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FULL PAPER

Genetic analysis of modifiers for the hooded phenotype in the rat

Daisuke Torigoe[1)], Atsushi Asano[1, 2)], Hideto Yamauchi[1)], Dang Ruiha[1)], Nobuya Sasaki[1)] and Takashi Agui[1,*)]

[1)]Laboratory of Laboratory Animal Science and Medicine, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

[2)]Laboratory of Biochemistry, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, Tottori 680–8553, Japan

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Abstract

The hooded phenotype is one of the coat color phenotype seen peculiarly in the rat. The hooded locus showing autosomal recessive inheritance is mapped to chromosome (Chr) 14 and that the hooded phenotype receives modification by hooded-modifier gene showing the linkage to the hooded locus. However, a gene responsible for either the hooded or hooded-modifier gene is not yet identified. To clarify genetic control of hooded phenotype, we carried out genetic linkage studies using BN and LEA rats. For determination of phenotypic variation, we measured ratio of pigmented coat area in parental and their F_1 and F_2 rats. We, then, conducted a genome-wide scan on 152 F_2 rats for linkage with ratio of pigmented coat area for the dorsal, ventral, and total regions. A major quantitative trait locus (QTL), *D14Got40*, showing highly significant linkage contributing 70–90% of the variance for hooded phenotype was detected on Chr 14, which may be correspondent to the hooded locus. In addition, another QTL, *D17Rat2*, showing highly significant linkage was also detected on Chr 17 in dorsal region phenotype as well as a QTL showing suggestive linkage on Chr15 in ventral region phenotype. We, further, investigated a genome-wide scan for epistatic interactions and detected significant interactions between *D14Got40* and *D20Mit1*, and between *D14Got40* and *D17Rat2* in the dorsal region phenotype. These results suggest that a major QTL in Chr 14, which is possibly correspondent to the hooded locus, mainly regulates the hooded phenotype with some modifier loci, two of which show epistatic interactions with the hooded locus.

Key words: epistatic interaction, hooded, QTL analysis, rat coat color

*Corresponding author: Takashi Agui, Laboratory of Laboratory Animal Science and Medicine, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan
Phone & Fax: +81–11–706–5106. E-mail: agui@vetmed.hokudai.ac.jp

Introduction

The genetic studies of the coat color phenotype in rodents have been performed for more than 100 years because of its visible and attractive phenotype. At present, more than 130 loci with nearly 1,000 different alleles are known to affect coat color in the mouse[1,18]. The hooded phenotype, which is peculiar in the rat, shows that colored coat covers head and mid-dorsal regions. Hooded phenotype, however, has many alleles such as hooded (h), hooded Irish (h^i), hooded notch (h^n), hooded extreme (h^e), and hooded restricted (H^{re}), which cause different extents of pigmented coat area[6,13,15,16]. Homozygotes with hooded alleles show the quantitative order of colored coat area as follows; $h^i > h > h^n > h^e > H^{re}$. The h^i allele shows pigmented coat covering almost whole surface except for a ventral white spot between or behind front legs[3]. The h^n allele resembles h allele except that the area of pigmentation is further restricted so that the hooded region in the head is smaller than that of h allele without mid-dorsal pigmented stripe. All these three alleles are completely recessive to the wild type and the h is also recessive to the h^i allele[2]. The hooded locus has been reported to show linkage to plasma protein markers, Gl-1, Gc protein, and albumin loci, all of which are now mapped to chromosome (Chr) 14[12,22,23]. Although the hooded locus was mapped to Chr 14 and various hooded alleles were identified and studied by many investigators, a gene responsible for the hooded phenotype is not yet identified. Furthermore, hooded modifier locus such as Hm^l for a long dorsal hooded pattern and Hm^s for a short dorsal hooded pattern, are reported to influence the hooded phenotype. The Hm locus is linked to the h locus, but a gene responsible for Hm is not yet identified either[19].

Pigmentation of the coat is achieved by melanocytes in the skin. Melanocytes are derived from neural crest cells and migrate through the skin in dorsal and caudal directions during embryogenesis. Therefore, studying the mechanism of generation of the hooded phenotype facilitates to understand the mechanism of migration and development of neural crest cells and melanocytes.

In the course of pathogenesis study of *Erysipelothrix rhusiopathiae* in rats, we found strain difference in resistance or susceptibility to the pathogen. Namely, the LEA rat, possessing h allele for hooded phenotype, is resistant to the *Erysipelothrix rhusiopathiae* infection, whereas the BN rat, possessing h^i allele, is sensitive. We, then, generated F_2 progenies from both strains to be used genetic study for resistance to the *Erysipelothrix rhusiopathiae* infection. In this study, we found that the hooded phenotype was not segregated into 3 : 1 ratio for the h^i and h phenotypes as following the Mendel's law, but distributed various and consecutive extents of pigmented regions. Thus, this attractive phenomenon led us to elucidate the genetic mechanism of it. Here, we describe the presence of genetic modifiers for the hooded phenotype in the rat as a consequence of extensive quantitative trait locus (QTL) analysis.

Materials and Methods

Animals: BN rats were purchased from Japan SLC (Shizuoka, Japan). LEA rats were provided from the National Bio Resource Project for Rat (Kyoto, Japan). F_1 progenies were obtained from female LEA rats mated with male BN rats. F_2 progenies were produced by mating F_1 progenies randomly. Animals were maintained in specific pathogen-free conditions with feeding and drinking *ad libitum*. In the experimental animal care and handling, the investigators adhered to the Regulation for the Care and Use of Laboratory Animals, Hokkaido University.

Measurement of pigmented coat ratio: Photographs of both dorsal and ventral side of rats were taken with COOLPIX 4500 digital

camera (Nikon, Tokyo, Japan). Pigmented and non-pigmented area was traced manually, separated with two colors (Fig. 1A right panel of each photograph), and calculated as pixels using histogram function of Photoshop Elements 4.0 (Adobe Systems, California, USA). Area of four foots was excluded from the calculation. To control for variations in size among animals, percentage of pigmented area (pigmented area/total area $\times$ 100) was calculated separately for dorsal, ventral, and total body surfaces of each animal. These values were used for quantitative traits. For pigmented area measurements, data were presented as means $\pm$ SEM, compared each other using the Student's *t*-test, and considered to be significantly different at $p < 0.05$.

Genotyping of microsatellite markers: Extraction of genomic DNA from tail clips was performed by the standard methods. A total of 116 microsatellite markers showing polymorphisms between BN and LEA rats were used for genetic study (Table 1). The average interval of adjacent microsatellite markers was 15.1 cM. The map positions of microsatellite loci were based on information from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). PCR was carried out on a Bio-Rad PCR thermal cycler (iCycler, California, USA) with the cycling sequence of 95°C for 1 min (one cycle), followed by 35 cycles consisting

Table 1. Microsatellite markers used for genotyping F_2 progenies.

Microsatellite Markers	Posision (Mbp)	Microsatellite Markers	Posision (Mbp)	Microsatellite Markers	Posision (Mbp)	Microsatellite Markers	Posision (Mbp)	Microsatellite Markers	Posision (Mbp)
D1Rat250	13	*D3Got34*	86	*D7Mgh11*	2	*D12Rat14*	29	*D17Rat14*	33
D1Rat403	41	*D3Rat21*	110	*D7Rat31*	28	*D12Rat22*	46	*D17Rat13*	34
D1Mit1	59	*D3Mit4*	131	*D7Rat51*	50	*D13Rat59*	31	*D17Rat15*	37
D1Rat344	116	*D3Rat8*	132	*D7Rat112*	90	*D13Mgh4*	38	*D17Arb7*	71
D1Mgh19	155	*D3Mit3*	136	*D7Rat128*	121	*D13Mit2*	62	*D17Rat47*	85
D1Arb29	180	*D3Mgh3*	155	*D8Rat164*	28	*D13Mit4*	90	*D18Mit1*	12
D1Rat169	229	*D3Arb15*	170	*D8Rat188*	44	*D14Mit2*	18	*D18Rat132*	25
D1Rat90	267	*D4Rat5*	9	*D8Mgh4*	86	*D14Rat36*	33	*D18Rat91*	61
D2Rat309	21	*D4Rat231*	88	*D8Rat90*	114	*D14Got40*	35	*D18Rat5*	77
D2Rat184	40	*D4Rat78*	105	*D8Rat3*	126	*D14Rat12*	42	*D19Rat17*	12
D2Arb7	57	*D4Rat273*	133	*D9Rat46*	6	*D14Rat15*	44	*D19Arb1*	14
D2Rat19	58	*D4Arb28*	154	*D9Rat30*	20	*D14Rat17*	59	*D19Rat68*	43
D2Rat74	70	*D4Rat203*	161	*D9Mit3*	55	*D14Rat94*	89	*D19Rat107*	48
D2Rat203	76	*D5Rat121*	9	*D9Rat90*	73	*D14Rat38*	99	*D20Mgh5*	9
D2Mit17	109	*D5Rat190*	25	*D9Rat4*	90	*D15Rat5*	22	*D20Rat37*	32
D2Mit8	148	*D5Mit10*	57	*D9Rat108*	103	*D15Rat6*	32	*D20Rat54*	41
D2Rat40	163	*D5Rat107*	94	*D10Rat51*	11	*D15Rat11*	51	*D20Mit1*	48
D2Mit13	182	*D5Rat95*	130	*D10Mgh6*	67	*D15Mgh4*	83	*DXRat82*	19
D2Rat52	202	*D5Rat33*	143	*D10Mgh3*	100	*D15Rat106*	106	*DXRat5*	25
D2Rat61	227	*D5Rat99*	157	*D10Rat7*	105	*D16Mgh4*	18	*DXRat102*	133
D3Rat52	14	*D6Rat105*	18	*D11Rat21*	18	*D16Rat65*	61		
D3Mgh7	36	*D6Rat136*	48	*D11Rat5*	37	*D16Rat55*	76		
D3Mgh6	68	*D6Rat11*	115	*D11Rat43*	85	*D17Rat2*	10		
		D6Rat3	145	*D12RAt58*	4	*D17Rat102*	27		

of denaturation at 95°C for 30 sec, primer annealing at 58°C for 30 sec, and extension at 72°C for 30 sec. PCR mixture and enzymes were purchased from TaKaRa (Ex Taq DNA Polymerase, Otsu, Japan). The amplified samples were electrophoresed with 10–15% polyacrylamide gel (Wako, Osaka, Japan), stained with ethidium bromide, and then photographed under an ultraviolet lamp.

QTL analysis: QTL analysis was performed with Map Manager QTXb20 software program[10]. In this program, linkage probability was examined by interval mapping. Genome-wide significance thresholds were set, as suggested previously, at the 37th ("suggestive"), 95th ("significant"), and 99.9th ("highly significant") percentiles, which correspond to the chance of finding 1 false-positive linkage 0.63, 0.05, and 0.001 times, respectively[8,9]. For each chromosome, the likelihood ratio statistic (LRS) values were calculated by 5,000 random permutations of the trait values relative to genotypes of the maker loci. For the quantitative trait of dorsal region, suggestive, significant, and highly significant values were 10.0, 17.2, and 27.1, respectively. For the quantitative trait of ventral region, suggestive, significant, and highly significant values were 10.1, 17.1, and 27.0, respectively. For the quantitative trait of total region, suggestive, significant, and highly significant values were 10.0, 16.7, and 24.5, respectively. Confidence intervals (CI) were estimated by bootstrap analysis[24,25] instead of the classic 1-LOD support interval[8], since it has been shown to be more reliable over all QTL strengths[5].

Possible interactions between all pairs of marker loci were primarily screened with QTXb20 software program. This program searches for digenic epistasis by testing all pairs of marker loci for both main effects and interaction effects using regression. Pairs of loci must pass two tests in order to be reported as having a significant interaction effect. First, the total effect of the two loci must have a p-value less than 10^{-5} (software suggestion). Second, the interaction effect itself must have a p-value less than 0.01. For each significant two-locus interaction, QTX software calculates LRS for each main effect, the interaction effect, and the total effect. In addition, this software allows us to run permutation tests for two-locus interactions for given data set to determine threshold LRS for declaring significance. We used 2,000 permutation tests of our data set for declaring a significant two-locus interaction. After screening epistatic interactions with QTX software program, statistic analysis of detected interactions of microsatellite loci including near loci was performed with ANOVA program to confirm substantial epistatic interactions.

Results

Phenotyping of parental strains and their F_1 and F_2 progenies

Initially, we measured pigmented area for dorsal, ventral, and total regions in parental and F_1 rats (Fig. 1A). The ratios for dorsal region were 1.00 in BN rats ($n = 5$), 0.960 ± 0.009 in LEA rats ($n = 7$), and 0.997 ± 0.0004 in F_1 rats ($n = 8$) (Fig. 1B upper panel). The ratios for ventral region were 0.997 ± 0.002 in BN rats, 0.395 ± 0.02 in LEA rats, and 0.987 ± 0.003 in F_1 rats (Fig. 1C upper panel). The ratios for total region were 0.998 ± 0.001 in BN rats , 0.678 ± 0.012 in LEA rats, and 0.992 ± 0.002 in F_1 rats (Fig. 1D upper panel). The ratios in F_1 rats were similar to those of BN rats in all three regions. There was significant difference between BN and LEA rats and between F_1 and LEA rats ($p < 0.01$). These results were consistent with previous study that the h^i allele is dominant to the h allele[3]. Next, we measured the phenotype of F_2 ($n = 152$) progenies (Figs. 1A and 1B–D lower panel). These values were not segregated into two groups such as BN and LEA types with 3 : 1 ratio, but showed consecutive values in all

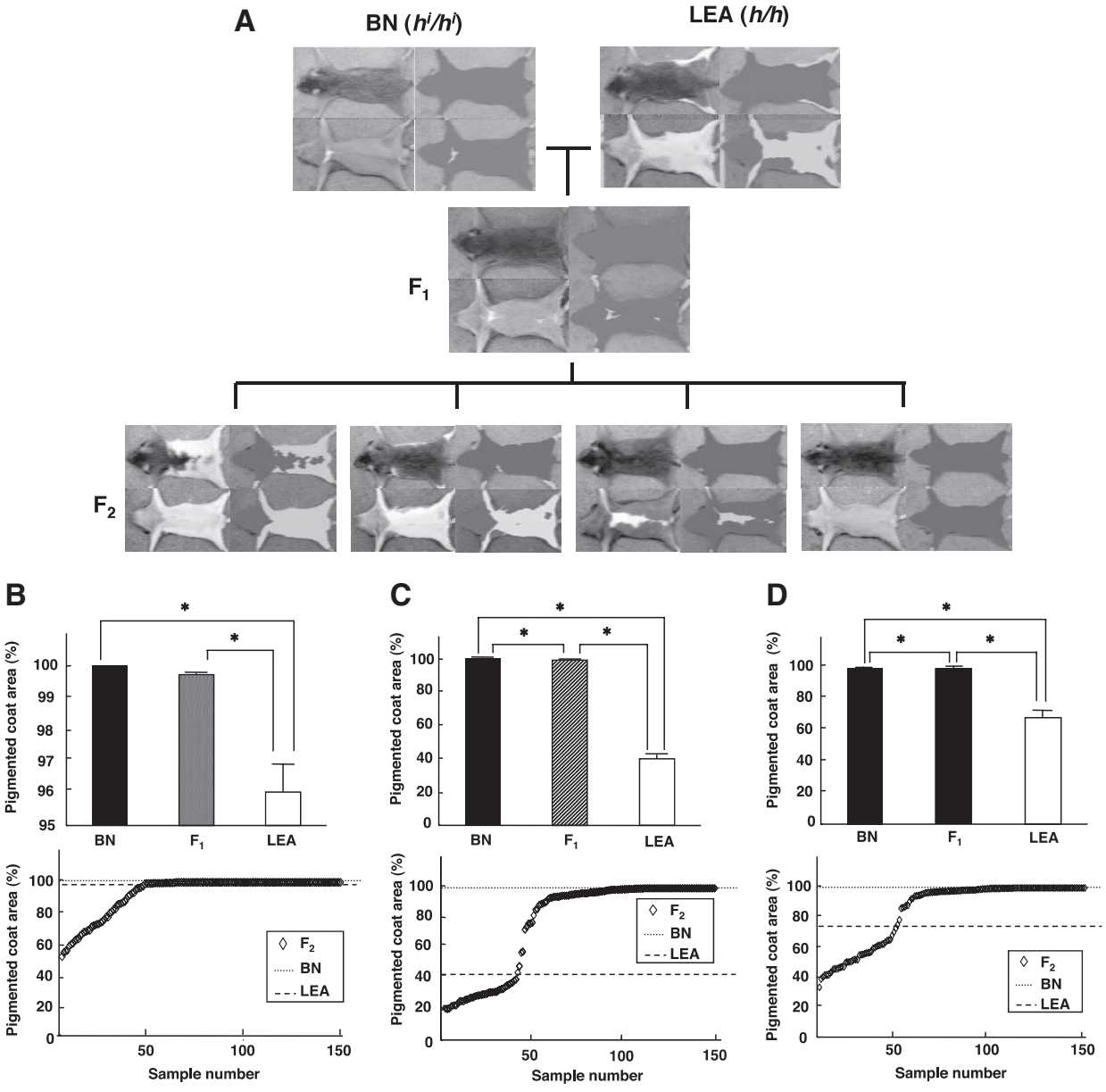

Fig. 1. Ratio of pigmented coat area of parental strains, F_1, and F_2 progenies. A, Photographs of representatives of each generation. Right side panels show the tracing patterns for pigmented area. B, C, and D show the ratios of pigmented coat area for dorsal, ventral, and total regions, respectively. The upper panels show the ratio of pigmented area in parental and F_1 rats, and the lower panels show the ratio of pigmented area in F_2 rats. The number of rats for BN, LEA, F_1, and F_2 rats are 5, 7, 8, and 152, respectively. Data represent the mean $\pm$ SEM and asterisks indicate $p < 0.01$ in the upper panels.

three regions. These data suggest that the regulation of hooded phenotype was under multigenic control.

Genome-wide scan for mapping hooded-modifier loci in F_2 progenies

To clarify the association between the variety of hooded phenotype in F_2 rats, we performed genome-wide scan using 116 microsatellite markers found polymorphisms between BN and LEA rats with Map Manager QTXb20 software program. In this QTL analysis, bootstrap analysis was performed to detect CI of the QTL. Only peak located in the CI was

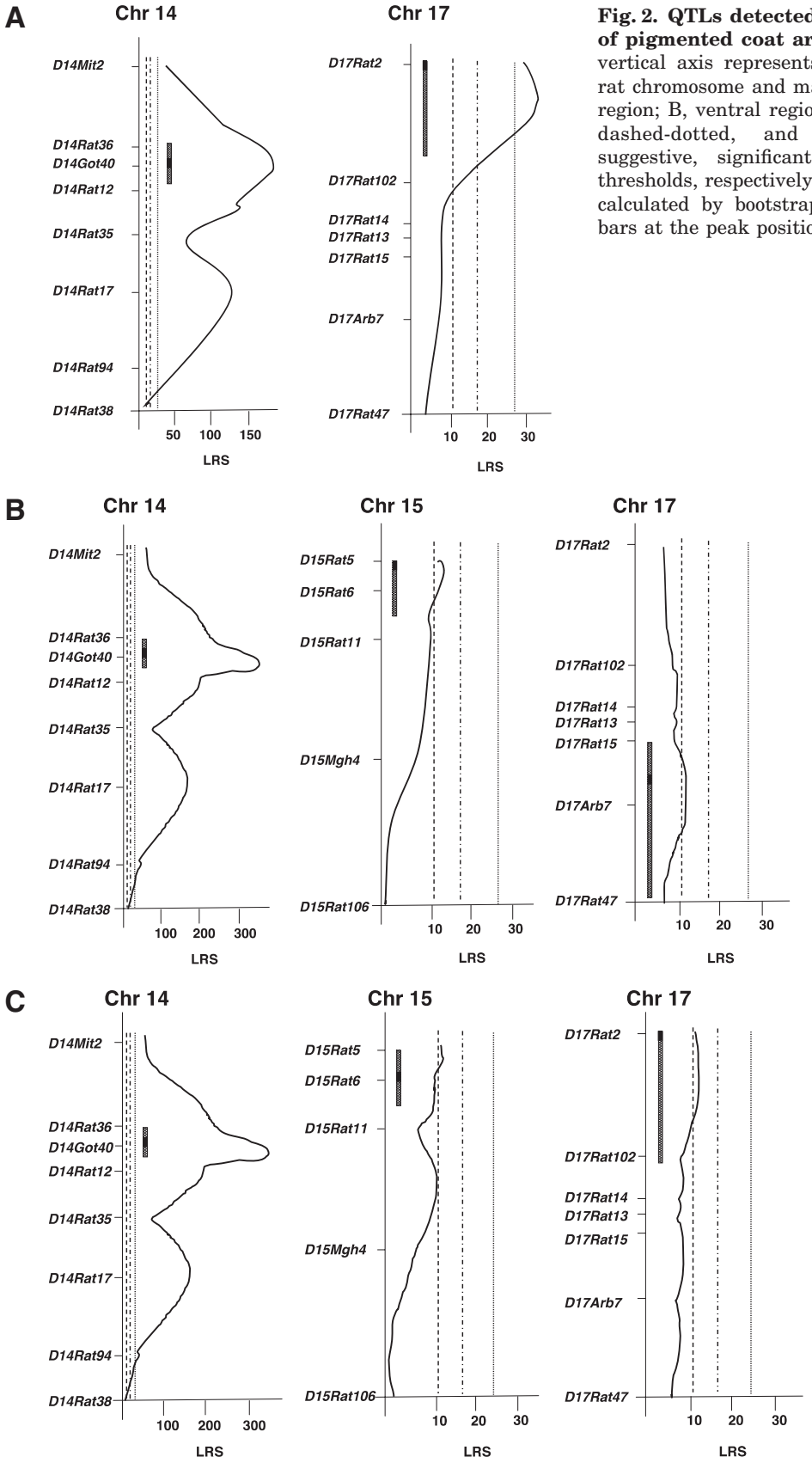

Fig. 2. QTLs detected to influence the extent of pigmented coat area in F_2 progenies. Each vertical axis represents the genetic map for the rat chromosome and markers in F_2 rats. A, dorsal region; B, ventral region; C, total region. Dashed, dashed-dotted, and dotted lines indicate suggestive, significant, and highly significant thresholds, respectively. Shaded bars represent CI calculated by bootstrap analysis with the black bars at the peak position of each QTL.

Table 2. Characteristics of QTLs detected with Map Manager QTX for extent of pigmented coat area.

regions	Markers	Position[a)]	LRS	%[b)]	CI[c)]	BN/BN[d)]	BN/LEA[d)]	LEA/LEA[d)]
Dorsal	D14Got40	22.5	183.5	70	5	100	99.6 ± 0.2	75.8 ± 2.0***†††
Dorsal	*D17Rat2*	6.0	30.1	18	19	83.4 ± 3.4	94.6 ± 1.1***	97.9 ± 0.8***†
Ventral	*D14Got40*	22.5	367.0	91	4	99.9 ± 0.05	93.0 ± 1.4***	32.1 ± 1.6***†††
Ventral	*D15Rat5*	15.4	11.0	7	50	86.5 ± 3.7	67.3 ± 4.4**	79.9 ± 4.3†
Ventral	*D17Arb7*	54.9	13.7	9	41	75.9 ± 5.2	67.9 ± 4.2	88.3 ± 3.7†††
Total	*D14Got40*	22.5	355.3	90	4	99.9 ± 0.02	96.3 ± 0.8***	54.0 ± 1.5***†††
Total	*D15Rat5*	15.4	10.4	6	56	91.5 ± 2.5	78.8 ± 3.0**	86.1 ± 3.1
Total	*D17Rat2*	6.0	10.6	7	52	76.0 ± 4.8	86.7 ± 2.2*	90.2 ± 2.4**

[a)]Expressed in cM according to recombination fraction by Map Manager QTX software.
[b)]Percentage of total variance attributable to locus.
[c)]95% confidence interval of QTL location as calculated by QTX software.
[d)]Mean phenotypic value ± SEM for rats homozygous for the BN allele (BN/BN), heterozygous (BN/LEA), and homozygous for the LEA allele (LEA/LEA).
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, vs. BN/BN.
$^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ vs. BN/LEA.

recognized as a substantial QTL. Two highly significant chromosomal regions regulating hooded phenotype for dorsal region were detected on Chr 14 and 17 (Fig. 2A). A highly significant chromosomal region regulating hooded phenotype for ventral and total was also detected on Chr 14 and two suggestive chromosomal regions were detected on Chr 15 and 17 (Figs. 2B and C). Table 2 summarizes microsatellite markers linked to the phenotype, showing LRS values, genetic effects, CIs, and phenotypic values in each genotype. The highly significant QTL (*D14Got40*) on Chr 14 was detected as a common locus with fur exceeding LRS threshold value and BN-dominant effect in QTL analyses using quantitative traits of dorsal, ventral, and total regions. This QTL (*D14Got40*) contributed 70–90% variance for hooded phenotype of dorsal, ventral, and total regions (Table 2). Two suggestive QTLs detected on Chr 15 and 17 had slightly exceeding threshold LRS value in ventral and total regions. A QTL on Chr 15 showed non-additive and non-dominant trait, whereas a QTL on Chr 17 showed LEA-dominant trait.

Identification of epistatic interactions involved in hooded phenotype

To find epistatic interactions among microsatellite loci, we performed analysis using the interaction function of Map Manager QTX. Permutation tests in this software program for the interaction analysis in dorsal region showed that LRS values of 33.4, 45.1, and 59.4 were necessary for suggestive, significant, and highly significant interactions, respectively. Similarly, LRS values of 31.0, 45.1, and 59.4 in ventral region, and 31.3, 40.9, and 50.1 in total region are necessary for suggestive, significant, and highly significant interactions, respectively. Pairwise testing across all 116 markers revealed significant interaction between Chr 14 (*D14Rat36*) and Chr 20 (*D20Mit1*) and suggestive interaction between Chr 14 (*D14Rat17*) and Chr 17 (*D17Rat102*) in dorsal region. In total region, suggestive interaction between Chr 14 (*D14Rat17*) and Chr 17 (*D17Rat2*) was detected. Next, we performed ANOVA analysis with those microsatellite loci including loci locating in the vicinity to confirm substantial epistatic interaction. The ANOVA analysis revealed that several microsatellite loci locating in the vicinity of the above microsatellite loci also showed significant

interaction. Thus, we considered that *D14Got40* locus could be representative of maicrosatellite loci showing significant interaction on Chr14, because it locates in a peak position of QTL on Chr 14 by bootstrap analysis. Similarly, *D17Rat2* locus was considered to be representative of microsatellite loci showing significant interaction on Chr 17. Thus, we showed epistatic interaction between *D14Got40* and *D20Mit1* loci in Fig. 3A, and between *D14Got40* and *D17Rat2* loci in Fig. 3B. Both *D20Mit1* and *D17Rat2* loci show significant effect, only when the genotype of *D14Got40* locus is homozygous for LEA. It is noteworthy that the extent of pigmented coat is maximal, when the genotype of *D17Rat2* locus is homozygous for LEA.

Discussion

The hooded phenotype is peculiarly expressed in the rat and there are many alleles reported. A typical hooded phenotype, *h* allele, shows that pigmented coat covers head and mid-dorsal region. However, *h* allele shows the variation in the extent of pigmented area, for example, the LEA rat possessing *h* allele shows that pigmented coat covers more widely in the dorsal region than that of the typical *h* allele rat strain. In the BN rat, on the other hand, pigmented coat covers almost whole surface except for a ventral small white spot between or behind front legs, which is categorized as h^i allele. When BN and LEA rats were mated, F_1 rats showed the same phenotype as the BN rat, consistent with previous observation that the h^i allele is dominant to the *h* allele. However, phenotypes of F_2 progenies did not follow the

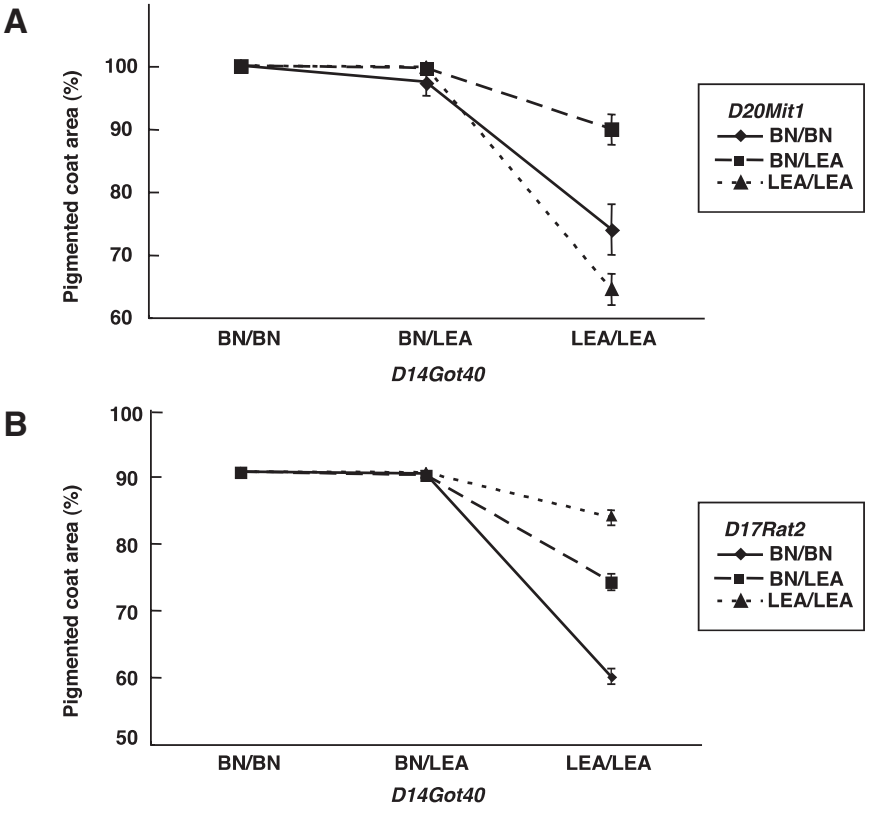

Fig. 3. Significant epistatic interactions detected to influence the extent of pigmented coat area in F_2 progenies. Two significant epistatic interactions were detected in the dorsal region phenotype between *D14Got40* and *D20Mit1* loci (A) and between *D14Got40* and *D17Rat2* loci (B). Error bars represent the SEM.

Mendel's law. The extent of pigmented coat was not segregated into 3 : 1 ratio, but varied with consecutive values. This suggests that the extent of coat color pigmentation receives multigenetic control, which led us to perform QTL analysis.

QTL analysis revealed a main QTL located closely to *D14Got40* showing extremely high LRS value, which is possibly corresponding to the hooded locus. A gene responsible for the hooded phenotype is not yet identified. Possibly h^i and *h* alleles cause polymorphisms on the hooded gene, which should be identified in future. In this study we succeeded to identify several other QTLs located closely to *D15Rat5*, *D17Rat2,* and *D17Arb7*, all of which possibly affect the extent of pigmented coat cooperatively. Among these QTLs, the *D17Rat2* locus is of interest. The *D17Rat2* locus provided highly significant LRS value by itself in the QTL analysis in the dorsal region phenotype (Fig. 2A). Further, the *D17Rat2* locus showed epistatic interaction with the hooded locus, *D14Got40* (Fig. 3B). It is noteworthy that the ability of increasing the extent of pigmented coat in the dorsal region is depending on the genotypes of the *D17Rat2* locus, when the hooded locus, *D14Got40*, is homozygous for LEA allele. The extent of pigmented coat is the highest in the F_2 rats possessing LEA-homozygous genotype of the *D17Rat2* locus followed by BN/LEA-heterozygous and BN-homozygous genotypes. This result proposes the reason why the LEA rat, which possesses the *h* allele, shows the wider pigmented coat area in the dorsal region than that of the typical hooded rat strain possessing the same *h* allele. The *D20Mit1* is another locus showing significant epistatic interaction with the *D14Got40* locus (Fig. 3A). The *D20Mit1* locus by itself never affects the extent of pigmented coat area because of not being detected in QTL analysis, whereas it affects the extent of pigmented coat area, only when the hooded locus, *D14Got40*, is homozygous for LEA allele. The effect of *D20Mit1* locus is maximal in BN/LEA-heterozygous genotype followed by BN-homozygous and LEA-homozygous genotypes. Both *D17Rat2* and *D20Mit1* loci may be relating to the *Hm* locus reported previously[19].

Generally, coat color pigmentation depends on the amount of melanin produced by melanocytes derived from neural crest cells[4,17]. Until now, a large number of coat color loci involved in the development of melanocytes and mutation of those genes have been reported[14]. The hooded phenotype is considered to be a defect in melanocyte migration, development, and/or melanin production. Therefore, a gene responsible for the hooded phenotype may be attributed to one of these genes, because knockout or natural mutation in these genes cause the defect in migration, proliferation, or survival of melanocytes, resulting in a loss of pigmentation in coat[7,11,21]. In fact, it has been raised a possibility that the white pattern in hooded rat is due to the delay of melanoblast migration[26]. Therefore, genes responsible for hooded modifiers as well as the hooded phenotype itself may be involved in migration, development, and survival of melanocytes. However, genes for responsible for the hooded phenotype are not yet identified[12,20,22,23]. Further study is necessary for the identification of genes affecting the hooded phenotype.

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