，紫外線(ultraviolet light, UVL)照射，能促進黑色素的生成。在適當的刺激下，黑色素細胞內的 tyrosinase mRNA 會在數天內上昇 2~3 倍，同時 tyrosinase 的催化活性能增加 20~100 倍。α 促黑激素(α melanocyte stimulating hormone, αMSH)與 Agouti protein 是另外二個重要的 melanogenic regulators(圖 3)。αMSH 能增加 tyrosinase 的活性，影響黑色素形成的種類。它由腦下垂體後葉(posterior pituitary)分泌，與黑色素細胞表面的黑素皮質受器(melanocortin 1 receptor, Mclr)結合，會活化黑色素細胞內的 protein kinase A 促使細胞中的 cAMP 大量增加，促進 tyrosinase 的活化(使 tyrosinase gene 表現量提高 20 倍)，促使黑色素細胞內的 tyrosine 合成真黑色素，造成毛色偏黑。另一方面，細胞外的 Agouti protein 會與 αMSH 競爭 Mc1r，當 Agouti 與 Mc1r 結合時，會降低細胞內的 cAMP，進而同時降低 tyrosinase、*Tyrp1*、*Tyrp2* (*Dct*)、*silver* 和 *pinkeyed-dilution* 等基因的表現(>50%)，使得黑色素細胞內的 tyrosine 合成僞黑色素，造成毛色偏黃。

1.4 毛色基因

有關動物毛色的遺傳研究，和遺傳學本身的歷史幾乎一樣久遠。遠在 20 世紀初期，也就是孟德爾遺傳定律再被發現後不久，W.E. Castle 和他的學生就已經在研究老鼠、兔子和豬等動物毛色的遺傳特性(Silvers, 1979)。甚至遠從 1866 年開始，孟德爾(Gregor Mendel 1822-1884)，就已經在研究老鼠的毛色，只是後來受教會的禁止才改做豌豆的實驗(Henig, 2000)。經過一個世紀的知識累積，我們已知黑色素細胞(Melanocyte)主導了毛色的變化。毛髮生長過程中，位於毛囊的黑色素細胞表面有 Mc1r (Melanocortin-1 receptor)受器(圖 3)，當 Mc1r 受到促黑激素(MSH, melanocyte-stimulating hormone)刺激時，會促進黑色素細胞分泌黑色素。黑色素有 2 類，一類爲產生黑色、褐色的真黑色素，另一類造成毛髮呈現黃色、紅色的

僞黑色素。*Mc1r* 基因在毛色形成過程，扮演關鍵性角色，當 Mc1r 受到 MSH 刺激，真黑色素的分泌量會增加。相反的，若 MSH 的刺激量不足，或 Mc1r 受到 Agouti protein 抑制時，僞黑色素的分泌量則會增加。動物身上的毛色變化，主要就是由真黑色素和僞黑色素的數量以及分佈情形決定。現今有許多研究皆証實 *Mc1r* 的 gain-of-function 突變，會導致毛色黑化(毛髮中真黑色素的量增加)，相反的，loss-of-function 突變會導致毛色黃化或紅化(僞黑色素的量增加)。

飼養動物中，已有許多物種發現 *Mc1r* 當特定位置發生點突變(point mutation)，就能造成毛色的改變，包括小家鼠(Robbins et al., 1993)、牛(Klungland et al., 1995; Joerg et al., 1996)、馬(Marklund et al., 1996; Rieder et al., 2001)、豬(Kijas et al., 1998)、羊(Vage et al., 1999; Vage et al., 2003)、狐狸(Vage et al., 1997)、狗(Everts, Rothuizen & van Oost, 2000; Newton et al., 2000; Schmutz et al., 2003; Kerns et al., 2004)、貓(Eizirik et al., 2003)、雞(Takeuchi et al., 1996b; Kerje et al., 2003; Ling et al., 2003)等(表 1)。進一步於野生動物亦發現 *Mc1r* 基因會影響多種動物的毛色、羽色或體色，包括口袋鼠(*Chaetodipus intermedius*) (Hoekstra & Nachman, 2003; Nachman et al., 2003)、海灘鼠(*Peromyscus polionotus*) (Hoekstra et al., 2006)、美洲豹(*Panthera onca*)、南美豹貓(*Herpailurus yaguarondi*) (Eizirik et al., 2003)、黑熊(*Ursus americanus*) (Ritland, Newton & Marshall, 2001)和猛獁象(*Mammuthus primigenius*) (Rompler et al., 2006)。綜上所述，*Mc1r* 基因在哺乳類(Majerus & Mundy, 2003)、鳥類(Mundy et al., 2003)和爬蟲類(Rosenblum et al., 2004)的毛色(羽色、體色)形成過程具有關鍵性影響，這也是我們挑選 *Mc1r* 做爲研究毛色表型-基因型關聯性研究的候選基因(candidate gene)的原因之一。

除了 *Mc1r* 基因外，目前已知有超過 100 種以上的基因，會影響小家鼠(*Mus musculus*)的毛色變化(Jackson, 1994; Barsh, 1996; Nakamura et al., 2002; Bennett & Lamoreux, 2003)，其中更有超過 50 種基因已被選殖(clone)。這些基因分別在黑色素細胞生成的各個步驟產生影響，包括影響黑色素細胞從神經脊(neural crest)遷移的發育過程的基因(*i.e.*, *Kit*, *Kitl*, *Edn3*, *Ednrb*)、影響黑色素(melanin)合成的基因(*i.e.*, *Tyr*, *Tyrp1*, *Dct*)，控制黑化(melanogenesis)的基因(*i.e.*, *Pomc1*, *Mc1r*, *Agouti*, *Mitf*)，影響黑色素體生成的基因(*i.e.*, *Silver*, *Pink-eyed dilution*, *Ap3*)，和運輸黑色素體有關的基因(*i.e.*, *Mlph*, *Myo5a*, *Rab27a*)。其中特別以控制黑化的基因(*i.e.*,

Mc1r, Agouti)，對野生族群的毛色變化扮演關鍵性角色。而且依前人的研究結果，雖然上述許多基因都 會影響毛色的變化，但在自然情況下，野生小家鼠的毛色變異，只由 *Mc1r* 和 *Agouti* 這 2 個有上位作用(epistatic interaction)關係的基因，控制色素(pigment)的形成，它們能決定僞黑色素和真黑色素的多寡以及分佈(Silvers, 1979)。因此，接下來將更詳細介紹 *Mc1r* 以及 *Agouti* 這二個目前已知最可能影響動物毛色變化的基因。

人類和小家鼠的 *Mc1r* 最早於 1992 年被選殖出來(Mountjoy et al., 1992)，它是具有 7 個 transmembrane domain 的 G-coupled receptor protein。基因結構簡單，僅有 1 個 exon。許多動物的基因型變化已被證實與毛色變化有關(Klungland & Vage, 2000; Rees, 2000; Sturm, Teasdale & Box, 2001; Klungland & Vage, 2003; Majerus & Mundy, 2003; Mundy et al., 2003)，甚至在不同物種的毛色改變，是由同一個非同義突變(nonsynonymous mutation)所造成。例如小家鼠(*Mus musculus*)、雞(*Gallus gallus*)和曲嘴森鶯(*Coereba flaveola*)的 *Mc1r* 基因，同樣由 Glu92Lys 的突變造成毛色黑化(Robbins et al., 1993; Takeuchi et al., 1996a; Takeuchi et al., 1996b; Theron et al., 2001; Kerje et al., 2003; Ling et al., 2003)。又如口袋鼠(*Perognathus flavescens*)和北極賊鷗(*Stercorarius parasiticus*)在同源位置的突變(口袋鼠爲: His233Gln; 北極賊鷗爲: Arg230His)，皆和毛色變化有關(Hoekstra & Nachman, 2003; Nachman et al., 2003; Hoekstra, Drumm & Nachman, 2004; Mundy et al., 2004; Hoekstra, Krenz & Nachman, 2005; Nachman, 2005)。若我們找到的非同義突變(nonsynonymous mutation)和別種動物在相同位置上有類似的變化，即可爲基因型–表型相關結果，提供具有因果關係的佐證。表 1 即爲現今關於 *Mc1r* 以及 *Agouti* 的序列變異與動物毛色變化之整理。

鼠類的 Agouti 蛋白僅在表皮表現，是由黑色素細胞旁的 dermal papillae cells 分泌，只作用於毛囊的微環境，是 Mc1r 的 antagonist(Lu et al., 1994)，藉此影響鼠類的毛色。相反的人類 *Agouti* 基因(*Agouti*-signaling protein, ASIP)表現範圍很廣，包括脂肪組織、睪丸、卵巢、心臟、包皮、腎臟和肝臟等組織均會表現，但卻不影響人類的毛色，目前它在人類身上的功能仍然不是很清楚(Dinulescu & Cone, 2000)。

Agouti protein 只有 131 個胺基酸，無任何 transmembrane domains，由三個部

份組成，包括 N-terminal region、Pro-rich 的 central region 和 Cys-rich 的 C-terminal region，其中 C-terminal 是和 Mc1r 產生 antagonism 的主要部位。Agouti 爲高度 glycosylated 的蛋白質，且於高溫環境下仍具穩定性。它的基因結構相對 *Mc1r* 而言較爲複雜，包含四個 exons，其中 exon2、exon3、和 exon4 爲 coding exons，而 exon1 爲 non-coding exon，且又被細分爲 4 個部份，分別爲 exon1A、exon1A'、exon1B 和 exon1C (Bultman, Michaud & Woychik, 1992; Vrieling et al., 1994)。*Agouti* 基因 5 端的 transcript 帶有 1B 或 1C 的 untranslated exon，會影響鼠類整體的毛色。另一方面 5 端的 transcript 是否帶有 1A 和 1A'的 5'-untranslated exons 會影響腹部毛色的變化(Vrieling et al., 1994)。目前，我們已知於小家鼠的毛色變異上，*Agouti* 會受到 *Mc1r* 的影響(即上位作用)，當顯性的 *Mc1r* allele 造成毛色黑化時，Agouti 將失去 antagonist 的功效，此時不論 *Agouti* 基因呈現何種基因型，都不會造成明顯的毛色變化。

1.5 研究問題與目的

本論文以在亞洲採集到的小家鼠(*Mus musculus*)和在台灣採集到的黑腹絨鼠(*Eothenomys melanogaster*)爲研究材料，透過量化的方式描述野生族群的毛色變化，並與環境因子(雨量)做相關，嘗試解釋自然族群中毛色變化的部份原因。進一步分析小家鼠毛色基因 *Agouti* gene intron 1 的保守區域，描述其於野生族群中的變異，包含 retrotranspon 造成的插入或缺失等基因型變化。最後分析黑腹絨鼠毛色基因 *Mc1r*，包含 regulatory 和 coding regions 的序列變異；除了與毛色做相關外，亦分析 5'UTR 的序列特徵。

表 1、各種動物毛色的表型-基因型關聯性

Animal	Gene	Mutant	Association	Reference
House mice	*Mc1r*	His183Gln	***	Robbins, et al. 1993
	Mc1r	Leu98Pro	***	
	Mc1r	Glu92Lys	***	
Tobacco mice	*Mc1r*	Ser69Leu	***	Robbins, et al. 1993
Pocket mice	*Mc1r*	Cys18Arg	***	Hoekstra, et al. 2004
	Mc1r	Trp109Arg	***	Hoekstra & Nachman,
	Mc1r	Trp160Arg	***	2003
	Mc1r	His233Gln	***	Nachman, et al. 2003
Cat	*Agouti*	△123-124	***	Eizirik, et al. 2003
Jaguar	*Mc1r*	△301-315	***	Eizirik, et al. 2003
Jaguarundi	*Mc1r*	△283-306	*	Eizirik, et al. 2003
Dog	*Mc1r*	Arg306ter	***	Newton, et al. 2000
	Mc1r	Arg306ter	***	Schmutz, et al. 2002
	Mc1r		-	Kerns, et al. 2003
	Agouti	Arg96Cys	***	Kerns, et al. 2004
	Agouti		-	Kerns, et al. 2003
Fox	*Mc1r*	Cys125Arg	***	Vage, et al. 1997
	Agouti	△exon2	***	
Pig	*Mc1r*	Ala240Thr	***	Kijas, et al. 1998
	Mc1r	Leu99Pro	***	
	Mc1r	Asp121Asn	*	
Cattle	*Mc1r*	△771 or △772	***	Joerg, et al. 1996
Sheep	*Mc1r*	Met73Lys	***	Vage, et al. 1999
	Mc1r	Met73Lys	***	Vage, et al. 2003
	Mc1r	Asp121Asn	***	Vage, et al. 2003
Horse	*Mc1r*	Ser83Phe	***	Marklund, et al. 1996
	Mc1r	C901T	*	Rieder, et al. 2001
	Agouti	△2174-2184	***	Rieder, et al. 2001
Black bear	*Mc1r*	Tyr298Cys	***	Ritland et al, 2001

Chicken	*Mc1r*	Glu92Lys	***	Takeuchi, et al. 1996a,b
	Mc1r	Cys33Try	***	Takeuchi, et al. 1996b
	Mc1r	Glu92Lys	*	Kerje, et al. 2003
White-winged fairy-wrens	*Mc1r*	Thr16Ala	***	Doucet, et al. 2004
	Mc1r	Asp38Ile	***	
	Mc1r	Ile111Val	***	
	Mc1r	Arg157Gln	***	
	Mc1r	Ile166Val	***	
Arctic skua	*Mc1r*	Arg230His	*	Mundy et al, 2004
Bananaquit	*Mc1r*	Glu92Lys	***	Theron, et al. 2001
Lesser snow geese	*Mc1r*	Val85Met	*	Mundy et al, 2004
Eastern fence lizard	*Mc1r*	His208Tyr	*	Rosenblum, et al. 2004
Lesser earless lizard	*Mc1r*	Val168Ile	*	Rosenblum, et al. 2004
Little striped whiptail	*Mc1r*	Thr170Ile	*	Rosenblum, et al. 2004
Desert horned lizard	*Mc1r*		-	Rosenblum, et al. 2004
California legless lizard	*Mc1r*		-	Rosenblum, et al. 2004
Side-blotched lizard	*Mc1r*		-	Rosenblum, et al. 2004
Common gartersnake	*Mc1r*		-	Rosenblum, et al. 2004

註：*表示基因型與表型有顯著相關，***表示基因型與表型存在完全(100%)相關，-表示基因型與表型無顯著相關。

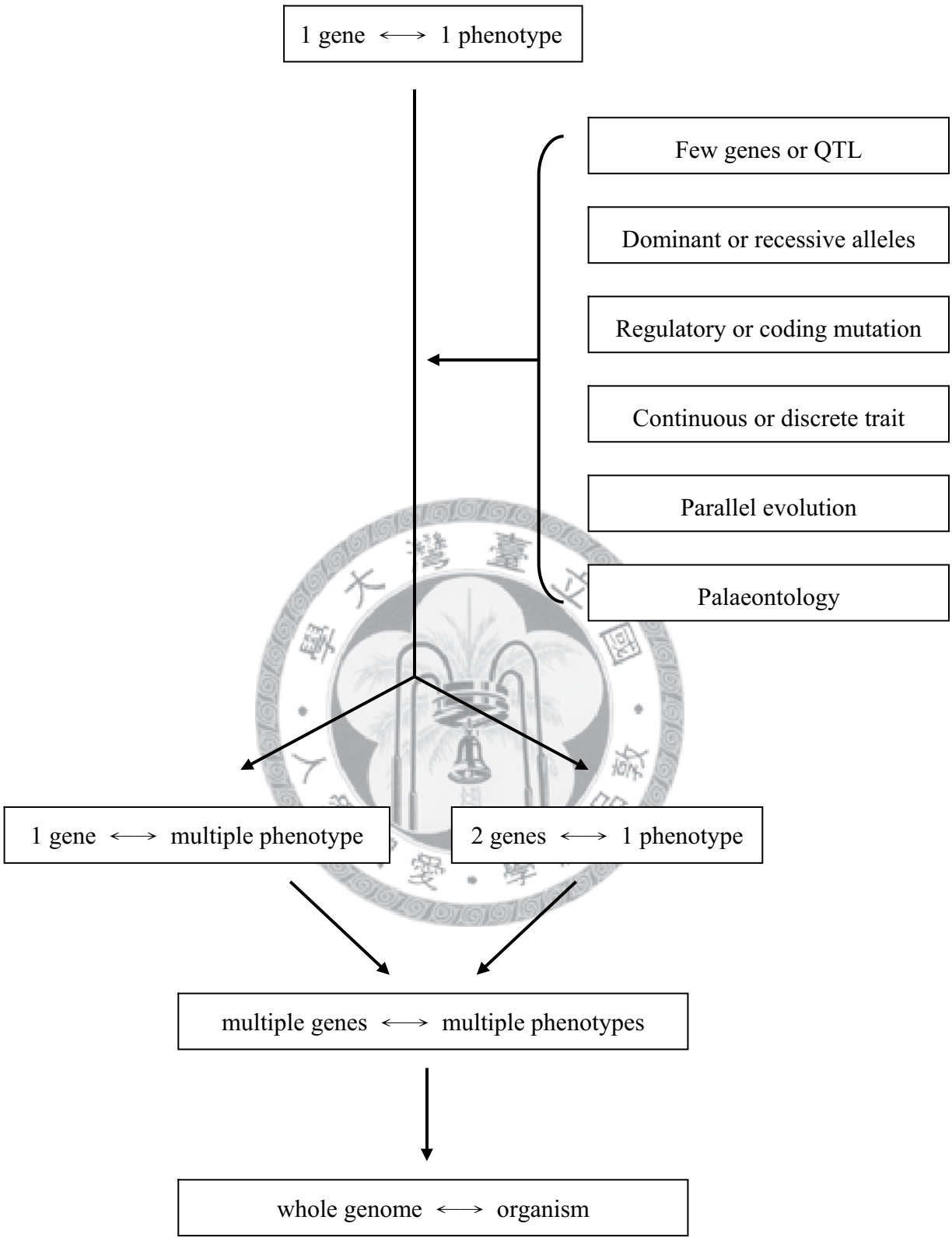

圖 1 適應性演化特徵，表型和基因型相關研究的發展史

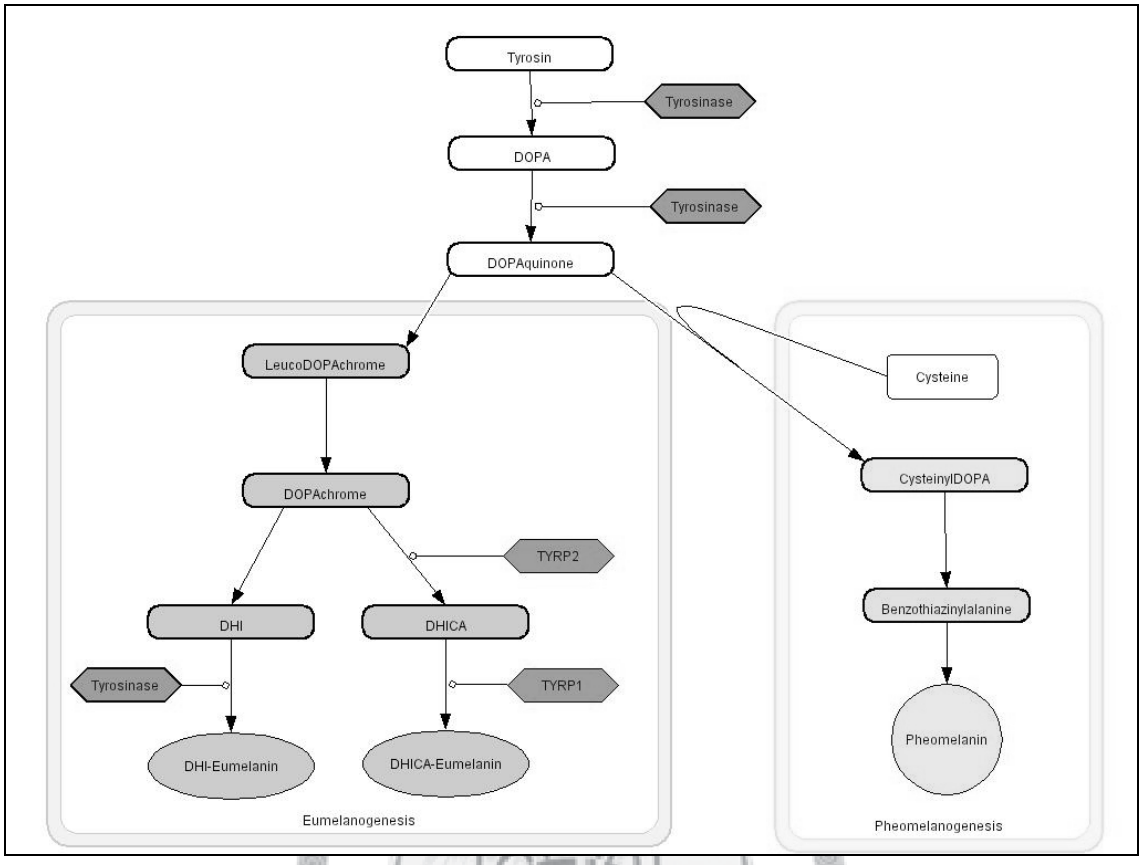

圖 2 黑色素(Melanin)生成圖。

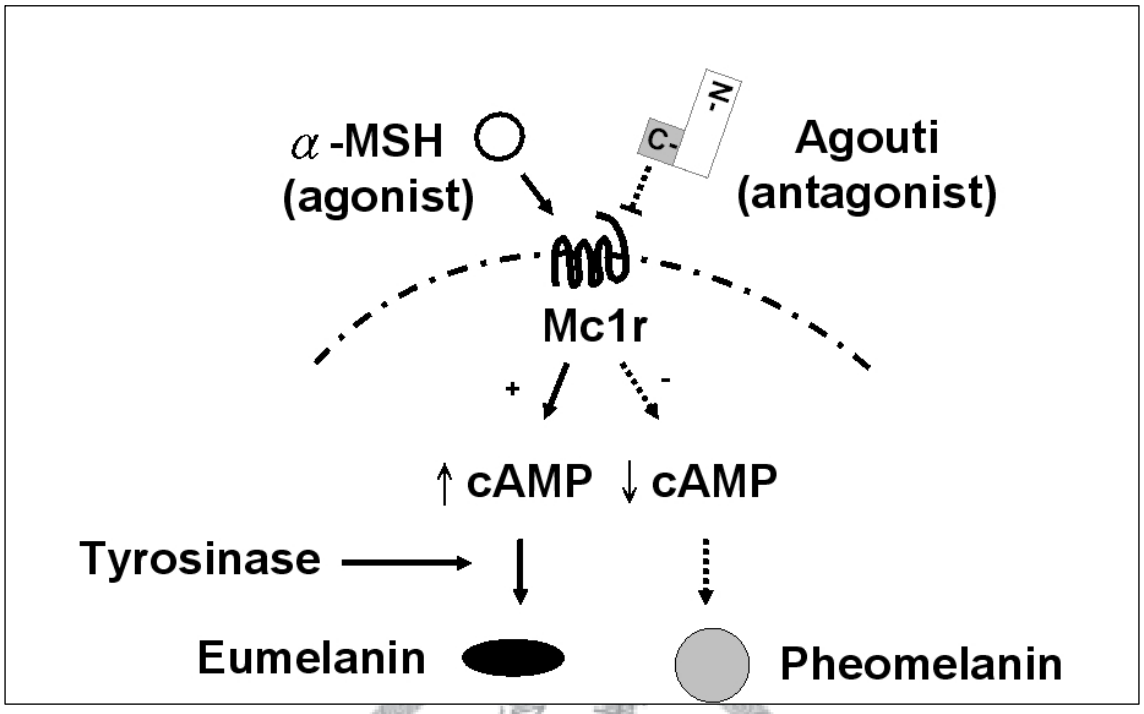

圖 3 黑色素生成(melanin synthesis)的調節機制。

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第二章、野生小家鼠的毛色變異

我研究日本國立遺傳研究所(National Institute of Genetics, NIG)、國立自然科學博物館、國立臺灣博物館典藏之小家鼠毛皮標本。發現小家鼠毛色與雨量變化有顯著相關，推測毛色具有保護色的功能，故推論小家鼠的毛色變異爲一種適應性演化特徵。此部份的研究結果如下，且已在 Journal of Zoology 發表(附錄一)。

Title: Variation of coat color in house mice (*Mus musculus*) throughout Asia

Abstract

Coat color variation due to melanin pigment synthesis in house mice (*Mus musculus*) in Asia is described and found to be consistent with Gloger's Rule which states that individuals of endothermic animals are darker in humid habitats than those in drier habitats. Three properties of coat color (hue, value and chroma) were measured, and a lightness variable was derived from a principal components analysis using 428 skin specimens representing three subspecies from 85 localities. Dorsal coat color ranged from yellow through brown to black, whereas ventral coat color ranged from white to black. Dorsal coat color varied less than the ventral color. In our samples, the variation in coat color in natural populations was far less than that has been observed in the laboratory. We found a significant correlation between the lightness variable of dorsal coat color and precipitation. Dark coat color was observed in more

humid and closed habitats (darker background color), and pale coat color in drier, more open habitats (lighter background color). This result might imply the role of concealment as a selective force affecting dorsal coat color in house mice. We also discussed other selective forces that could affect the coat color variation in house mice, like resistance to bacterial degradation and thermoregulation. In addition, the color spectra of the dorsal pelage among the three subspecies were different, the major distinction being the environmental background color of the habitats in which they are distributed.

Keywords: Gloger's rule, coat color, crypsis, *Mus musculus*, protective color

Introduction

Coat color is an important phenotypic characteristic in mammals because it is an intermediary for an individual to interact with environments and with other animals. Therefore coat color is tightly associated with an individual's survival and fitness. Adaptive significance of coloration in animals can be explained by several selective forces (Burtt, 1981; Cloudsley-Thompson, 1999). Yet, for mammals, many of the working hypotheses concerning the adaptive value of coat color were proposed more than 100 years ago and the field has progressed little since then (Caro, 2005). Recently, these hypotheses have attracted interest, and are again being explored and

tested (e.g., Ortolani, 1999; Stoner, Bininda-Emonds & Caro, 2003a; Stoner, Caro & Graham, 2003b; Nachman, 2005; Hoekstra, 2006; Hoekstra et al., 2006). The three most important adaptive factors influencing coat coloration in mammals are concealment, thermoregulation, and communication. For example, after removing the confounding effects of shared ancestry, pale coloration of lagomorphs has been strongly associated with open habitats serving the purpose of protective coloration (Stoner et al., 2003a). Similarly, coat color patterns provide crypsis for carnivores (Ortolani, 1999). Coloration may also be related to thermoregulation. Stoner et al. (2003a) found that dark coloration on extremities in lagomorphs might help conserve body heat in cold environments. In addition, coat color plays a role in animal communication. For example, dark ear tips in lagomorphs (Stoner et al., 2003a) and carnivores (Ortolani, 1999) have been shown to be useful signals for individual recognition, whereas conspicuous tail colors offer a similar function in artiodactyls (Stoner et al., 2003b).

Despite the fact that many coat color variants due to melanin synthesis and distribution have been well documented in laboratory mice (Silvers, 1979; Bennett & Lamoreux, 2003), we know little about coat color variation in wild house mice from which laboratory strains were originally derived. We know even less about the adaptive significance of coloration in wild mice. Taking advantage of a large series of wild mouse specimens housed in the National Institute of Genetics in Japan, we

document coat color variation in natural house mouse populations collected from areas spanning a large geographic range across Asia and evaluate in the house mice the applicability of Gloger's rule which demonstrates that mainly in birds the darker pigmented individuals tend to reside in more humid regions and the paler ones in drier areas (Gloger, 1833; Zink & Remsen, 1986). In addition, we explore the potential role of coat color variation as it relates to an environmental factor (precipitation) throughout the geographic range of these specimens.

Materials and Methods

We analyzed 428 specimens of house mice (*Mus musculus*) housed in the National Institute of Genetics (NIG) in Japan. The specimens were collected from 1980 to 1997 from 85 localities distributed throughout 16 Asian countries (Fig. 1). These localities lie between latitude 60° N and 7° S, and between longitude 60° E and 151° E. Countries, number of localities in each, and sample sizes are as follows: China (42 sites, 242 mice), India (four sites, 26 mice), Indonesia (two sites, four mice), Iran (one site, two mice), Japan (five sites, 22 mice), South Korea (two sites, 11 mice), Mongolia (one site, five mice), Nepal (two sites, seven mice), Pakistan (three sites, 13 mice), the Philippines (two sites, 11 mice), Russia (eight sites, 53 mice), Sri Lanka (four sites, five mice), Taiwan (one site, two mice), Thailand (one site, one mouse), Uzbekistan (four

sites, 11 mice), and Vietnam (three sites, 13 mice).

Two researchers (Y-C Lai and H-T Yu) independently determined the coat color of each mouse skin by comparing them to Munsell Soil Color Charts. In case of disagreement between the two researchers, a consensus was reached by re-examining the coat color together. The Munsell Soil Color Charts use tristimulus color scores (hue, value, and chroma) to depict colors. Hue indicates whether a color looks Red, Yellow, Green, Blue, or Purple; value indicates the color's lightness, and chroma indicates its strength or departure from a neutral color of the same lightness. The Munsell system is based on human perception, and therefore the outcome may not reflect actual visual effects, either among individual mice or between the mice and their predators (Endler, 1990; Bennett, Cuthill & Norris, 1994). Nevertheless, the standardized color schemes are still very useful for studies to analyze the color variation (Taylor, Meester & Rautenbach, 1990; Holt, Maples & Savok, 2003; Taylor, Kumirai & Contrafatto, 2005). For a quantitative analysis, we converted the three Munsell readings to numerical values following a method developed for forensic purposes (Sugita & Marumo, 1996). The conversion primarily affects hue which uses discrete integers to represent specific hues (see Table 1 in Sugita & Marumo, 1996). To further characterize the coat color, principal components analysis (PCA) was used to reduce the three color variables (hue, value and chroma) into a single variable (PC1) that represents

the largest proportion of variation in coat color and lightness of the coat color (also see results).

To evaluate Gloger's rule, which predicts that animals in more humid areas tend to be darker, we used correlation analysis to analyze coat color (PC1) in relation to precipitation, a climate factor that is known to be involved in the evolution of coat color (Gloger's rule; see in Zink & Remsen, 1986). Precipitation data were taken from the CPC Merged Analysis of Precipitation (CMAP; http://www.cdc.noaa.gov/cdc/data.cmap.html) (Xie & Arkin, 1997). The data set is grid by latitude and longitude (2.5° × 2.5°), and covers from 88.75° N to 88.75° S and from 1.25° E to 358.75° E.

Subspecies designations were recorded from museum specimen labels. However, only three subspecies were recognized for purposes of analyses, *i.e.*, *M. musculus musculus*, *M. musculus castaneus*, and *M. musculus bactrianus*, and we did not further distinguish more subspecies under *M. musculus musculus* proposed by Tsuchiya et al. (1994). Moreover, because the nuclear genome of *M. musculus molossinus* originated from *M. musculus musculus*, we assigned the hybrid subspecies (Yonekawa et al., 1994) distributed in Japan to *M. musculus musculus*. In addition, we recorded the sex from specimen labels, yielding 224 males, 197 females, and 7 specimens of unknown sex. We used Kruskal-Wallis test to examine the difference in coat color distribution among

the three subspecies described above. In addition, multiple regression analysis was used to account for the variance in lightness among mice, based on the precipitation and subspecies variables. Two indicator variables (Montgomery & Peck, 1982), subsp1 (coded 1 for *M. musculus castaneus* and 0 for others) and subsp2 (coded 1 for *M. musculus bactrianus* and 0 for others) will be required to incorporate the three levels of subspecies. Partial R^2 was used to distinguish the relative importance of the two independent variables.

Results

Overall dorsal coat showed fewer color types (21 types or direct Munsell readings) than ventral coat color (33 types) in the mice we examined. Dorsal color variation ranged from yellow through brown to black whereas ventral color varied from white to black (Fig 2). This trend held true even at a single locality. We found 1.54 ± 0.94 (mean ± S.D.) color types on the dorsum and 1.98 ± 1.95 types on the ventrum for the 85 localities. This difference is significant (t-test, $t_{168} = -2.36$, $p = 0.019$).

From the perspective of direct Munsell readings, value and chroma contained much more variation than hue, as reflected by the standard deviation (S.D.) and the coefficient of variation (CV) of the three Munsell readings: value, S.D. = 1.021, CV = 31.5%; chroma, S.D. = 1.096, CV = 26.6%; hue, S.D. = 0.096, CV = 2.4%. The

results suggest that value and chroma contribute to the majority of variation in house mouse coat color. PCA reduced the three Munsell readings into a single variable (PC1 = 0.93 × value + 0.92 × chroma + 0.26 × hue) that represents the largest proportion of the variation (59.3%) in coat color (Table 1). Taken together, PC1, in general, can be interpreted as lightness of coat color. The higher the value of PC1, the higher the Munsell scores for value and chroma (*i.e.*, more light-yellow); the lower the value of PC1, the lower the Munsell score for value and chroma (*i.e.*, more dark brown).

The standardized PC1 variable (lightness) between male and female were not significantly different (t-test, $t_{419} = 0.729$, $p = 0.466$). We, therefore, analyzed the data combining two sexes. The correlation between the standardized PC1 variable (lightness) and precipitation was highly significant ($r = -0.47$, $p<0.0001$) (Fig. 3). Precipitation explained 21.6% of the variation ($R^2 = 0.216$) in coat color. Paler coats were found in dry habitats, darker coats in more humid environments. Even within subspecies, the relationship was still significant (*M. musculus musculus:* $r = -0.22$, $p = 0.026$; *M. musculus castaneus*: $r = -0.34$, $p < 0.0001$; *M. musculus bactrianus*: $r = -0.81$, $p < 0.0001$). The pattern corresponds to Gloger's rule, which can be simply stated as animals in relatively humid environments are darker than their conspecifics in relatively dry areas.

Dorsal coat colors among the three subspecies were significantly different from

one another (Fig. 4, Kruskal-Wallis test χ^2_2=71.47, p< 0.0001). Among the three subspecies, *M. musculus castaneus* and *M. musculus bactrianus* occupy the darker end and the lighter end of the spectrum, respectively, whereas *M. musculus musculus* shows an intermediate distribution in color pattern.

The standardized multiple regression model, PC1 (lightness) = -0.37 × precipitation -0.14 × subsp1 + 0.09 × subsp2, indicated that the precipitation variable can explain more of the variation (21.59 %) in coat lightness in mice (partial R^2 = 0.2159) than the two subspecies indicator variables (1.24 % variance for subsp1, and 0.81% variance for subsp2). However, the regression coefficients of all three variables are significant (precipitation, t_{424} = -6.86, p<0.0001; subsp1, t_{424} = -2.54, p = 0.011; subsp2, t_{424} = 2.13, p = 0.034).

Discussion

The variation in coat color among wild house mice, as demonstrated here, is substantial. Furthermore, we have shown that house mouse coat color variation follows Gloger's rule. While the Gloger's rule is verified in many endothermic species, especially in birds (Gloger, 1833; Zink & Remsen, 1986; Hayes, 2001; Hayes, 2003), the causes were not readily known so far. Several non-mutually exclusive hypotheses can account for the plumage color variation in birds consistent with

Gloger's rule (see in discussion in Burtt & Ichida, 2004). Here we explore some explanations for the coat color variation in wild house mice.

The protective coloration can be one of most compelling explanations for the pattern despite a potential anthropogenic bias in analyzing the color perception (Endler, 1990; Bennett, Cuthill & Norris, 1994). The concealment effect has been demonstrated true in small rodents, such as, pocket mice (*Chaetodipus intermedius*) (Hoekstra & Nachman, 2003; Hoekstra, Drumm & Nachman, 2004) and oldfield mice (*Peromyscus polionotus*) (Smith, Carmon & Gentry, 1972; Belk & Smith, 1996) that their coat colors resemble soil background colors, supporting this hypothesis. These cases are convincing because predation experiments were conducted in field enclosures and confirmed that background color matching could increase survival rate in rodents (Dice, 1947; Kaufman, 1974). Furthermore, in *Mus musculus*, experimental evidence shows that both aerial (Kaufman & Wagner, 1973) and terrestrial (Brown, 1965) predators selectively prey on conspicuously colored individuals. Here, we adopt a conventional notion that precipitation reflects the environmental background color. Higher precipitation means higher vegetation density (*i.e.*, shade) and darker soil color (*i.e.*, saturated with moisture), both contributing to a darker background color. In contrast, lower precipitation means a lighter background color. Consequently, the significant correlation between coat color and precipitation ($r = -0.47$, $p<0.0001$) (Fig.

3), suggests that coat color variation in wild house mice results, in part, from a selective effect of crypsis. Additionally, less variation in dorsal color among individuals also suggests that the dorsal color is the major target for predation. To sum up, background matching will minimize differences between an animal's coloration and its surroundings; therefore, we consider that it is one of the rational explanations for the variation in coat color that we observed in the wild-caught house mice.

Recently the concealment explanation was found to be confounded by bacterial resistance in bird (Burtt & Ichida, 2004). Because bacteria are more abundant and active in humid environments and because the dark pigment, eumelanin resists bacterial degradation better than light pigment, pheomelanin (Hearing, 2000; Burtt & Ichida, 2004; Goldstein et al., 2004), the coat color variation in wild house mice following the Gloger's rule, likewise, might be a response to the selection to resist bacterial degradation. However, this explanation is less likely to be valid for the house mice because the color variation in the dorsum did not correspond to that of the venter. If the bacterial resistance had been an important factor, the selection force would have had similar effects on the dorsal and ventral coloration. However, rigorous experiments should be conducted to confirm the bacterial effect like in song sparrows (Burtt & Ichida, 2004).

Still, thermoregulation may play a partial role on the coat color variation. The

endothermic animals in cold climate tend to be darker for maintaining body temperature, because the dark coat color can absorb solar radiation more effective than the pale one (Cloudsley-Thompson, 1999; Caro, 2005). If the thermoregulation argument were true, a negative correlation may exist between the lightness of coat color (standardized PC1) and latitude, which inversely reflects annual temperature. This is only true in the subspecies of *M. musculus musculus* ($r = -0.183$, $p<0.0001$, data not shown), and yet the latitude (indirectly temperature) factor can account just 3.3% of the variation ($R^2 = 0.033$). Therefore, the thermoregulation argument is uncertain for the mouse mice, perhaps because animals can employ tactics without involving radiation to maintain body temperature.

Differences in coat color among the three subspecies examined (Fig . 4) are consistent with differences in precipitation throughout the areas in which the mice were collected (Fig. 1). The darkest subspecies, *M. musculus castaneus*, is distributed in humid areas and the lightest subspecies, *M. musculus bactrianus*, occurs in arid areas. The third subspecies, *M. musculus musculus*, shows an intermediate pattern (Fig. 4) and its distribution (Fig. 1) is broadest spanning from humid to arid areas. This pattern is supported by our multiple regression analysis which showed that the precipitation variable explains much more of the variation (21.59 %) in coat color than the subspecies variables (subsp1: 1.24% and subsp2: 0.81 %) do. Therefore, we suggest that the

differences in coat color among the three subspecies reflect parallel differences in levels of precipitation and thus environmental background colors of their habitats.

Since coat color variation, which can be explained by precipitation, accounts for only 21.59 % of the variation observed, some other environmental factors must be involved (e.g., microhabitats or factors associated with the animals' commensalism with humans). Because the precipitation data that CMAP provided are only a rough estimation, actual precipitation in microhabitats may deviate from the estimated data. Furthermore, the levels of predation pressure and other environmental parameters within microhabitats are unknown. All of these factors may contribute to the residual variation in coat color which can not be explained by precipitation. For example, the quality of habitats can affect animal color (Veiga & Puerta, 1996; Griffith, 2000; Fitze & Richner, 2002; Parker et al., 2003; McGraw, 2007). Some experiments also confirmed that the environmental stress was associated with the variation of feather color and the eumelanin could signal "good genes" (Johnston & Janiga, 1995; Roulin et al., 2000; Roulin et al., 2001; Roulin et al., 2003). Finally, the house mice are primarily commensal with human habitation, such as granaries and buildings. Although the house mice can easily disperse between human and natural habitats (Pocock, Hauffe & Searle, 2005), their coat colors, at most are only partially affected by natural selection (Merilaita, Tuomi & Jormalainen, 1999) and may be neutral when in

commensal habitats. Therefore, commensalism may be another factor that may contribute to the residual variation between coat color and precipitation, because polymorphic coat color can be maintain within a population (Roulin, 2004). Nevertheless, without information about other environmental factors, the highly significant correlation between the single indirect environmental factor (precipitation) and coat color variation may indicate that the selection pressure of background matching must be strong.

Like pocket mice (Nachman, Hoekstra & D'Agostino, 2003; Nachman, 2005), lesser snow geese and arctic skuas (Mundy et al., 2004), some genetic factors are responsible for variation in the coat color of house mice. Research on coat color genetics is almost as old as the science of genetics itself (Silvers, 1979). There are more than 100 loci and 800 phenotypic alleles of coat color known in laboratory mice today (Bennett & Lamoreux, 2003). However, house mice in natural populations have much less color variation than that has been observed in laboratory populations. In fact, many phenotypes which emerged from the laboratory, such as spotted, complete lack of pigmentation, mottled, belted, piebald, and albino (Jackson, 1994; Nakamura et al., 2002; Bennett & Lamoreux, 2003), are unlikely to be seen in the wild. We surmise that if coat color is constrained by selection (background matching) in natural populations, the alleles that act on variation in coat color of wild mice must be much

fewer than those in laboratory mice. When alleles are lethal or pleiotropically deleterious, the chances of being retained in natural populations are slim.

In conclusion, many of the mutant coat color alleles that have been observed in laboratory mice were induced by radiation or chemical treatments (Nakamura et al., 2002). These mutations are unlikely to happen spontaneously in natural populations. The major genes and alleles that have been found to act on coat color in other mammals (Majerus & Mundy, 2003), like *Mc1r*, *agouti*, etc., may still be the major candidate genes responsible for coat color variation in wild mice. A future attempt to associate the genotypes of some candidate loci with phenotypes as we clarified would shed light on the adaptive coloration in wild house mice.

Table 1 Variable loadings and percent variance explained by PCA.

Variables	PC1	PC2	PC3
Hue	0.257	0.965	0.044
Value	0.933	-0.072	-0.352
Chroma	0.917	-0.179	0.346
Percent variance explained	59.30%	32.50%	8.20%

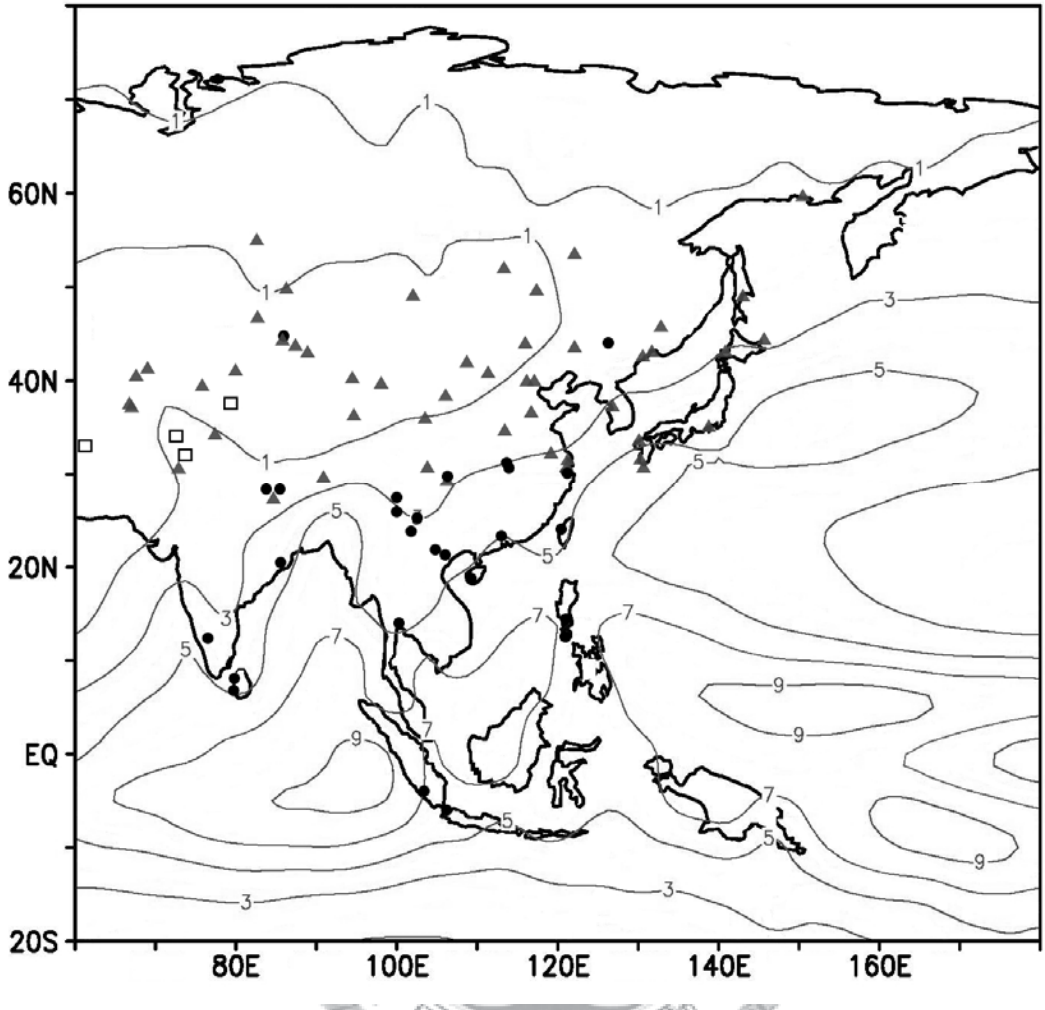

Fig. 1 Map showing localities from which specimens of *Mus musculus* used in this study were collected. Dark circles indicate *M. musculus castaneus* localities; gray triangles indicate *M. musculus musculus* localities; open rectangles indicate *M. musculus bactrianus* localities. Contour values represent mean annual precipitation from 1993 to 2002 (unit: mm/per day).

(a)

(b)

Fig. 2 Representative variation in coat color in wild house mice (*Mus musculus*).

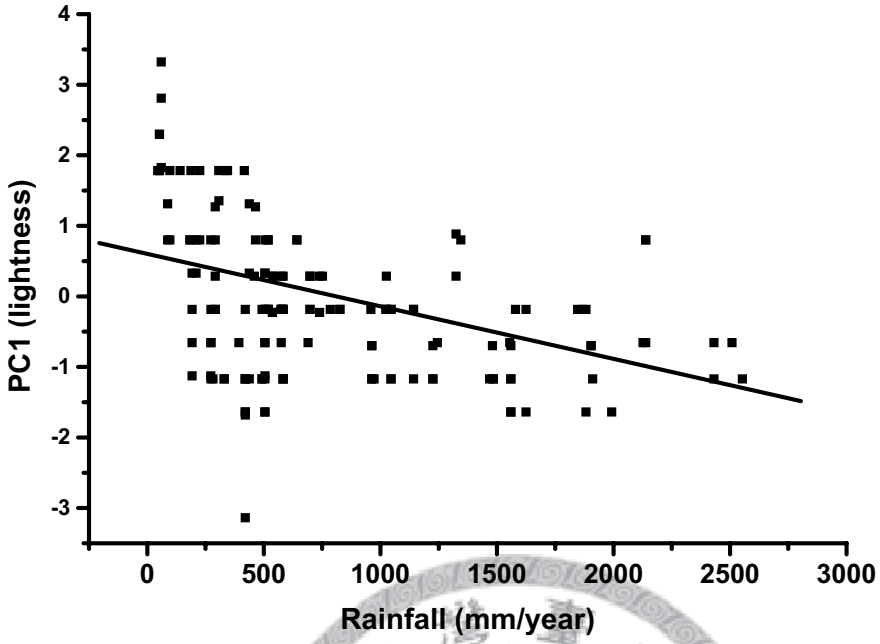

Fig. 3 PC1 scores plotted against mean annual precipitation ($r = -0.47$, $p<0.0001$) showing the relationship between coat color and precipitation.

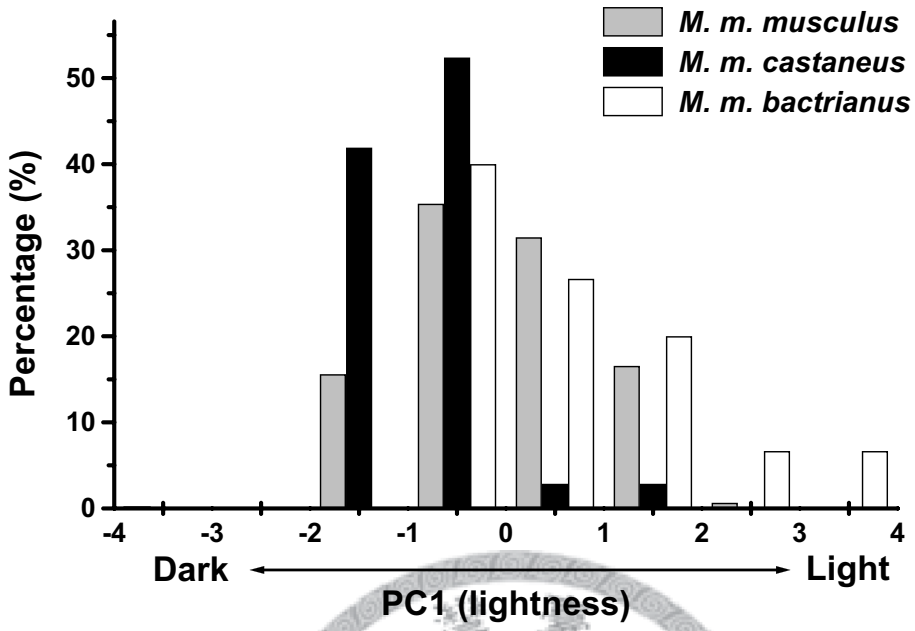

Fig. 4 Frequency distribution of PC1 among three subspecies of *M. musculus*.

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第三章、小家鼠 *Agouti* 基因 intron 1 保守區域的 insertional mutation 在亞洲的頻率與分佈

前言

Agouti 基因位於小家鼠(*Mus musculus*)的第二號染色體 89.0 cM 位置上，它是以南美豚鼠(*Dasyprocta aguti*)之種名爲名，因爲南美豚鼠的毛色能充份代表黑色毛髮上有 subapical yellow 橫條的顏色。*Agouti* 基因包含 4 個 exons，其中 exon 1 爲 noncoding exon，exon 1 可再細分爲 exon 1A, 1B, 1C, 1D (Siracusa, 1994)。*Agouti* 下游的 exon 2, 3, 4 組成含 131 個胺基酸的單一蛋白質，其中包含疏水性的 signal sequence，中間的 basic domian 和富含 cysteine 的 C 端等三個區段；沒有 transmembrane domain，符合 secreted protein 的特性，且基因體中尚未找到其它的同源基因(Bultman, Michaud & Woychik, 1992)。

正常情況下 *Agouti* 僅在毛囊的黑色素細胞表現，爲影響毛色形成的關鍵基因(Dinulescu & Cone, 2000)。Agouti 和毛囊中黑色素細胞表面的 melanocortin 1 receptor (Mc1r) 受器結合，抑制促黑激素(α melanocyte stimulating hormone, αMSH)與 Mc1r 的結合，促使黑素色素細胞產生的黑色素，從真黑色素(eumelanin, black to brown)轉成僞黑色素(pheomelanin, yellow to red)，造成毛色從黑色到黃色之間的變化(Lu et al., 1994; Kobayashi et al., 1995)。*Agouti* 突變除了會影響毛色外，亦與肥胖、糖尿病、腫瘤、胎兒致死有關聯(Hayssen, 2001)。

根據 Mouse Genome Informatics (http://www.informatics.jax.org/)的資料庫表列顯示，*Agouti* 基因的 spontaneous mutation allele 已有 57 個。*Agouti* 基因在實驗小鼠身上的 spontaneous mutation rate 非常快(Schlager & Dickie, 1966; Schlager & Dickie, 1967; Dickie, 1969)。因此，*Agouti* 基因在自然界的基因型以及分佈情況非常值得我們去了解，進一步與實驗小鼠相比較，是否在野生族群中的 *Agouti* 亦有如此高的突變率，能幫助我們更加了解 *Agouti* 在自然族群中所扮演的角色。

許多 *Agouti* mutation 都是 insertion 造成的，例如 intermediate yellow (A^{iy})、sienna yellow (A^{sy})、viable yellow (A^{vy})、intercisternal A-particle yellow (A^{iapy})、nonagouti (a)、black-and-tan (a^{t}) 和 white-bellied *Agouti* (A^{w})(Bultman et al., 1994; Duhl et al., 1994; Michaud et al., 1994; Siracusa et al., 1995)。其中 *a* allele 是 retroviral insertion 在 intron 1 造成的 spontaneous mutation，之後 retrovirus 經由

homologous recombination 掉出 *Agouti* 基因後會再 reverse 成 a^t 和 A^w(Bultman et al., 1994)。如此的情況非常特殊，實驗小鼠目前僅知有三個基因：*Agouti*、*dilution*、*hairless*，會有 endogenous retroviruses 插入後，再自發產生 reverse mutation 的例子(Maksakova et al., 2006)。同時 *Agouti* wild type 突變成 *a* 再變回 a^t 或 A^w 的突變速率非常快，故本研究的重點即鎖定 *Agouti* 基因 intron 1 的 insertional mutation，想嘗試了解野生族群，是否存在與實驗小鼠相同的遺傳變異機制，或者 retriviral insertion 無論 forward 和 reverse 的高突變率，僅是在缺乏天擇作用之下的實驗室環境，才能觀察到。最後，研究野生族群的小家鼠，有機會找到新的 allele，是實驗室未曾發現的，如此有助於我們對 *Agouti* 基因有更深入的了解，亦爲本研究目的之一。

方法

本研究於 16 個樣區共取得 103 隻小家鼠進行分析(圖 1)，採集範圍從北緯 47 度到 22 度，東經 82 度到 120 度，採集點橫跨中國大陸 6 個省份 13 個縣市以及台灣 3 個縣。各樣區的樣本數以及對應圖 1 中的採集點分述如下: 新疆省和丰縣(5 隻，圖 1 點 1)、新疆省烏魯木齊市(5 隻，圖 1 點 2)、新疆省于田縣(5 隻，圖 1 點 3)、山西省大同市大同縣(11 隻，圖 1 點 4) 、山西省長治市沁縣(4 隻，圖 1 點 5)、山西省長治市長治縣(11 隻，圖 1 點 6)、陝西省延安市富縣(6 隻，圖 1 點 7)、陝西省漢中市寧強縣(5 隻，圖 1 點 8)、河南省洛陽新野縣(1 隻，圖 1 點 9)、河南省南陽市伊川縣(3 隻，圖 1 點 10)、湖北省襄樊市棗陽縣(2 隻，圖 1 點 11)、湖北省孝感市安陸市(17 隻，圖 1 點 12)、雲南省昆明市(14 隻，圖 1 點 13)、台灣金門縣(5 隻，圖 1 點 14)、台灣彰化縣(4 隻，圖 1 點 15)、台灣屏東縣(5 隻，圖 1 點 16)，平均一個縣市採集 6 隻個體。採集時記錄小家鼠的體重(單位：g)，精確至小數點第一位。捕捉到的個體剝製成攤開式毛皮標本(flat skin specimen)後，使用 Munsell 土壤比色卡(Munsell soil color chart)量化小家鼠的毛色。我們首先利用比色卡比對標本的背部毛色(Holt, Maples & Savok, 2003)，該比色卡的每張色卡都有三個值，分別爲色調(hue，代表一個顏色相對於紅、黃、綠、藍、紫等顏色的關係)、明暗(value，代表一個顏色的明暗程度)，以及濃度(chroma，代表某個顏色的深淺程度)。進一步，我們使用 Sugita and Marumo (1996)的方法，將比色卡的三個值數量

化。最後使用到的色調(hue)僅一個值(10YR)，因此僅用數量化的明暗，以及濃度進行主成份分析(Principal Component Analysis，簡稱 PCA)，求得最大變異的主成份 1 (PC 1，表 1)，可以代表毛色的最大變異，用以比較不同個體的毛色。

我們從 NCBI 上小家鼠(B6)的 whole genome 下載有關 *Agouti* 基因的序列，依據 Bultman et al.(1994)的實驗結果，針對 *Agouti* intron 1 中的 insertion site 自行設計一對引子如下，MmA_F2：5'-CTCTCTTCGGTTCTGACTTGATTCT-3'、MmA_R2: 5'-CTGCCCACCTATCACCTTTAGA-3'(圖 2)，利用 Phusion DNA polymerase (Finnzymes)對 *Agouti* 基因 intron 1 做 PCR 反應。PCR 反應條件爲：(1) 98℃ 30 sec；(2) 98℃ 10 sec, 58℃ 10sec, 72℃ 1~5min, 30 個循環 (3) 72℃ 10min。PCR 產物使用 ABI 自動定序儀跑毛細管電泳，進行 5'及 3'端的定序。其中~6 kb 的 PCR 產物使用 TOPO XL PCR cloning kit (Invitrogen)做 cloning 後，再定序兩端。定序的結果使用 Sequencher 4.1.4 軟體做序列整理。最後使用 t-test 比較不同 genotype 個體的體重與毛色是否有差異。

結果

於 16 個採集點中，共 5 個採集點發現有 insertional mutation 的個體(表 2)，包括新疆烏魯木齊市(1 隻)、山西長治市沁縣(2 隻)、山西長治市長治縣(1 隻)、陝西延安市富縣(4 隻)，和陝西漢中市寧強縣(1 隻)。上述 5 個採集樣點，平均含 insertional mutation 個體數的比例爲 29%。比例最高的地點爲陝西延安市富縣(67%)，捕獲 6 隻小家鼠中，就有 4 隻含有 insertional mutation；比例最低的地點爲山西長治市長治縣(9%)，採集到的 11 隻個體中，僅 1 隻小家鼠有 insertional mutation。發現有 insertion 個體的採集分佈頗爲分散，沒有集中分佈的情形(圖 1)，不過南方的 *Mus musculus castaneus* 亞種，包括雲南昆明市(圖 1 點 13)、台灣金門縣(圖 1 點 14)、台灣彰化縣(圖 1 點 15)、台灣屏東縣(圖 1 點 16)，共 28 隻個體，均未發現含 insertional mutation 的個體。

在所有採集的 103 隻小家鼠中，有 9 隻個體的 *Agouti* intron 1 有發現 insertional mutation (9 %)，另外 93 隻個體(91 %)則無(表 3)。Insertion 的大小分爲兩型，一型爲 674 bp，另一型長度約爲 5.5 kb。其中 674 bp 的 insertion 與 murine leukemia virus (MuLV) VLeco long terminal repeat (LTR) 相似，674 bp insertion 從第 36 ~

671 bp 序列，共 636 bp 中有 601 bp 與 MuLV LTR 相同，nucleotide identity 高達 94%(圖 3)。約 5.5 kb 的 insertion 兩端爲與 764 bp 相同的序列，中間的序列因爲存在 repeat 的關係，尚未定序完全。9 隻有 insertonal mutation 的個體，有 2 隻基因型爲 674 bp 的同型合子、1 隻爲~5.5 kb 的同型合子，1 隻爲 674 bp 和無 insertion 的異型合子、2 隻爲~5.5 kb 和無 insertion 的異型合子，3 隻爲 674 bp 和~5.5 kb 的異型合子。其中 2 個 alleles 皆爲 insetional mutation 的小家鼠有 6 隻，只有 3 隻個體僅其中一個 allele 爲 insertional mutation。9 隻個體共 18 個 alleles 中，674 bp 和 ~5.5 kb 各占 8 個和 7 個 alleles，數量相當。另外，*Agouti* 基因 intron 1 的 insertion site 非常保守，除了 08007 個體找不到 insertion site 外，多數個體在 insertion site 往前 47 bp 與往後 61 bp，共 108 bp 的序列皆一模一樣(圖 4)。

雲南昆明採集的一隻個體(08007)，雖然亦沒有 insertional mutation，但 PCR product size 僅爲 502 bp，較其它沒有 insertional mutation 的 PCR product 長度(582 bp)來得短。將 08007 個體與其它個體的 consensus 序列比較後發現，它主要是少了一段 79 bp 的序列，且其中包含了 insertion site 的位置(圖 4)。

進行毛色比較時刪除 3 隻體重小於 6 g 的幼體(04295: 4.9g, 04081: 5g, 04309: 5.5g)，剩下 100 隻小家鼠做比較。結果發現無論 insertional mutation 之有無，毛色無顯著差異(t_{98} = 1.66, p = 0.10)。進一步比較 2 個 alleles 皆爲 insertional mutation 的個體，毛色與其它個體亦無顯著差異(t_{98} = 1.75, p = 0.08)。

進行體重比較時，除刪除體重小於 6 g 的幼體外，另刪除 21 隻採集時體重資料不完整的個體，剩下 79 隻小家鼠做比較。結果發現不論 insertional mutation 之有無，體重無顯著差異(t_{77} = 1.47, p = 0.15)。進一步比較 2 個 alleles 皆爲 insertional mutation 的個體，體重與其它個體亦無顯著差異(t_{77} = 0.29, p = 0.77)。

討論

Agouti 基因 intron 1 有 insertional mutation 的 9 隻個體，包含 2 種 insertional alleles，較短 allele 的 insertion 長度爲 674 bp，序列和 murine leukemia virus (MuLV) VLeco long terminal repeat (LTR)非常相近(圖 3)，同樣的序列在較長 allele 的兩端又重覆出現，且兩種 insertion 的插入點皆相同，故我們認爲 674 bp 的 insertion 是某種 retrovirus 的 LTR。進一步推測~5.5 kb 的 insertion 應該是完整的

retrotransposon，但需等序列完全解讀後才能確認。目前已知 endogenous retrovirus mutations (ERVs)的 reversion 例子非常少(Maksakova et al., 2006)，*Agouti* 是僅知的 3 個基因之一。Bultman et. al.(1994)研究 *Agouti* spontaneous forward and reverse mutation 結果顯示，一段 11 kb 序列(5.5 kb retrovirus-like transposable elements containing 5.5 kb additional internal sequence)會選擇 *Agouti* 基因 intron 1 的固定位置插入，此時 *Agouti* 會從 wild type 變成 *nonagouti* (*a*) allele (參考圖 2)。接著，當 retrovirus-like transposable elements 於基因重組過程中因爲 homologous recombination，藉由兩端 LTR 產生 self recombination 而掉出，最後只留下單一 LTR 存在於當初插入的 insertion site，此時 *Agouti* 會從 *nonagotui* 變回 white-bellied *agouti* (A^w)。如果我們在野外族群中發現的 insertional mutation 和 Bultman et al.(1994)的研究有類似的機制存在，合理推測~5.5 kb 的 insertion 爲一開始插入的 retrotransposon，它的兩端皆帶有 674 bp 的 LTR。而 674 bp 的 insertion 爲 retrotransposon 藉由兩端 LTR 產生 self recombination 掉出 *Agouti* 基因後，在 intron 1 原來插入位置上(insertion site)遺留下來的單一 LTR。進一步，retrotransposon 具有辨識基因中特定位置插入的功能，能解釋爲何我們在不同地區找到的 insertional mutations，以及不同 size 的 insertions，皆發生在相同的 insertion site 上。

雲南省昆明市採集的一隻個體(08007)，具有特別的 allele，雖然 *Agouti* 基因的 intron 1 中無 insertional mutation，但是在極保守的 insertion site 卻發生了 79 bp 的 deletion，是前人沒有報導過的新 allele。如此的結果，對於在野生族群中找尋新的 allele 具有鼓勵作用，相信經過更大範圍以及樣本數的檢測，於野生個體中應能找到更多過去在實驗室未曾發現的新 allele，對於 *Agouti* 或其它基因在自然界的功能與作用，能有更深入與全面的了解。同時新 allele 的發現亦更加突顯 screen 小家鼠野生族群 genotype 的重要性，能補足實驗室分析上的不足。

總共 9 隻具 insertional mutation 的野生個體，它們的背部毛色、體重與其它個體沒有顯著差異。然而，實驗小鼠已被證實 *Agouti* 基因的突變會影響個體的毛色與體重，若能再分析 *Agouti* 基因 intron 1 外的其它區域，仍有可能找到 *Agouti* 基因和毛色或體重的相關。事實上，毛色和體重都不只受一個基因的影響(Cheverud et al., 1996; Corva & Medrano, 2001; Bennett & Lamoreux, 2003)，若分析 *Agouti* 以外的其它基因，例如 *Mc1r* (Majerus & Mundy, 2003; Nachman, Hoekstra &

D'Agostino, 2003; Steiner, Weber & Hoekstra, 2007)，相信將能更加了解影響野生小家鼠毛色與體重變化的基因型組合。除了其它基因的影響之外，其它像是 null allele、sampling error、genetic background 的影響，與環境的影響等，都可能是造成我們找不到 phenotype-genotype association 的影響因子。

我們總共分析 103 隻野生小家鼠，其中 9%的個體 *Agouti* 基因 intron 1 有 insertional mutation。實驗室小家鼠研究結果顯示 *Agouti* 基因的 spontaneous mutation rate 非常高(Schlager & Dickie, 1966; Schlager & Dickie, 1967)，應可解釋爲何野生族群中有如此高比例的 retro elements 存在。然而我們發現 *Agouti* 基因 intron 1 的 insertion site 非常保守(圖 4)，且插入的 insertion 又爲相同的 retrotransposable element。進一步，9 隻突變的小家鼠中，高達 6 隻個體(67%，表 3)是 2 個 alleles 均爲 retroelement 的基因型組合，推測此 insertional mutation 可能爲 neutral mutation，對個體沒有明顯的不利影響，因此自然族群中能維持如此高的比例。已知 *Agouit* 基因突變亦會影響其它基因的表現，例如 MCM6 (minichromosome maintenance protein)、ITF2 (immunoglobulin transcription factor 2, a basic helix-loop-helix transcription factor) 等(Furumura et al., 1998)，而 homozygte 的 lethal yellow (A^y) 和 lethal light-bellied *nonagouti* (a^x) allele 更是會造成個體的死亡(Silvers, 1979)，故不排除存在天擇作用的可能性，值得更深入研究，幫助我們了解野生小家鼠 *Agouti* 爲何有如此高比例的 insertional mutation。

不同 retroviral elements 在不同品系的小家鼠，會有不同的 insertional mutation rate。例如大多數的 intracisternal A particle (IAP) insertions 都發生在 C3H/HeJ 品系上(Rakyan et al., 2003; Ishihara et al., 2004)，這或許可以解釋爲何我們在 *Mus musculus castaneus* 亞種身上未發現任何的 insertional mutation。然而我們取樣的數目不夠大，*M. m. castaneus* 亞種的實際採集範圍僅在台灣和雲南昆明 2 地，沒有找到 insertional mutation 亦可能是 sampling error 的結果。未來更大範圍與數量的採集，將有助於釐清不同亞種的小家鼠，是否因爲 genetic background 的不同，在 *Agouti* 基因 intron 1 的 insertional mutation rate 有明顯差別。

我們在野生族群中找到~5.5 kb 的 retroviral transposon，已利用 colony PCR 以及確認 plasmid size 的方式，確認其長度約爲 5.5 kb，且已成功 clone 全長。但它的兩端有 LTR，無法直接定序整個 clone 的序列，目前尚不知全部的序列。未來我們

將利用 nested deletions 或 *in vitro* transposition sequencing (例如 Epicentre 的 EZ-Tn5 <oriV/KAN-2> insertion kit)等技術，逐步將~5.5 kb retroviral transposon 的序列解讀出來，相信有助於了解它在野生族群中扮演的功能。

表 1、小家鼠背部毛色主成份分析結果

Variables	PC1	PC2
Chroma	0.894	0.447
Value	0.894	-0.447
Percent variance explained	80%	20%

表 2、*Agouti* intron 1 的基因型分佈

	地名	基因型	隻數	Insertion
新疆	和丰縣	582bp/582bp	5	無
	烏魯木齊市	582bp/582bp	4	無
		582bp/1256bp	1	有
	于田縣	582bp/582bp	5	無
山西	大同市大同縣	582bp/582bp	11	無
	長治市沁縣	582bp/582bp	2	無
		1256bp/1256bp	1	有
		6kb/6kb	1	有
	長治市長治縣	582bp/582bp	10	無
		582bp/6kb	1	有
陝西	延安市富縣	582bp/582bp	2	無
		1256bp/1256bp	1	有
		582bp/6kb	2	有
		1256bp/6kb	1	有
	漢中市寧強縣	582bp/582bp	4	無
		1256bp/6kb	1	有
河南	洛陽伊川縣	582bp/582bp	3	無
	南陽市新野縣	582bp/582bp	1	無
湖北	襄樊市棗陽縣	582bp/582bp	2	無
	孝感市安陸市	582bp/582bp	17	無
雲南	昆明市	582bp/582bp	13	無
		502bp/502bp	1	無
台灣	金門縣	582bp/582bp	5	無
	彰化縣	582bp/582bp	4	無
	屏東縣	582bp/582bp	5	無

表 3、*Agouti* 不同基因型的個體數與百分比

基因型	隻數	百分比
502bp/502bp	1	1%
582bp/582bp	93	90%
582bp/1256p	1	1%
1256bp/1256bp	2	2%
1256bp/6kb	3	3%
582bp/6kb	2	2%
6kb/6kb	1	1%

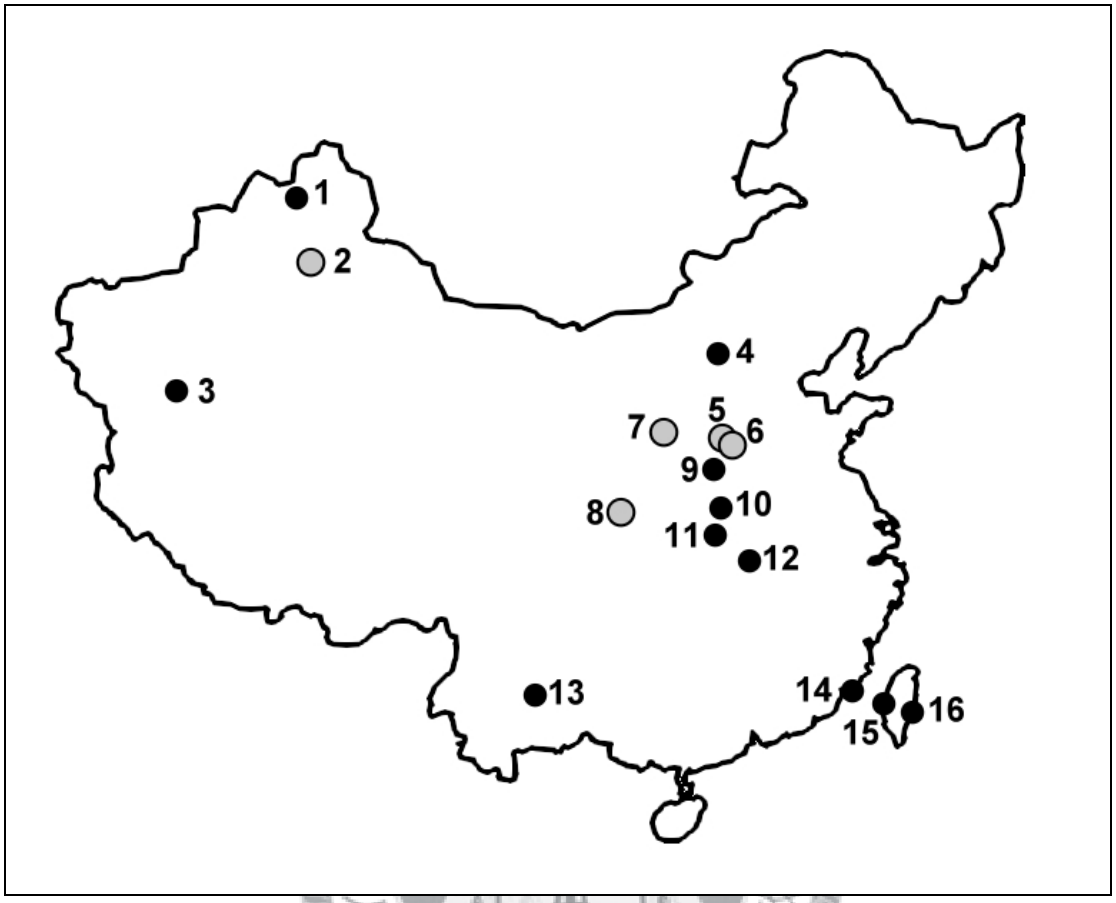

圖 1、採集樣區。地點說明：1 (新疆省和丰縣)、2 (新疆省烏魯木齊市)、3 (新疆省于田縣)、4 (山西省大同市大同縣)、5 (山西省長治市長沁縣)、6 (山西省長治市長治縣)、7 (陝西省延安市富縣)、8 (陝西省漢中市寧強縣)、9 (河南省洛陽伊川縣)、10 (河南省南陽市新野縣)、11 (湖北省襄樊市棗陽縣)、12 (湖北省孝感市安陸市)、13 (雲南省昆明市)、14 (台灣金門縣)、15 (台灣彰化縣)、16 (台灣屏東縣)。灰色的點表示族群中有個體的 *Agouti* 基因 intron 1 有 insertional mutation。

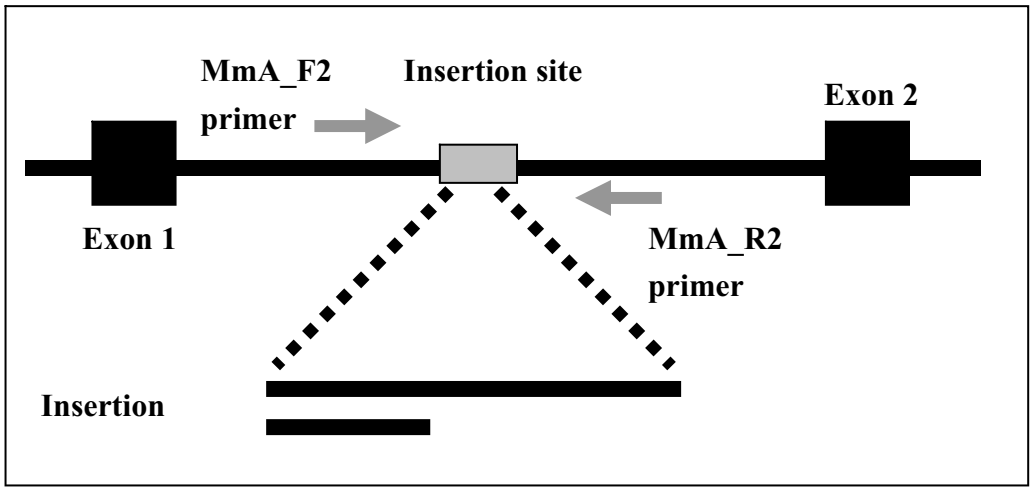

圖 2、PCR primers(MmA_F2, MmA_R2)設計示意圖。黑色的方塊爲 exon，灰色的長方塊爲 intron 1 的 insertion site，灰色箭頭爲自行設計的 primer，insertion site 下方，由虛線擴大的橫線爲不同大小的 insertion。實驗小鼠的研究顯示當 insertion size 爲 11 kb 時，*Agouti* 基因變成 *a* allele、6 kb 時成爲 a^{t} allele，0.6 kb 時成爲 A^{W} allele。

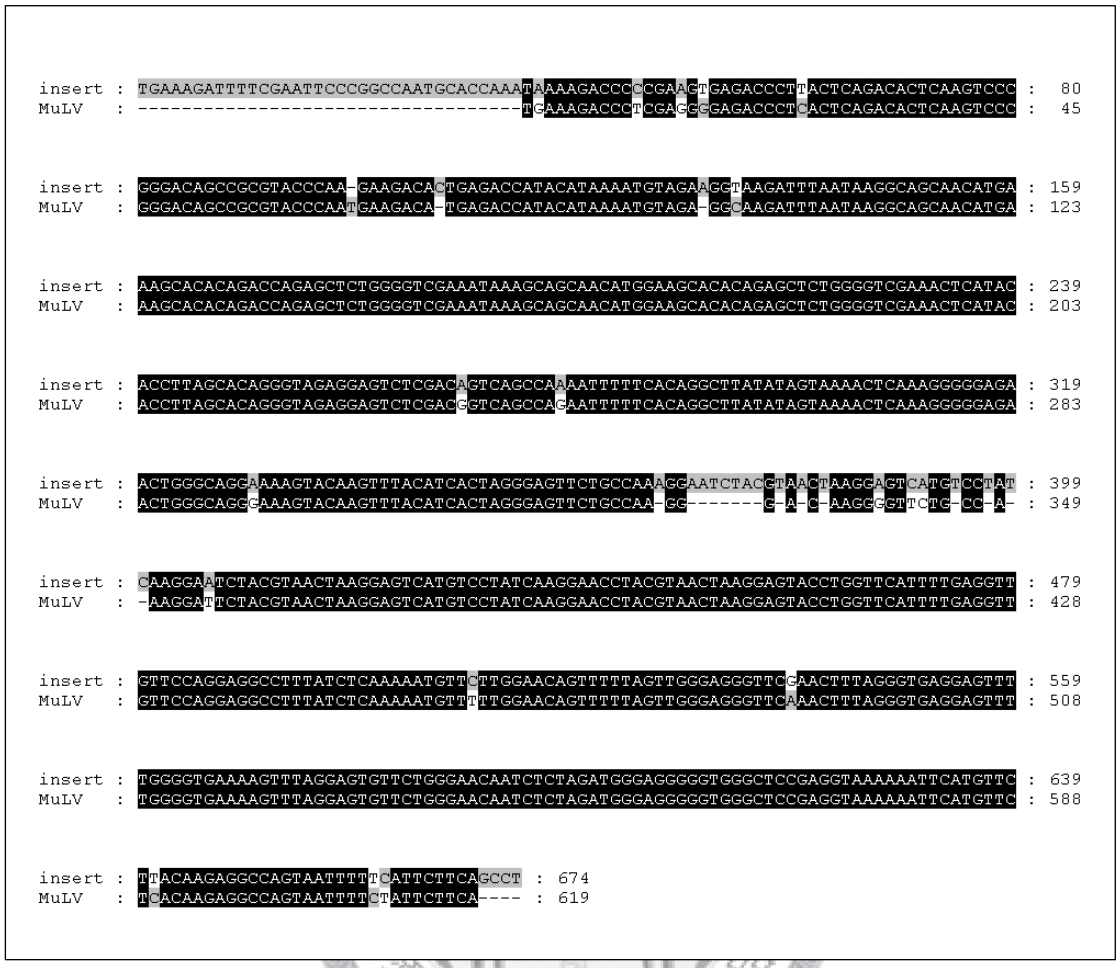

圖 3、*Agouti* gene intron 1 的 764 bp insertion (上)與 Murine leukemia virus (MuLV) VLeco long terminal repeat (下)的比較。黑色方塊爲兩者相同的核苷酸序列。

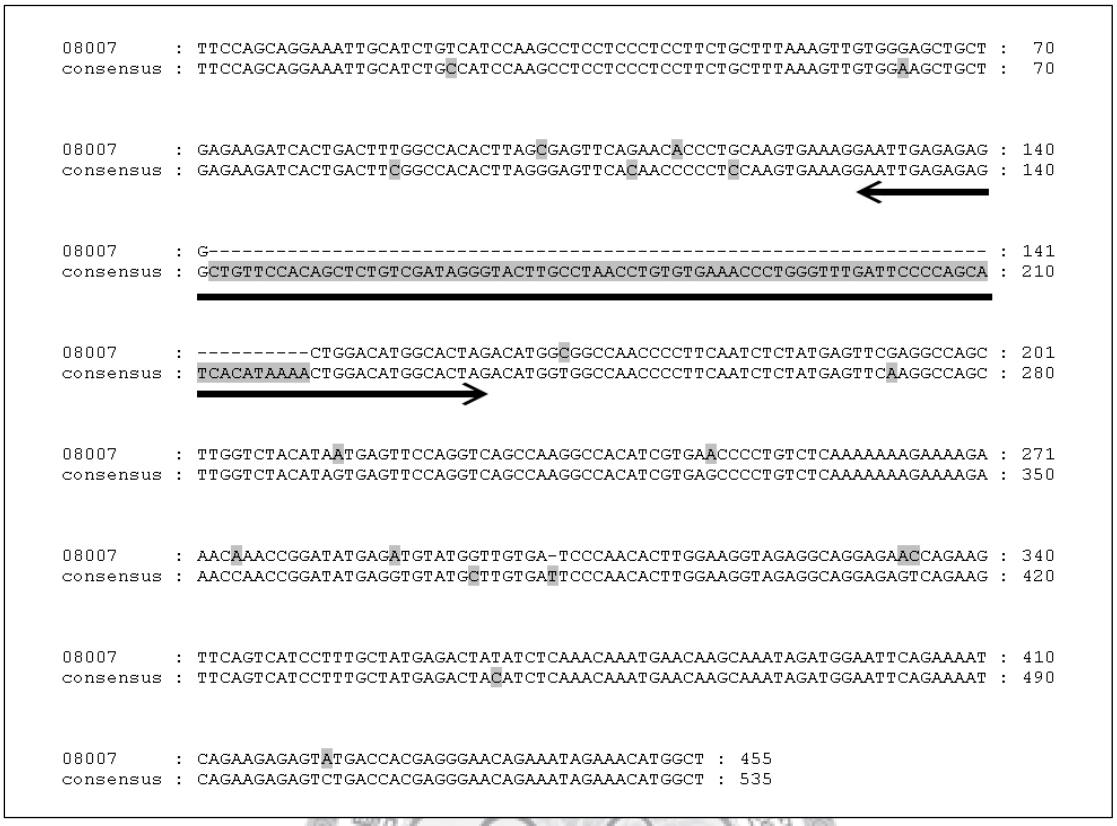

圖 4、08007 個體(PCR size = 502 bp)與其它無 insertion 個體(PCR size = 582 bp)的 consensus 序列比較。上面爲 08007 個體的序列，下面爲另外 92 隻無 insertional mutation 個體的 consensus 序列，兩者皆已去除 primer 序列，故 08007 剩 455 bp、consensus 剩 535 bp。灰色的方塊表示兩者有差異的位置，黑色箭頭代表除 08002 個體外，其它個體序列皆相同的 insertion site 保守區域。

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第四章、黑腹絨鼠毛色候選基因: *Mc1r* 的編碼與非編碼序列變異與毛色變異無關

我分析自己野外採集、林良恭老師實驗室收藏，以及國立臺灣博物館收藏的黑腹絨鼠毛色變異，同樣發現毛色變化與雨量有相關。進一步定序毛色基因(*Mc1r*) regulatory 以及 coding regions 的序列變異，發現 *Mc1r* 的序列變異與毛色無關，表示黑腹絨鼠的毛色可能是受其它的基因控制，此部份的研究結果如下。

Title: Sequence variation in the coding and non-coding region at the melanocortin-1 receptor gene (*Mc1r*) is not associated with coat colour variation in the Pére David's vole (*Eothenomys melanogaster*)

Summary

The molecular genetic changes associated with adaptive morphology remain an interesting puzzle in evolutionary biology. Previous studies have shown that mutations in the coding regions of the coat colour candidate gene, melanocortin 1 receptor (*Mc1r*) gene underlie adaptive coat colour variation in a wide range of species. However, the evolution of regulatory regions of *Mc1r* still remains unclear. In this study, we obtain the upstream sequences of the *Mc1r* gene from Pére David's vole (*Eothenomys melanogaster*). Our results demonstrate that the coat colour in Pére David's vole is an adaptive trait, with individuals of black colour living in a more humid environment and

brown colour forms in a drier area. However, no association was found between the coat colour and the polymorphisms in either regulatory or coding sequences. This implies that there may be other genes, acting alone or in concert with *Mc1r*, underlie coat colour variation in Pére David's voles.

Keywords: *Mc1r*, coat colour, adaptation, *Eothenomys melanogaster*, 5'RACE, regulatory evolution

Introduction

One of the principal goals in evolutionary biology is to elucidate the genetic mechanisms underlying an adaptive trait. Whether the evolution of an adaptive phenotype is a result from mutations in the *cis*-regulatory elements or coding regions of structural genes still remains controversial (Hoekstra & Coyne, 2007; Wray, 2007). Coat colour in vertebrates has been the most prominent case among adaptive morphological traits to suggest a clear relationship between phenotypes and genotypes (Hoekstra, 2006). A large number of genes have been known to affect vertebrate pigmentation. For example, in mice, more than 100 loci and 800 phenotypic alleles have been identified (Bennett & Lamoreux, 2003). Among them, the melanocortin-1 receptor (*Mc1r*) gene, a critical regulator in pigmentation synthesis, has been shown to contribute to colour polymorphism in many species (Majerus & Mundy, 2003).

Mc1r is a G-protein coupled receptor with seven transmembrane domains, and is specifically expressed in the melanocytes, the melanin-producing cells. In mammalian melanocytes, there are two basic types of melanin: (1) eumelanins, which are black or brown, and (2) pheomelanins, which are yellow or red. Typically both types of melanin are mixed in various proportions and result in coat colour variations in animals. The expression of the *Mc1r* gene is regulated by the agonist α-Melanocyte-stimulating hormone (α-MSH) and the antagonist agouti protein. Binding of *Mc1r* by α-MSH results in an increase in the synthesis of eumelanins while *Mc1r* bound by agouti protein will increase the production of pheomelanins (Hearing, 2000).

The *Mc1r* gene has been shown to be responsible for coat colour differences for a wide range of species (Theron et al., 2001; Ling et al., 2003; Nachman, Hoekstra & D'Agostino, 2003; Mundy et al., 2004; Rosenblum, Hoekstra & Nachman, 2004; Hoekstra et al., 2006; Nadeau, Minvielle & Mundy, 2006). All mutations in the gene identified were in the coding region (Hoekstra, 2006; Steiner, Weber & Hoekstra, 2007), in part because the *Mc1r* gene has only one small exon (~1 kb) that has been conserved in different species. Although the regulatory mechanisms (Rouzaud & Hearing, 2005) have been addressed, the evolution of *Mc1r* gene and its cis-regulatory elements still remain unclear.

In this study, we used a rapid amplification of 5'-cDNA end (5'-RACE) method

to obtain the 5'-untranslated region (5'-UTR) sequences of the *Mc1r* gene in Pére David's vole (*Eothenomys melanogaster*), a species that exhibits a dark-brown pattern of coat colour. To test the hypothesis of regulatory evolution, we examined the relationship between coat colour and the *Mc1r* genotype in both regulatory and coding sequences to determine if the causal mutations that underlie the coat colour polymorphism observed in this species occur in the regulatory or coding regions?

Materials and methods

Samples and measurements of coat colour

Pére David's vole (fig. 1) is a species widely distributed in China and Taiwan (Luo et al., 2004), inhabiting in middle-high altitude montane areas from 1700 to 2700 meters in elevation (Yu, 1995). A total of 37 voles were collected from 4 locations in Taiwan. The sample size for each population is as follows: 14 from Alishan, 8 from Tataka, 6 from Guanwu, and 9 from Wuling. The voles were trapped from the field and brought back to process in the lab. Skin specimens were made and tissue samples were preserved.

In order to test the Gloger's rule which predicts that animals in more humid areas tend to be darker, we used regression analysis to test the relationship between the coat colour and an environmental factor, *i.e.*, precipitation. The coat colours of the

specimens are classified into melanic, intermediate, or brown forms (fig. 1), and scored as 1, 0.5, and 0, respectively. The mean annual precipitation for Alishan, Tataka, Guanwu, and Wuling were 3988 mm, 2418 mm, 2500 mm, and 1488 mm, respectively.

5'RACE and primer design

Poly $(A)^+$ RNAs from skin and tail tissues were extracted according to the Trizol protocol (Invitrogen). The 5'-UTR sequences and the transcription initiation sites of the *Mc1r* gene of Pére David's vole were determined using a 5' RACE kit (Roche). We obtained the exon sequences of the *Mc1r* gene by a pair of primers (mMc1r-1-for and mMc1r-2-rev) specific for house mice (*Mus musculus*) (Wada et al., 2005) for amplifying. The amplification was successful and the products were sequenced. Based on exon sequences obtained, we designed three gene specific-primers for 5'RACE: EmMc1r_SP1 (5'-CCA GAC AGC AGA TGA AGC AA-3') for synthesis of first strand cDNA using AMV reverse transcriptase, EmMc1r_SP2 (5'-GAG GCC ATC TGG GAT AGA CA-3') for first PCR to amplify the adaptor-ligated cDNA, and EmMc1r_SP3 (5'-GGT AGC CAG TCC AAG GTG AG-3') for second PCR. Thereafter we successfully obtained the 5'UTR sequences. In order to obtain the sequences encompassing 5'UTR and exon, a new primer pair, EmMc1r_F1 (5'-CTA CGG GGG CTT TGA ACA C-3') and EmMc1r_R1 (5'- TGG TCC CAG GCA GTT

TGT G-3'), was designed to amplify a 1714-bp fragment in the voles.

Genomic DNA extraction, PCR cloning, and sequencing

Genomic DNA was extracted from frozen muscle, liver or kidney tissues using commercial kits (Lamda). PCR amplification were performed in thermal cyclers (ABI 2720 or Bio-Rad PTC 200) in 100 µl total volume containing 1.0 unit of Phusion DNA polymerase (Finnzymes), 20 µl 5× Phusion HF buffer, 0.2mM for each dNTP and 1.5 mM $MgCl_2$. PCR thermocycling condition were as follows: (1) an initial denaturing step of 98℃ for 30 s; (2) 30 cycles of the following: 10 s at 98℃, 10 s at 58-61℃, 2 min at 72℃; and (3) a 10 min extension step at 72℃. The 1714 bp PCR product was purified using a QIAquick gel extraction kit (Qiagen). After A-tailing with 10mM dATP at 72℃ for 30 min, TA cloning was performed using the pGEM-T vector system (Promega) according to manufacturer's instructions. At least five clones were sequenced for each vole.

Data analysis

Sequences were edited and aligned manually by Sequencher, v.4.1.4 (Genecodes). Associations between single nucleotide polymorphisms and colours were tested by the Chi-square test. All *Mc1r* sequences generated in this study have been deposited in

GenBank. To define the boundary of coding region, *E. melanogaster Mc1r* nucleotide sequences were aligned with those of house mouse (*M. musculus*) and pocket mouse (*Chaetodipus intermedius*) by ClustalW. In addition, the transmembrane regions were predicted by four web tools: *i.e.*, HMMTOP 2.0 (http://www.enzim.hu/hmmtop/), SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/), TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). Potential transcription factor binding sites were predicted by Transcription element search system (TESS; http://www.cbil.upenn.edu/tess). A parsimony haplotype network based on *Mc1r* sequences from all alleles was constructed by TCS 1.21 (Clement, Posada & Crandall, 2000). The evidence for selection at *Mc1r* was provided by Tajima's D statistic (Tajima, 1989) using the programme DnaSP 4.10.9.

Results

Characteristics of the 5'UTR and coding regions of the *Mc1r* gene

A region of 1714 bp of the *Mc1r* gene was determined for Pére David's vole, including the entire coding region (954 bp) and 5' (588 bp) and 3'(172 bp) UTR. The 5'UTR sequence shows 71.7% nucleotide identity to that of house mouse (Adachi et al., 2000). The transcription initiation sites identified using the 5'-RACE analysis were

stretched over a GC rich (57.4%) region of approximately 500 bp upstream the *Mc1r* coding region (fig. 2, position at 1, 199, 256, 271, 276, 293, 302, 305, 318, 378, and 474). No apparent groups of transcriptional initiation sites were noted, in contrast to the human pattern (Moro, Ideta & Ifuku, 1999). We predict three and one putative binding sites for the transcription factor SP-1 and AP-2, respectively by TESS. In addition, five CANNTG motifs, which have been experimentally identified in the house mouse *Mc1r* promoter (Adachi et al., 2000), were also found. Neither TATA nor CAAT box were found in this proximal region.

The nucleotide positions 589-1542 (954 bp), from start codon (ATG) to stop codon (TGA), correspond to the exon sequence of the *Mc1r* gene of the house mouse and the pocket mouse. The exon of Pére David's vole shows 89.0% and 82.8% nucleotide identity to that of house mouse (Wada et al., 2005) and of pocket mice (Nachman et al., 2003), respectively. All of the four web tools predict that the exon we sequenced contain seven putative transmembrane domains, a feature of G protein coupled receptor.

Mc1r sequence variation is not associated with coat colour polymorphism

Eleven polymorphic sites were detected, three within the 5'UTR, seven within the exon, and one within the 3'UTR (table 1). Within the exon, five of the substitutions

were synonymous and two were nonsynonymous. The two nonsynonymous mutations, amino acid position 109 and 231, occur in the second extracellular region and in the third intracellular region, respectively. None of the eleven substitutions showed any association with the coat colour variation (table 1). That is, we did not observe any variant unique to melanic, intermediate, or brown coat colour in any population. Thus, we suggest that *Mc1r* is not a principal determinant of coat colour polymorphism in *E. melanogaster*, at least in the four populations we sampled.

Phylogenetic relationships and testing for selection

Sequence variation at *Mc1r* was largely consistent with phylogeographic patterns inferred from mitochondrial cytochrome B sequence variation. Three polymorphic sites (table 1: 1104, 1279, and 1607) support the split between the southern (including population Alishan and Tataka in this study) and northern (including population Guanwu and Wuling in this study) lineages, which is the basal split inferred from mtDNA (Chang, 2007). The genealogy of *Mc1r* haplotypes (fig. 3) is very similar in topology to those generated from mitochondrial cytochrome B, nuclear IRBP, and X chromosome-link *G6pd* gene for Pére David's voles (Chang, 2007). Besides, there are only two nonsynonymous substitution in *Mc1r* sequence, one occur in heterozygotes (ID: Yu2087, nucleotide position 913, amino acid position 109), and the other separate

the North and South populations (nucleotide position 1279, amino acid position 231).

Tajima's D test did not differ significantly from zero (0.87142, $p > 0.10$), which means the coat colour related gene, *Mc1r*, is not subject to selection.

Melanic coat colour is associated with the amount of precipitation

The standardized regression coefficient for the precipitation variable was 0.54 ($p < 0.001$). It was to say that the environmental factor (*i.e.* precipitation) can explained 27% of the variation in coat colour (adjusted $R^2 = 0.27$). In addition, the most melanic population (Alishan) stays in the most humid environment, the least melanic population (Wuling) inhabits in the driest habitat. The pattern corresponds to the Gloger's rule (Gloger, 1833), which states that individuals of endothermic animals in humid habitats are darker than those in drier habitats, implying the coat colour is an adaptive phenotype.

Discussion

We cloned and characterized the promoter region of *Mc1r* in Pére David's voles. Gene expression of most eukaryotes are under the control of regulatory elements, especially the promoter region which are usually located upstream of transcription initiation sites. Our result showed that the ~ 500 bp GC-rich region upstream the *Mc1r*

coding region seemed to be the main promoter. Not only all of the detected transcription initiation sites are located in this region, but also the features contained in region, (including GC-rich, TATA-less, and SP-1 transcription factor binding sites) are all consistent with those of G-protein-coupled receptors. In addition, the first two CANNTG motifs of Pére David's vole are completely identical in sequence to the promoter region of house mouse , which are recognized by the transcription factor of basic-helix-loop-leucine zipper (bHLH-Zip) protein family (Adachi et al., 2000). Therefore, the two CANNTG motifs upstream from most of transcription initiation sites are important candidate core promoters, although it still needs to be confirmed by further experiment, such as gel shift assay. In short, our result is the first report for characterizing the *Mc1r* promoter region of *E. melanogaster*.

In this study, the pattern of coat colour variation in Pére David's voles corresponds to Gloger's rule (Gloger, 1833; Zink & Remsen, 1986), which states that animals in more humid environments tend to be darker, implying the coat colour is an adaptive trait. In other words, the percentage of melanic forms among the four vole populations completely corresponds with the amount of precipitation, implying the coat colour variation, at least in part, is formed by a selective effect of crypsis and/or bacterial resistance. One possibility is the melanic forms are more cryptic in a darker background caused by higher vegetation density (*i.e.*, shade) and darker soil colour (*i.e.*,

saturated with moisture) which are associated with the habitats of higher precipitation (Belk & Smith, 1996; Lai et al., 2008). An alternative explanation is that bacteria are more abundant and active in humid environments, and the melanic form with more eumelanin pigments resists bacterial degradation better than brown form caused by pheomelanin pigments (Burtt & Ichida, 2004; Goldstein et al., 2004).

In contrast to a wide range of taxa in which *Mc1r* mutations underlie coat colour polymorphism (review in Majerus & Mundy, 2003; Mundy, 2005; Hoekstra, 2006), our finding is one of few studies which found no association between the *Mc1r* genotype and the colour variation in wild animals (MacDougall-Shackleton, Blanchard & Gibbs, 2003; Cheviron, Hackett & Brumfield, 2006; Haitina et al., 2007; Wlasiuk & Nachman, 2007). Furthermore, we found no association between coat colour variation and both in cis-regulatory mutations and coding mutations. Therefore, other genes, like *Agouti* (Steiner et al., 2007) or *Tyrp1* (Schmutz, Berryere & Goldfinch, 2002) might underlie the coat colour variation. Alternatively, because both enhancers and silencers can be up to 100 kb away from their core promoter region, it is difficult to identify them. In addition, *Mc1r* mutation may underlie coat colour variation in other populations which we did not screen. For example, pocket mice had been found association between coat colour variation and *Mc1r* substitution only in one population, but not in other three populations (Hoekstra & Nachman, 2003). Therefore, our data could not thoroughly

rule out the potential responsibility of *Mc1r* gene. However, the consistent patterns between *Mc1r*-based and cytochrome B-based phylogenies and no significant selection detected by the Tajima's D test suggest that the differences at *Mc1r*, including gene regulation and gene structure region, might simply reflect population divergence rather than a different adaptive evolution.

Although we did not found the phenotype and genotype association both in gene regulatory and gene structure region of the *Mc1r* gene, we attempt to distinguish whether the *cis*-regulatory mutation or the coding mutation play a crucial role in adaptive evolution which is an exciting outset for the evolutionary biology. In the future, it might require more decades to obtain enough evidence to confirm the hypothesis of regulatory evolution (Carroll, 2005).

Table 1 Nucleotide polymorphism at *Mc1r* gene (Numbers in the top row correspond to nucleotide positions. Dots represent identity with respect to the first sequence. For heterozygous sites, the genotype is indicated.)

population	coat colour	specimen	88	89	374	750	913	1104	1279	1323	1518	1524	1607
Guanwu (Gu)	melanic	Yu2086	C	C	G	G	C	T	A	C	G	G	A
	melanic	Yu2088	.	.	.	.	.	.	.	.	G/A	.	.
	intermediate	Yu2091	.	.	.	.	.	.	.	.	.	.	.
	intermediate	Yu2092	.	.	.	.	.	.	.	.	.	.	.
	intermediate	Yu2093	.	.	.	.	.	.	.	.	.	.	.
	intermediate	Yu2087	.	.	.	.	C/G	.	.	.	.	.	.
Wuling (Wu)	brown	Yu2036	.	.	.	.	.	.	.	.	.	.	.
	brown	Yu2041	.	.	.	.	.	.	.	T	.	.	.
	brown	Yu2037	.	.	.	A	.	.	.	T	.	.	.
	brown	Yu2014	.	.	.	G/A	.	.	.	T	.	.	.
	brown	Yu2040	.	.	.	G/A	.	.	.	T	.	.	.
	brown	Yu2039	.	.	.	.	.	.	.	C/T	.	.	.
	brown	Yu2044	.	.	.	.	.	.	.	C/T	.	.	.
	brown	Yu2038	.	.	.	G/A	.	.	.	C/T	.	.	.
	brown	Yu2042	.	.	.	G/A	.	.	.	C/T	.	.	.
Tataka (Ta)	melanic	Yu2022	A	G	.	.	.	C	G	.	.	.	G
	melanic	Yu2072	A	G	.	.	.	C	G	.	.	.	G
	melanic	Yu2071	.	.	A/G	.	.	C	G	.	.	G/A	G
	melanic	Yu2064	C/A	C/G	.	.	.	C	G	.	.	G/A	G
	intermediate	Yu2076	.	.	.	.	.	C	G	.	.	A	G
	brown	Yu2082	.	.	A	.	.	C	G	.	.	.	G
	brown	Yu2020	A	G	.	.	.	C	G	.	.	.	G
	brown	Yu2066	C/A	C/G	.	.	.	C	G	.	.	G/A	G
Alishan (Al)	melanic	Yu2045	.	.	A	.	.	C	G	.	.	.	G
	melanic	Yu2046	.	.	A	.	.	C	G	.	.	.	G
	melanic	Yu2050	.	.	A	.	.	C	G	.	.	.	G
	melanic	Yu2051	.	.	A	.	.	C	G	.	.	.	G
	melanic	Yu2053	.	.	A	.	.	C	G	.	.	.	G
	melanic	Yu2058	.	.	A	.	.	C	G	.	.	.	G
	melanic	Yu2059	.	.	A	.	.	C	G	.	.	.	G
	melanic	Yu2049	.	.	.	.	.	C	G	.	.	.	G
	melanic	Yu2055	.	.	A/G	.	.	C	G	.	.	.	G
	intermediate	Yu2056	.	.	A	.	.	C	G	.	.	.	G
	intermediate	Yu2057	.	.	A	.	.	C	G	.	.	.	G
	brown	Yu2048	.	.	A	.	.	C	G	.	.	.	G
	brown	Yu2052	.	.	A	.	.	C	G	.	.	.	G
	brown	Yu2054	C/A	C/G	A/G	.	.	C	G	.	.	.	G
Gene structure			5'UTR			Exon							3'UTR
Amino acid							Arg		Ala				
							Gly		Thr				
Amino acid position						54	109	172	231	245	310	312	

Fig. 1 Variation in coat colour of Pére David's voles (*Eothenomys melanogaster*).

Samples from left to right are melanic, intermediate, and brown forms, respectively.

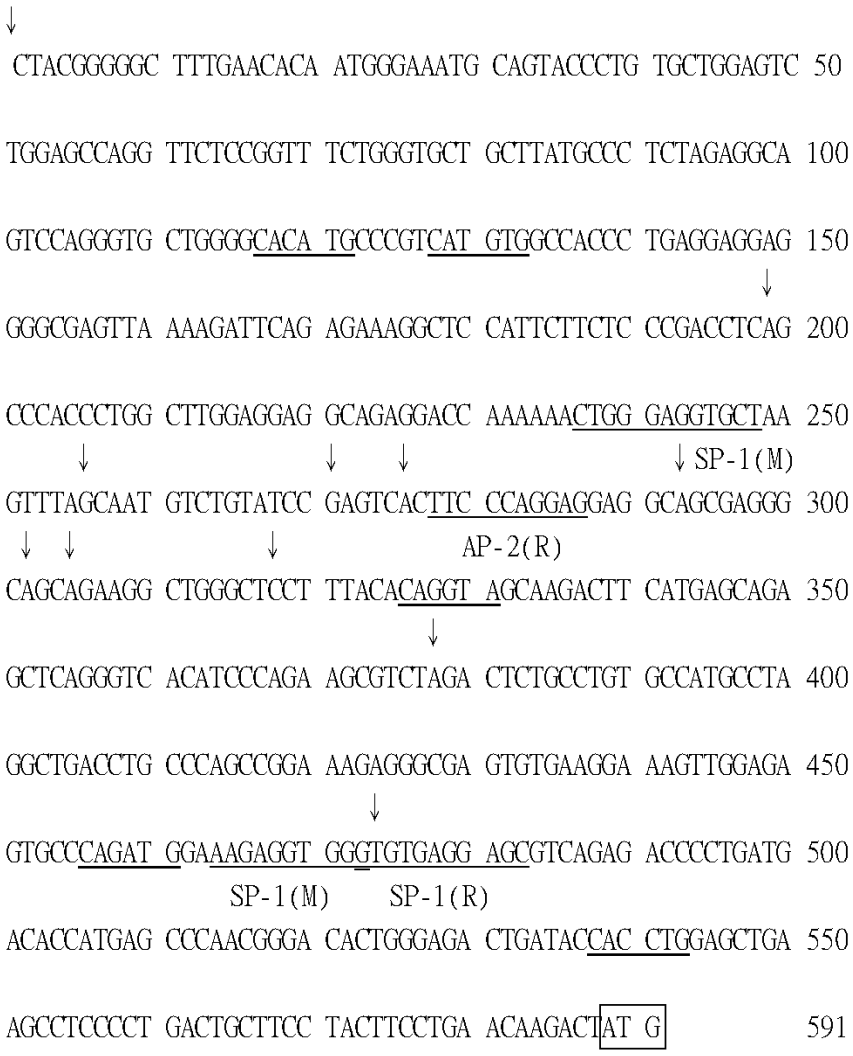

Fig. 2 The nucleotide sequence of 5' UTR of the *Mc1r* gene. The maximum length of 5'UTR is 588 bp. The first ATG codon is boxed. Multiple transcriptional initiation sites are indicated by arrows. Consensus binding sites for SP-1 and AP-2 transcription factor are underlined. The parentheses refer to the database from mouse (M) or rat (R). The CANNTG motifs based on mice experiment are thickly underlined.

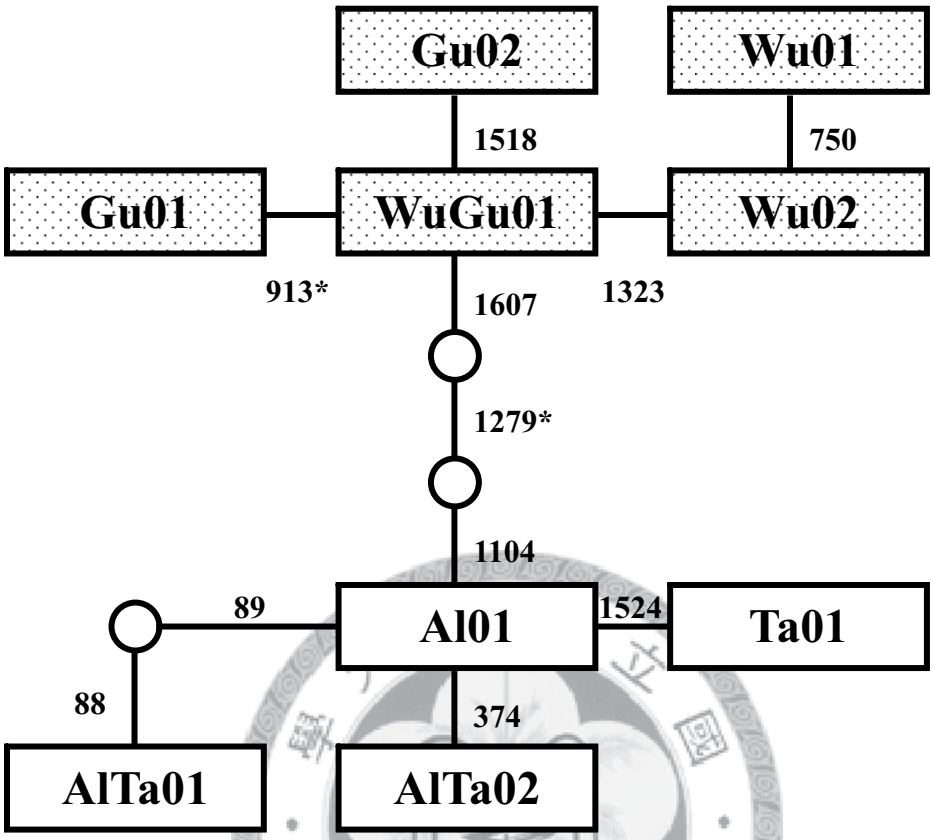

Fig. 3 Statistical parsimony network based on *Mc1r* haplotypes. Each line between haplotypes indicates one substitution with the positions of the nucleotide substitutions noted above. * indicates nonsyonymous substitution. Gu01 is one of alleles from Yu2087. Al, Ta, Gu, and Wu indicate alleles from the Alishan, Tataka, Guanwu, and Wuling population, respectively. AlTa indicates alleles from the Alishan and Tataka populations. WuGu indicated alleles from Guanwu and Wuling populations. The reticular background indicates the two north populations, and white background indicates the two south populations.

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第五章、研究結論與建議

(一) 鼠類之毛色變異

本研究發現野生鼠類族群，無論是小家鼠(*Mus msuculus*)或黑腹絨鼠(*Eothenomys melanogaster*)，背部毛色皆有很大的變異，進一步發現小家鼠的毛色與雨量顯著相關，符合 Gloger's rule，可能是一種適應性演化的特徵。未來有關毛色變異的研究，若能擴展到更多的鼠類物種，相信是很值得深入研究的有趣課題。

(二) 本研究之發現與改進策略

(a) 小家鼠的毛色與體重都和 *Agouti* 基因型無顯著相關

(b) 黑腹絨鼠的毛色變異與 *Mc1r* 基因型無相關

(c) 無論毛色或體重，都是多基因遺傳的表型特徵，只分析一個候選基因找不到表型與基因型的關聯性是可理解的結果。未來的研究，若能一起分析多個基因，且同時分析 regulatory 以及 coding regions 的變異，相信會有更高的機會，找到表型和基因型之間的關聯性。

(三)未來工作及展望

尋找適應性演化特徵表型和基因型的關聯性，是演化生物學無法避免的重要研究課題，因爲它可以幫助我們了解天擇作用在什麼基因上，以及現今的物種多樣性是如何演變而來。此研究領域的方向，從單一基因對單一表型開始，漸漸朝向單一基因對多個表型，以及多個基因對單一表型的方向發展。然而目前的適應性表型多集中在毛色變化(因爲分子機制清楚)，未來的研究方向，應會朝向更多源的適應性表型特徵，同時探討多基因與多表型的關聯性，最終朝向整個基因組對所有表型的關聯性，如此方能解釋基因的變化，如何造就世上這麼多物種的各類適應性特徵。

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Variation of coat color in house mice throughout Asia

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Keywords
Gloger's rule; coat color; crypsis; *Mus musculus*; protective color.

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Abstract

Coat color variation due to melanin pigment synthesis in house mice *Mus musculus* in Asia is described and found to be consistent with Gloger's rule, which states that individuals of endothermic animals are darker in humid habitats than those in drier habitats. Three properties of coat color (hue, value and chroma) were measured, and a lightness variable was derived from a principal components analysis using 428 skin specimens representing three subspecies from 85 localities. Dorsal coat color ranged from yellow through brown to black, whereas ventral coat color ranged from white to black. Dorsal coat color varied less than the ventral color. In our samples, the variation in coat color in natural populations was far less than that observed in the laboratory. We found a significant correlation between the lightness variable of dorsal coat color and precipitation. Dark coat color was observed in more humid and closed habitats (darker background color), and pale coat color in drier, more open habitats (lighter background color). This result might imply the role of concealment as a selective force affecting dorsal coat color that was observed in house mice. We also discussed other selective forces that could affect the coat color variation in house mice, such as resistance to bacterial degradation and thermoregulation. In addition, the color spectra of the dorsal pelage among the three subspecies were different, the major distinction being the environmental background color of the habitats in which they are distributed.

Introduction

Coat color is an important phenotypic characteristic in mammals because it is an intermediary for an individual to interact with environments and with other animals. Therefore, coat color is tightly associated with an individual's survival and fitness. Adaptive significance of coloration in animals can be explained by several selective forces (Burtt, 1981; Cloudsley-Thompson, 1999). Yet, for mammals, many of the working hypotheses concerning the adaptive value of coat color were proposed more than 100 years ago and the field has progressed little since then (Caro, 2005). Recently, these hypotheses have attracted interest, and are again being explored and tested (e.g. Ortolani, 1999; Stoner, Bininda-Emonds & Caro, 2003*a*; Stoner, Caro & Graham, 2003*b*; Nachman, 2005; Hoekstra, 2006; Hoekstra *et al*., 2006). The three most important adaptive factors influencing coat coloration in mammals are concealment, thermoregulation and communication. For example, after removing the confounding effects of shared ancestry, pale coloration of lagomorphs has been strongly associated with open habitats serving the purpose of protective coloration (Stoner *et al*., 2003*a*). Similarly, coat color patterns provide crypsis for carnivores (Ortolani, 1999). Coloration may also be related to thermoregulation. Stoner *et al*. (2003*a*) found that dark coloration on extremities in lagomorphs might help conserve body heat in cold environments. In addition, coat color plays a role in animal communication. For example, dark ear tips in lagomorphs (Stoner *et al*., 2003*a*) and carnivores (Ortolani, 1999) have been shown to be useful signals for individual recognition, whereas conspicuous tail colors offer a similar function in artiodactyls (Stoner *et al*., 2003*b*).

Despite the fact that many coat color variants due to melanin synthesis and distribution have been well documented in laboratory mice (Silvers, 1979; Bennett & Lamoreux, 2003), we know little about coat color variation in wild house mice from which laboratory strains were originally derived. We know even less about the adaptive significance of coloration in wild mice. Taking advantage of a large series of wild mouse specimens housed in the National Institute of Genetics (NIG) in Japan, we document coat color variation in natural house mouse populations collected from areas spanning a large geographic range across Asia and evaluate in the house mice the applicability of Gloger's rule, which demonstrates that mainly in birds the darker pigmented individuals tend to reside in more humid regions and the paler ones in drier areas (Gloger, 1833; Zink

& Remsen, 1986). In addition, we explore the potential role of coat color variation as it relates to an environmental factor (precipitation) throughout the geographic range of these specimens.

Materials and methods

We analyzed 428 specimens of house mice *Mus musculus* housed in the NIG in Japan. The specimens were collected from 1980 to 1997 from 85 localities distributed throughout 16 Asian countries (Fig. 1). These localities lie between latitude 60°N and 7°S, and between longitude 60°E and 151°E. Countries, number of localities in each, and sample sizes are as follows: China (42 sites, 242 mice), India (four sites, 26 mice), Indonesia (two sites, four mice), Iran (one site, two mice), Japan (five sites, 22 mice), South Korea (two sites, 11 mice), Mongolia (one site, five mice), Nepal (two sites, seven mice), Pakistan (three sites, 13 mice), the Philippines (two sites, 11 mice), Russia (eight sites, 53 mice), Sri Lanka (four sites, five mice), Taiwan (one site, two mice), Thailand (one site, one mouse), Uzbekistan (four sites, 11 mice) and Vietnam (three sites, 13 mice).

Two researchers (Y. -C. Lai and H. –T. Yu) independently determined the coat color of each mouse skin by comparing them with Munsell soil color charts. In case of disagreement between the two researchers, a consensus was reached by re-examining the coat color together. The Munsell soil color charts use tristimulus color scores (hue, value and chroma) to depict colors. Hue indicates whether a color looks red, yellow, green, blue or purple; value indicates the color's lightness; and chroma indicates its strength or departure from a neutral color of the same lightness. The Munsell system is based on human perception, and therefore the outcome may not reflect actual visual effects, either among individual mice or between the mice and their predators (Endler, 1990; Bennett, Cuthill & Norris, 1994). Nevertheless, the standardized color schemes are still very useful for studies to analyze the color variation (Taylor, Meester & Rautenbach, 1990; Holt, Maples & Savok, 2003; Taylor, Kumirai & Contrafatto, 2005). For a quantitative analysis, we converted the three Munsell readings to numerical values following a method developed for forensic purposes (Sugita & Marumo, 1996). The conversion primarily affects hue, which uses discrete integers to represent specific hues (see Table 1 in Sugita & Marumo, 1996). To further characterize the coat color, principal components analysis (PCA) was used to reduce the three color variables (hue, value and chroma) into a single variable (PC1) that represents the largest proportion of variation in coat color and lightness of the coat color (also see 'Results').

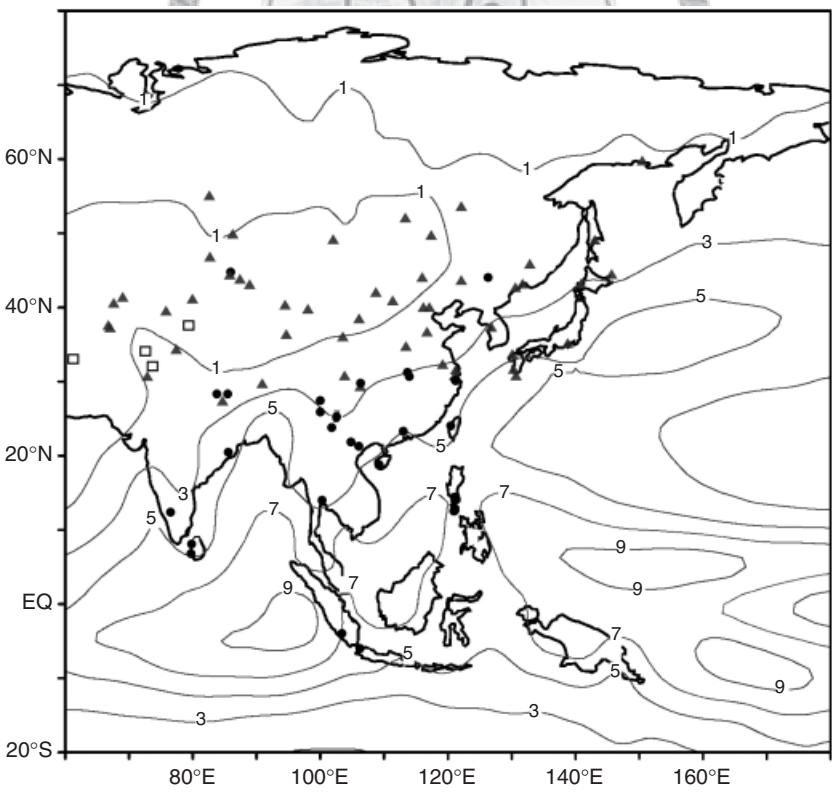

Figure 1 Map showing localities from which specimens of *Mus musculus* used in this study were collected. Dark circles indicate *Mus musculus castaneus* localities; gray triangles indicate *Mus musculus musculus* localities; open rectangles indicate *Mus musculus bactrianus* localities. Contour values represent mean annual precipitation from 1993 to 2002 (unit: mm day^{-1}).

Table 1 Variable loadings and percent variance explained by principal components analysis

Variables	PC1	PC2	PC3
Hue	0.257	0.965	0.044
Value	0.933	−0.072	−0.352
Chroma	0.917	−0.179	0.346
Per cent variance explained	59.30	32.50	8.20

To evaluate Gloger's rule, which predicts that animals in more humid areas tend to be darker, we used correlation analysis to analyze coat color (PC1) in relation to precipitation, a climate factor that is known to be involved in the evolution of coat color (Gloger's rule; see in Zink & Remsen, 1986). Precipitation data were taken from the CPC merged analysis of precipitation (CMAP; http://www.cdc.noaa.gov/cdc/data.cmap.html) (Xie & Arkin, 1997). The dataset is grid by latitude and longitude ($2.5^\circ \times 2.5^\circ$), and covers from 88.75°N to 88.75°S and from 1.25°E to 358.75°E.

Subspecies designations were recorded from museum specimen labels. However, only three subspecies were recognized for purposes of analyses, that is, *Mus musculus musculus*, *Mus musculus castaneus* and *Mus musculus bactrianus*, and we did not further distinguish more subspecies under *M. m. musculus* proposed by Tsuchiya *et al.* (1994). Moreover, because the nuclear genome of *Mus musculus molossinus* originated from *M. m. musculus*, we assigned the hybrid subspecies (Yonekawa *et al.*, 1994) distributed in Japan to *M. musculus musculus*. In addition, we recorded the sex from specimen labels, yielding 224 males, 197 females and seven specimens of unknown sex. We used the Kruskal–Wallis test to examine the difference in coat color distribution among the three subspecies described above. In addition, multiple regression analysis, was used to account for the variance in lightness among mice, based on the precipitation and subspecies variables. Two indicator variables (Montgomery & Peck, 1982), subsp1 (coded 1 for *M. m. castaneus* and 0 for others) and subsp2 (coded 1 for *M. m. bactrianus* and 0 for others), will be required to incorporate the three levels of subspecies. Partial r^2 was used to distinguish the relative importance of the two independent variables.

Results

Overall dorsal coat showed fewer color types (21 types or direct Munsell readings) than ventral coat color (33 types) in the mice we examined. Dorsal color variation ranged from yellow through brown to black whereas ventral color varied from white to black (Fig. 2). This trend held true even at a single locality. We found 1.54 ± 0.94 [mean ± standard deviation (SD)] color types on the dorsum and 1.98 ± 1.95 types on the ventrum for the 85 localities. This difference is significant (*t*-test, $t_{168} = -2.36$, $P = 0.019$).

From the perspective of direct Munsell readings, value and chroma contained much more variation than hue, as

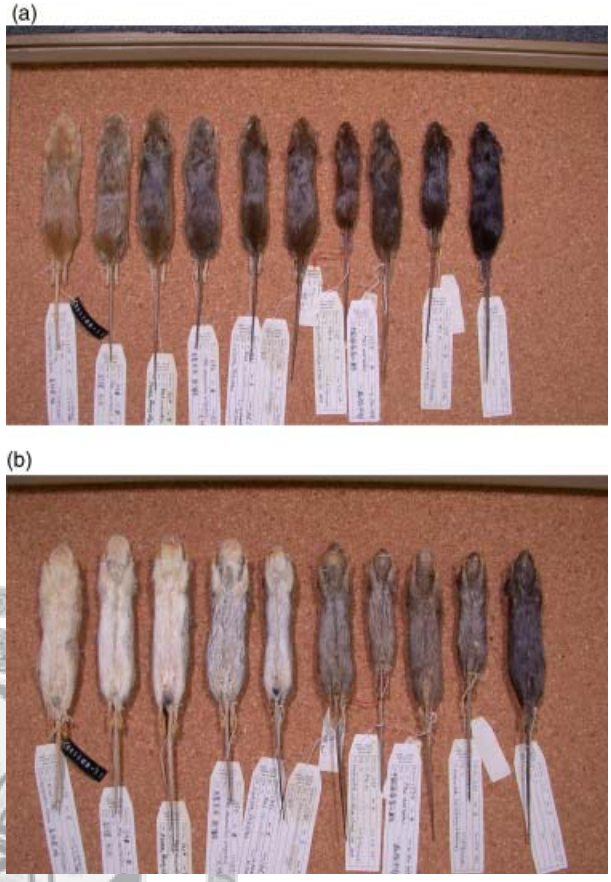

Figure 2 Representative variation in coat color in wild house mice *Mus musculus*.

reflected by the SD and the coefficient of variation (CV) of the three Munsell readings: value, SD = 1.021, CV = 31.5%; chroma, SD = 1.096, CV = 26.6%; hue, SD = 0.096, CV = 2.4%. The results suggest that value and chroma contribute to the majority of variation in house mouse coat color. PCA reduced the three Munsell readings into a single variable (PC1 = 0.93 × value + 0.92 × chroma + 0.26 × hue) that represents the largest proportion of the variation (59.3%) in coat color (Table 1). Taken together, PC1, in general, can be interpreted as lightness of coat color. The higher the value of PC1, the higher the Munsell scores for value and chroma (i.e. more light yellow); the lower the value of PC1, the lower the Munsell score for value and chroma (i.e. more dark brown).

The standardized PC1 variable (lightness) between male and female was not significantly different (*t*-test, $t_{419} = 0.729$, $P = 0.466$). We, therefore, analyzed the data combining two sexes. The correlation between the standardized PC1 variable (lightness) and precipitation was highly significant ($r = -0.47$, $P < 0.0001$) (Fig. 3). Precipitation explained 21.6% of the variation ($r^2 = 0.216$) in coat color. Paler coats were found in dry habitats and darker coats in more humid environments. Even within subspecies, the relationship was still significant (*M. m. musculus:* $r = -0.22$,

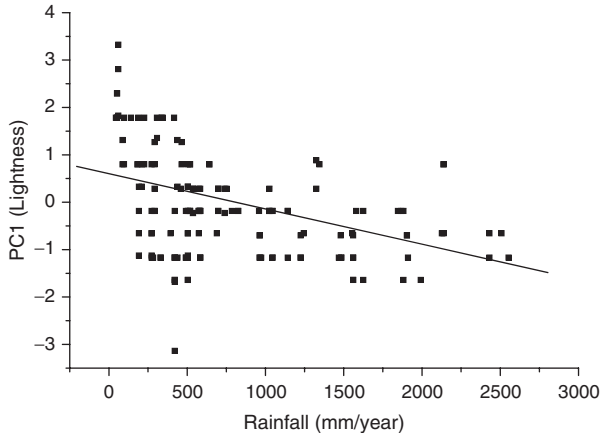

Figure 3 PC1 scores plotted against mean annual precipitation ($r = -0.47$, $P < 0.0001$) showing the relationship between coat color and precipitation.

$P = 0.026$; *M. m. castaneus*: $r = -0.34$, $P < 0.0001$; *M. m. bactrianus*: $r = -0.81$, $P < 0.0001$). The pattern corresponds to Gloger's rule, which can be simply stated as animals in relatively humid environments are darker than their conspecifics in relatively dry areas.

Dorsal coat colors among the three subspecies were significantly different from one another (Fig. 4, Kruskal–Wallis test $\chi^2_2 = 71.47$, $P < 0.0001$). Among the three subspecies, *M. m. castaneus* and *M. m. bactrianus* occupy the darker end and the lighter end of the spectrum, respectively, whereas *M. m. musculus* shows an intermediate distribution in color pattern.

The standardized multiple regression model, PC1 (lightness) $= -0.37 \times$ precipitation $-0.14 \times$subsp1 $+ 0.09 \times$subsp2, indicated that the precipitation variable can explain more of the variation (21.59%) in coat lightness in mice (partial $r^2 = 0.2159$) than the two subspecies indicator variables (1.24% variance for subsp1, and 0.81% variance for subsp2). However, the regression coefficients of all three variables are significant (precipitation, $t_{424} = -6.86$, $P < 0.0001$; subsp1, $t_{424} = -2.54$, $P = 0.011$; subsp2, $t_{424} = 2.13$, $P = 0.034$).

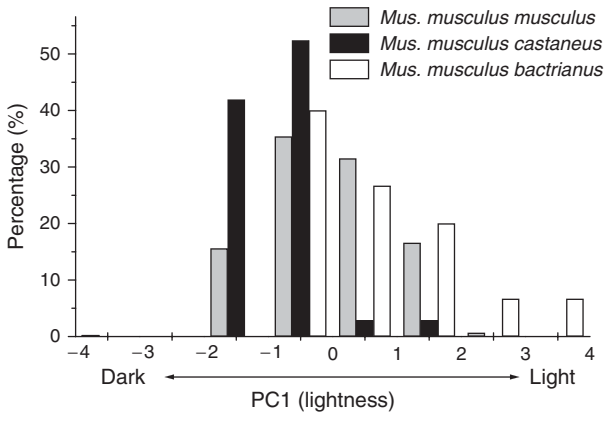

Figure 4 Frequency distribution of PC1 among three subspecies of *Mus musculus*.

Discussion

The variation in coat color among wild house mice, as demonstrated here, is substantial. Furthermore, we have shown that house mouse coat color variation follows Gloger's rule. While Gloger's rule is verified in many endothermic species, especially in birds (Gloger, 1833; Zink & Remsen, 1986; Hayes, 2001, 2003), the causes were not readily known so far. Several non-mutually exclusive hypotheses can account for the plumage color variation in birds consistent with Gloger's rule (see discussion in Burtt & Ichida, 2004). Here we explore some explanations for the coat color variation in wild house mice.

The protective coloration can be one of the most compelling explanations for the pattern despite a potential anthropogenic bias in analyzing the color perception (Endler, 1990; Bennett *et al.*, 1994). The concealment effect has been demonstrated to be true in small rodents, such as pocket mice *Chaetodipus intermedius* (Hoekstra & Nachman, 2003; Hoekstra, Drumm & Nachman, 2004) and oldfield mice *Peromyscus polionotus* (Smith, Carmon & Gentry, 1972; Belk & Smith, 1996) that their coat colors resemble soil background colors, supporting this hypothesis. These cases are convincing because predation experiments were conducted in field enclosures and confirmed that background color matching could increase survival rate in rodents (Dice, 1947; Kaufman, 1974). Furthermore, in *M. musculus*, experimental evidence shows that both aerial (Kaufman & Wagner, 1973) and terrestrial (Brown, 1965) predators selectively prey on conspicuously colored individuals. Here, we adopt a conventional notion that precipitation reflects the environmental background color. Higher precipitation means higher vegetation density (i.e. shade) and darker soil color (i.e. saturated with moisture), both contributing to a darker background color. In contrast, lower precipitation means a lighter background color. Consequently, the significant correlation between coat color and precipitation ($r = -0.47$, $P < 0.0001$) (Fig. 3) suggests that coat color variation in wild house mice results, in part, from a selective effect of crypsis. Additionally, less variation in dorsal color among individuals also suggests that the dorsal color is the major target for predation. To sum up, background matching will minimize differences between an animal's coloration and its surroundings; therefore, we consider that it is one of the rational explanations for the variation in coat color that we observed in the wild-caught house mice.

Recently the concealment explanation was found to be confounded by bacterial resistance in the bird (Burtt & Ichida, 2004). Because bacteria are more abundant and active in humid environments and because the dark pigment eumelanin resists bacterial degradation better than the light pigment pheomelanin (Hearing, 2000; Burtt & Ichida, 2004; Goldstein *et al.*, 2004), the coat color variation in wild house mice following Gloger's rule, likewise, might be a response to the selection to resist bacterial degradation. However, this

explanation is less likely to be valid for the house mice because the color variation in the dorsum did not correspond to that of the venter. If the bacterial resistance had been an important factor, the selection force would have had similar effects on the dorsal and ventral coloration. However, rigorous experiments should be conducted to confirm the bacterial effect in song sparrows (Burtt & Ichida, 2004).

Still, thermoregulation may play a partial role in coat color variation. The endothermic animals in cold climate tend to be darker for maintaining body temperature, because the dark coat color can absorb solar radiation more effectively than the pale one (Cloudsley-Thompson, 1999; Caro, 2005). If the thermoregulation argument were true, a negative correlation may exist between the lightness of coat color (standardized PC1) and latitude, which inversely reflects annual temperature. This is only true in the subspecies of *M. mus. musculus* ($r = -0.183$, $P < 0.0001$, data not shown), and yet the latitude (indirectly temperature) factor can account for just 3.3% of the variation ($r^2 = 0.033$). Therefore, the thermoregulation argument is uncertain for the mouse mice, perhaps because animals can employ tactics without involving radiation to maintain body temperature.

Differences in coat color among the three subspecies examined (Fig. 4) are consistent with differences in precipitation throughout the areas in which the mice were collected (Fig. 1). The darkest subspecies *M. m. castaneus* is distributed in humid areas and the lightest subspecies *M. m. bactrianus* occurs in arid areas. The third subspecies *M. m. musculus* shows an intermediate pattern (Fig. 4) and its distribution (Fig. 1) is broadest spanning from humid to arid areas. This pattern is supported by our multiple regression analysis, which showed that the precipitation variable explains much more of the variation (21.59%) in coat color than the subspecies variables (subsp1: 1.24%; subsp2: 0.81%) do. Therefore, we suggest that the differences in coat color among the three subspecies reflect parallel differences in levels of precipitation and thus environmental background colors of their habitats.

Because coat color variation, which can be explained by precipitation, accounts for only 21.59% of the variation observed, some other environmental factors must be involved (e.g. microhabitats or factors associated with the animals' commensalism with humans). Because the precipitation data that CMAP provided are only a rough estimation, actual precipitation in microhabitats may deviate from the estimated data. Furthermore, the levels of predation pressure and other environmental parameters within microhabitats are unknown. All of these factors may contribute to the residual variation in coat color, which cannot be explained by precipitation. For example, the quality of habitats can affect animal color (Veiga & Puerta, 1996; Griffith, 2000; Fitze & Richner, 2002; Parker *et al.*, 2003; McGraw, 2007). Some experiments also confirmed that the environmental stress was associated with the variation of feather color and the eumelanin could signal 'good genes' (Johnston & Janiga, 1995; Roulin *et al.*, 2000, 2001, 2003). Finally, the house mice are primarily commensal with human habitation, such as granaries and buildings. Although the house mice can easily disperse between human and natural habitats (Pocock, Hauffe & Searle, 2005), their coat colors at most are only partially affected by natural selection (Merilaita, Tuomi & Jormalainen, 1999) and may be neutral when in commensal habitats. Therefore, commensalism may be another factor that may contribute to the residual variation between coat color and precipitation, because polymorphic coat color can be maintained within a population (Roulin, 2004). Nevertheless, without information about other environmental factors, the highly significant correlation between the single indirect environmental factor (precipitation) and coat color variation may indicate that the selection pressure of background matching must be strong.

Like pocket mice (Nachman, Hoekstra & D'Agostino, 2003; Nachman, 2005), lesser snow geese and arctic skuas (Mundy *et al.*, 2004), some genetic factors are responsible for variation in the coat color of house mice. Research on coat color genetics is almost as old as the science of genetics itself (Silvers, 1979). There are more than 100 loci and 800 phenotypic alleles of coat color known in laboratory mice today (Bennett & Lamoreux, 2003). However, house mice in natural populations have much less color variation than has been observed in laboratory populations. In fact, many phenotypes that emerged from the laboratory, such as spotted, complete lack of pigmentation, mottled, belted, piebald and albino (Jackson, 1994; Nakamura *et al.*, 2002; Bennett & Lamoreux, 2003), are unlikely to be seen in the wild. We surmise that if coat color is constrained by selection (background matching) in natural populations, the alleles that act on variation in coat color of wild mice must be much fewer than those in laboratory mice. When alleles are lethal or pleiotropically deleterious, the chances of being retained in natural populations are slim.

In conclusion, many of the mutant coat color alleles that have been observed in laboratory mice were induced by radiation or chemical treatments (Nakamura *et al.*, 2002). These mutations are unlikely to happen spontaneously in natural populations. The major genes and alleles that have been found to act on coat color in other mammals (Majerus & Mundy, 2003), such as *mc1r*, *agouti*, etc., may still be the major candidate genes responsible for coat color variation in wild mice. A future attempt to associate the genotypes of some candidate loci with phenotypes as we clarified would shed light on the adaptive coloration in wild house mice.

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附錄二、小家鼠編號、採集地以及 *Agouti* 基因型

編號	採集地	*Agouti* 基因型
2247	新疆和丰縣	582 bp/582 bp
2248	新疆和丰縣	582 bp/582 bp
2249	新疆和丰縣	582 bp/582 bp
2250	新疆和丰縣	582 bp/582 bp
2251	新疆和丰縣	582 bp/582 bp
2225	新疆烏魯木齊市	582 bp/582 bp
2226	新疆烏魯木齊市	582 bp/582 bp
2227	新疆烏魯木齊市	582 bp/1256 bp
2228	新疆烏魯木齊市	582 bp/582 bp
2229	新疆烏魯木齊市	582 bp/582 bp
2242	新疆于田縣	582 bp/582 bp
2243	新疆于田縣	582 bp/582 bp
2244	新疆于田縣	582 bp/582 bp
2245	新疆于田縣	582 bp/582 bp
2246	新疆于田縣	582 bp/582 bp
04167	山西大同市大同縣	582 bp/582 bp
04168	山西大同市大同縣	582 bp/582 bp
04175	山西大同市大同縣	582 bp/582 bp
04177	山西大同市大同縣	582 bp/582 bp
04178	山西大同市大同縣	582 bp/582 bp
04179	山西大同市大同縣	582 bp/582 bp
04180	山西大同市大同縣	582 bp/582 bp
04181	山西大同市大同縣	582 bp/582 bp
04183	山西大同市大同縣	582 bp/582 bp
04184	山西大同市大同縣	582 bp/582 bp
04188	山西大同市大同縣	582 bp/582 bp
04207	山西長治市沁縣	1256 bp/1256 bp
04208	山西長治市沁縣	6 kb/6 kb
04214	山西長治市沁縣	582 bp/582 bp
04216	山西長治市沁縣	582 bp/582 bp
04222	山西長治市長治縣	582 bp/582 bp
04223	山西長治市長治縣	582 bp/582 bp
04224	山西長治市長治縣	582 bp/582 bp
04225	山西長治市長治縣	582 bp/582 bp

04226	山西長治市長治縣	582 bp/582 bp
04227	山西長治市長治縣	582 bp/582 bp
04228	山西長治市長治縣	1256 bp/6kb
04229	山西長治市長治縣	582 bp/582 bp
04234	山西長治市長治縣	582 bp/582 bp
04237	山西長治市長治縣	582 bp/582 bp
04239	山西長治市長治縣	582 bp/582 bp
04112	陝西延安市富縣	582 bp/6 kb
04114	陝西延安市富縣	582 bp/582 bp
04122	陝西延安市富縣	582 bp/6 kb
04124	陝西延安市富縣	582 bp/582 bp
04135	陝西延安市富縣	1256 bp/6 kb
04149	陝西延安市富縣	1256 bp/1256 bp
04051	陝西延安市寧強縣	582 bp/582 bp
04081	陝西延安市寧強縣	1256 bp/6 kb
04082	陝西延安市寧強縣	582 bp/582 bp
04083	陝西延安市寧強縣	582 bp/582 bp
04086	陝西延安市寧強縣	582 bp/582 bp
04176	河南洛陽伊川縣	582 bp/582 bp
04259	河南洛陽伊川縣	582 bp/582 bp
04260	河南洛陽伊川縣	582 bp/582 bp
04266	河南南陽市新野縣	582 bp/582 bp
04276	湖北襄樊市棗陽縣	582 bp/582 bp
04283	湖北襄樊市棗陽縣	582 bp/582 bp
04295	湖北孝感市安陸市	582 bp/582 bp
04297	湖北孝感市安陸市	582 bp/582 bp
04298	湖北孝感市安陸市	582 bp/582 bp
04299	湖北孝感市安陸市	582 bp/582 bp
04300	湖北孝感市安陸市	582 bp/582 bp
04301	湖北孝感市安陸市	582 bp/582 bp
04302	湖北孝感市安陸市	582 bp/582 bp
04303	湖北孝感市安陸市	582 bp/582 bp
04306	湖北孝感市安陸市	582 bp/582 bp
04307	湖北孝感市安陸市	582 bp/582 bp
04308	湖北孝感市安陸市	582 bp/582 bp
04309	湖北孝感市安陸市	582 bp/582 bp
04315	湖北孝感市安陸市	582 bp/582 bp

04316	湖北孝感市安陸市	582 bp/582 bp
04317	湖北孝感市安陸市	582 bp/582 bp
04318	湖北孝感市安陸市	582 bp/582 bp
04319	湖北孝感市安陸市	582 bp/582 bp
08001	雲南昆明市	582 bp/582 bp
08002	雲南昆明市	582 bp/582 bp
08003	雲南昆明市	582 bp/582 bp
08004	雲南昆明市	582 bp/582 bp
08005	雲南昆明市	582 bp/582 bp
08006	雲南昆明市	582 bp/582 bp
08007	雲南昆明市	502 bp/502 bp
08008	雲南昆明市	582 bp/582 bp
08009	雲南昆明市	582 bp/582 bp
08010	雲南昆明市	582 bp/582 bp
08011	雲南昆明市	582 bp/582 bp
08012	雲南昆明市	582 bp/582 bp
08013	雲南昆明市	582 bp/582 bp
08014	雲南昆明市	582 bp/582 bp
1197	台灣金門縣	582 bp/582 bp
1198	台灣金門縣	582 bp/582 bp
1199	台灣金門縣	582 bp/582 bp
1200	台灣金門縣	582 bp/582 bp
1201	台灣金門縣	582 bp/582 bp
832	台灣彰化縣	582 bp/582 bp
833	台灣彰化縣	582 bp/582 bp
837	台灣彰化縣	582 bp/582 bp
838	台灣彰化縣	582 bp/582 bp
1236	台灣屏東縣	582 bp/582 bp
1237	台灣屏東縣	582 bp/582 bp
1238	台灣屏東縣	582 bp/582 bp
1239	台灣屏東縣	582 bp/582 bp
1231	台灣屏東縣	582 bp/582 bp