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Genetic Dissection of Litter-size Quantitative Trait Loci Located on Murine Chromosome 7

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Abstract

A significant quantitative trait locus (QTL) for murine litter size, *Lsq1*, was previously identified on chromosome 7 in the female backcross progeny of ♀(♀C57BL/6J × ♂RR/Sgn) × ♂RR/Sgn (BRR mice). In the present study, QTL mapping analysis was performed for the same trait in the female backcross progeny of ♀(♀RR/Sgn × ♂C57BL/6J) × ♂RR/Sgn (RBR mice), and *Lsq1* was identified again. As litter size is significantly larger in BRR mice than that in RBR mice, the potential effect of grandparental cross direction was indicated. QTL mapping analysis of combined data from BRR and RBR mice showed that *Lsq1* was split into two loci: *Lsq1-1* and *Lsq1-2*. The RR/Sgn strain-derived allele was associated with reduced litter size at both loci. *Lsq1-1* and *Lsq1-2* exhibited significant effects on the number of stillbirths in RBR mice but not in BRR mice, and the RR/Sgn strain-derived allele was associated with increased stillbirths at both loci. Therefore, the effects of *Lsq1-1* and *Lsq1-2* suggest that the number of stillbirths depends on the grandparental cross direction and that the smaller litter size in RBR mice is a consequence of increased stillbirths. *Lsq1-1* and *Lsq1-2* are suggested to be novel QTL associated with murine litter size.

Discipline: Animal Science
Additional key words: grandparental cross direction, RR/Sgn mice, stillbirth

Introduction

Litter size, which is the number of pups born, is a representative quantitative trait relevant to female reproduction in mammals. Identifying the genes underlying litter size will help improve fecundity, especially in species with multiple births such as pigs, owing to its direct connection with prolificacy. Quantitative trait loci (QTL) mapping analyses (Distl 2007, Li et al. 2009) and genome-wide association studies (Bergfelder-Drüing et al. 2015, Guo et al. 2016, He et al. 2016, Ma et al. 2018, Wang et al. 2018) have identified many genomic regions contributing to pig litter size. However, only a few causal genes were identified in these studies. One of the major difficulties in identifying causal genes is the generally low heritability of litter size (Falconer & Mackay 1996, Nicholas 1996). It is important to minimize the effects of environmental factors to identify the genes underlying low-heritability traits (Li et al. 2020). Therefore, the analysis of the genetic basis of litter size of mice is rational because nonheritable effects may be eliminated to a considerable degree.

QTL mapping analysis for murine litter size in the female backcross progeny of ♀(♀C57BL/6J×♂RR/Sgn)×♂RR/Sgn (BRR mice) that identified a significant QTL (*Lsq1*) on chromosome 7 (Suto 2015a) was previously performed. However, it is necessary to confirm the presence of *Lsq1* in independent studies because of the low heritability of this trait. Therefore, litter size in an additional female backcrossed progeny of ♀(♀RR/Sgn × ♂C57BL/6J) × ♂RR/Sgn (RBR mice) was analyzed in this study. Furthermore, QTL mapping analysis of combined data from BRR and RBR mice was performed to narrow the 95% confidence interval (CI) for *Lsq1*, which was deemed too broad to propose candidate genes. Finally, the following three-step approach was taken to prioritize candidate genes on the assumption that the causal variants should be nonsynonymous single nucleotide variants (nsSNVs) or insertion and deletion (INDEL) mutations in their coding regions: (1) searching

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candidate genes located within the 95% CI of QTL via the Mammalian Phenotype Browser of Mouse Genome Informatics (MGI) database (http://www.informatics.jax.org), (2) seeking nsSNVs/INDELs within the candidate genes by performing a whole-exome sequencing analysis of the RR/Sgn strain genome, and (3) investigating the effect of nsSNVs/INDELs on litter size using a publicly available dataset for candidate genes with nsSNVs/INDELs in silico.

Materials and methods

1. Mice

Inbred mouse strains, RR/Sgn and C57BL/6J, were purchased from Riken BioResource Center (BRC No. RBRC01672, Tsukuba, Ibaraki, Japan) and Clea Japan Inc. (Tokyo, Japan), respectively. RR/Sgn females were crossed with C57BL/6J males to produce (RR/Sgn × C57BL/6J) F_1 mice. F_1 females were crossed with RR/Sgn males to generate a total of 228 (RR/Sgn × C57BL/6J) F_1 × RR/Sgn female backcross mice (RBR mice). Hereafter, the RR/Sgn and C57BL/6J strains are defined as having the R allele and B allele, respectively, throughout the genome.

All mice were maintained in a specific pathogen-free facility with a regular light cycle and controlled temperature and humidity conditions. Rodent chow (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and water were freely available throughout the experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee of the National Institute of Agrobiological Sciences, Ibaraki, Japan (authorization numbers H28-011 and R01-009).

2. Phenotyping

Only nulliparous RBR mice were used in this study, and litter-size data were obtained for 228 primiparous RBR mice by crossing them with male RBR mice (four to five female RBR mice were housed with one or two male RBR mice). Potential effects resulting from the heterogeneity of male gametes on litter size were not considered because litter size is thought to be determined by ovarian and uterine physiological processes (Ma et al. 2018). Thereafter, female RBR mice deemed to be pregnant were individually housed. The number of newborn offspring was scored once between 7:00 and 14:00 on the day of delivery.

The term "litter size" is used empirically to describe either the total number of pups born (TNB) or the number of pups born alive (NBA). In the present study, TNB and NBA were analyzed separately and are collectively referred to as "litter size." The number of stillbirths (NSB) was equivalent to the difference between TNB and NBA. As described below, all traits were analyzed via nonparametric interval mapping; some relevant fundamental statistics for traits in RBR mice are as follows: TNB (median 8, mode 8, min 0, max 12, skewness −1.05, and kurtosis 1.91); NBA (median 8, mode 8, min 0, max 12, skewness −1.15, and kurtosis 1.80); and NSB (median 0, mode 0, min 0, max 5, skewness 3.74, and kurtosis 16.80).

3. QTL mapping analysis

QTL mapping analysis was conducted using R/qtl version 1.33-7 (Broman et al. 2003, Broman & Sen 2009).

First, QTL mapping analysis was performed for 228 female RBR mice. All the trait values were not distributed normally after Box–Cox transformation; therefore, nonparametric interval mapping was performed by computing over at 1-cM step. The 228 female RBR mice were genotyped for the following 14 microsatellite markers on chromosome 7: *D7Mit340* (2.67), *D7Mit306* (6.42), *D7Mit76* (9.46), *D7Mit246* (17.40), *D7Mit308* (19.00), *D7Mit225* (21.36), *D7Mit247* (23.90), *D7Mit228* (25.61), *D7Mit229* (29.43), *D7Mit232* (33.06), *D7Mit195* (35.19), *D7Mit250* (45.00), *D7Mit62* (48.36), and *D7Mit220* (55.69) (figures in parentheses indicate chromosomal positions [cM], which were retrieved from the MGI database).

Next, QTL mapping analysis was performed in 255 female BRR mice. They were genotyped for additional six microsatellite markers on chromosome 7 because the BRR mice in the previous study were genotyped for only eight microsatellite markers on chromosome 7 (Suto 2015a). Consequently, BRR mice were genotyped for 14 microsatellite markers, which were the same as those genotyped in RBR mice.

Finally, QTL mapping analysis was performed on the population that combined 228 female RBR mice and 255 female BRR mice (Suto 2015a). The trait values from the 228 RBR mice and 255 BRR mice were merged to form 483 combined backcross mice after standardization within each backcross population.

Threshold logarithm of odds (LOD) scores for suggestive ($P < 0.63$) and significant ($P < 0.05$) linkages were determined by performing 1,000 permutations for each trait in 483 combined backcross mice (Lander & Kruglyak 1995). In this case, the genotypes of microsatellite markers outside chromosome 7 in the 228 RBR mice were labeled as missing data.

4. Whole-exome sequencing analysis

Genomic DNA was extracted from the tail clippings of a RR/Sgn mouse using a Wizard genomic DNA

purification kit (Promega, Madison, WI, USA). The isolated DNA samples were outsourced to Filgen Inc. (Nagoya, Aichi, Japan) for exome capture and DNA sequencing. Briefly, genomic DNA was subjected to agarose gel and OD ratio tests to confirm purity and concentration prior to Bioruptor (Diagenode, Inc., Denville, NJ, USA) fragmentation. Fragmented genomic DNAs were tested for size distribution and concentration using an Agilent Bioanalyzer 2100 and Nanodrop (Agilent Technologies, Wilmington, DE, USA). Illumina libraries were made from qualified fragmented genomic DNA using a SPRIworks HT reagent kit (Beckman Coulter, Inc., Indianapolis, IN, USA), and the resulting libraries were subjected to exome enrichment using the SureSelect XT Mouse All Exon Kit (Agilent Technologies) following the manufacturer's instructions. Enriched libraries were tested for enrichment by qPCR and for size distribution and concentration by an Agilent Bioanalyzer 2100. The samples were then sequenced on an Illumina HiSeq2000 (Illumina, San Diego, CA, USA), which generated paired-end reads of 90 or 100 nucleotides. The data were analyzed for data quality using FASTQC (Babraham Institute, Cambridge, UK). Sequence reads were mapped to the mouse reference genome (GRCm38, mm10). Read mapping and variant analyses were performed using CLC Genomics Workbench 7.0.4 and 8.5.1 (Filgen).

5. Candidate gene identification and prioritization of the candidate nsSNVs using a publicly available dataset

Candidate genes in the MGI database were explored by submitting a query termed "abnormal litter size." The presence of nsSNVs/INDELs was examined for all candidate genes within the 95% CI for *Lsq1* on chromosome 7.

Next, the significant effect of nsSNVs on litter size was determined (number of pups born in the second litter) using a publicly available dataset by Hadsell (https://phenome.jax.org/measures/49401). Briefly, litter-size data were obtained from the Hadsell 1 dataset for various inbred mouse strains, including 129S1/SvImJ (n = 10), A/J (n = 16), AKR/J (n = 7), BTBR T^{+} $Itpr3^{tf}$/J (n = 12), BUB/BnJ (n = 7), C3H/HeJ (n = 8), C57BL/6J (n = 7), C57L/J (n = 7), C58/J (n = 7), CBA/J (n = 11), DBA/1J (n = 8), DBA/2J (n = 8), FVB/NJ (n = 8), I/LnJ (n = 9), KK/HlJ (n = 17), LP/J (n = 5), NOD/ShiLtJ (n = 10), NZW/LacJ (n = 14), and SEA/GnJ (n = 8). In addition, nsSNVs data for these inbred strains were retrieved from the Mouse Phenome Database (https://phenome.jax.org/snp/retrieval). Subsequently, association tests were performed to further address whether the observed nsSNVs significantly impacted litter size in the murine population.

6. Statistics

All statistical analyses except for QTL mapping analyses were performed using JMP 13.0 (SAS Institute Inc., Cary, NC, USA). The normality of the trait values was assessed using the Shapiro–Wilk W test. If the trait data did not follow a normal distribution, Box–Cox transformation was applied to the raw trait data, and the normality of transformed data was re-assessed. Statistical differences between the two groups were analyzed using the Wilcoxon signed-rank test. *P*-value of <0.05 was considered statistically significant in all experiments unless otherwise noted.

Results

1. QTL mapping analysis in 228 female RBR mice

TNB, NBA, and NSB did not follow normal distribution according to the Shapiro–Wilk W test and could not be normalized; therefore, subsequent QTL mapping analyses were performed using a nonparametric method. Single-QTL scans (LOD score plots indicated by green lines in Fig. 1 (a)-(c)) showed that the peak LOD scores for TNB (3.7) and NBA (4.6) exceeded the significant threshold LOD scores. The R allele was associated with decreased TNB and NBA. A suggestive QTL for NSB was identified on chromosome 7. The R allele was associated with increased NSB.

2. QTL mapping analysis in 255 female BRR mice

Nonparametric single-QTL scans (LOD score plots indicated by blue lines in Fig. 1 (a)-(c)) showed that the peak LOD scores for TNB (2.7) and NBA (2.1) exceeded the significant and suggestive threshold LOD scores, respectively. The R allele was associated with decreased TNB and NBA. No suggestive/significant QTL was identified for NSB.

3. Fine mapping of *Lsq1* by combining BRR and RBR data

The means ± standard errors of TNB, NBA, and NSB in RBR mice (n = 228) were 7.76 ± 0.15, 7.49 ± 0.16, and 0.28 ± 0.06, respectively, compared with 8.51 ± 0.14, 8.18 ± 0.15, and 0.33 ± 0.06, respectively, in BRR mice (n = 255) (Suto 2015a). Thus, TNB and NBA were significantly smaller in RBR mice than those in BRR mice ($P < 0.0001$), whereas there was no significant difference in NSB between the BRR and RBR mice ($P > 0.65$) (although data are expressed as means for comprehensibility, statistical analyses were conducted according to the nonparametric method using the Wilcoxon signed-rank test).

TNB, NBA, and NSB did not follow normal

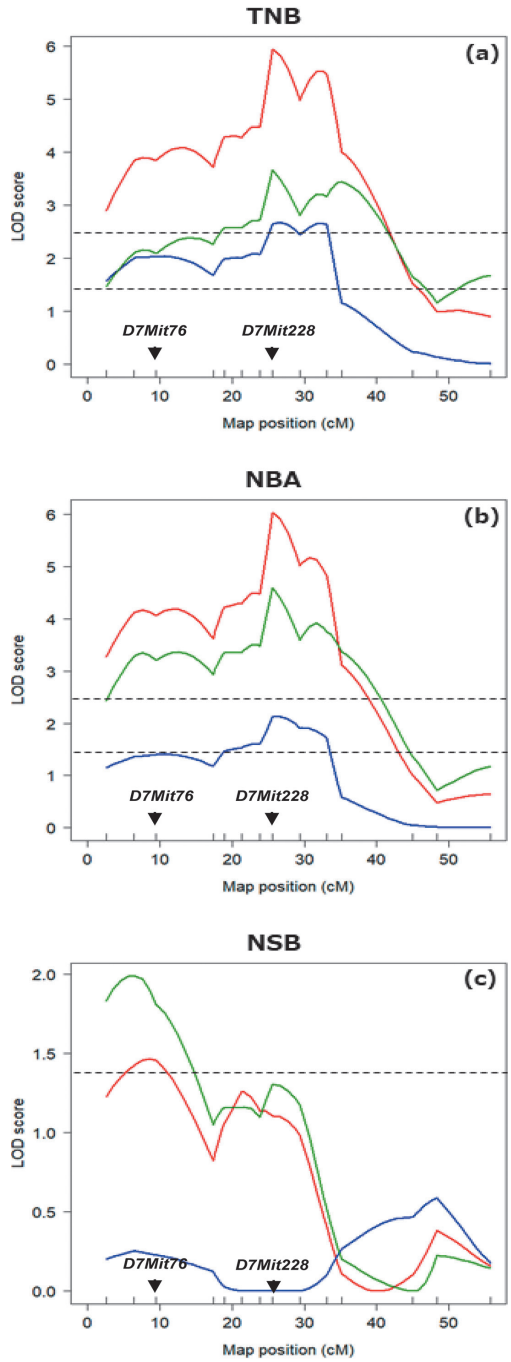

Fig. 1. Logarithm of odds (LOD) score plots for the total number of pups born (TNB) (a),number of pups born alive (NBA) (b), and number of stillbirths (NSB) (c) in RBR, BRR, and combined backcross mice on chromosome 7

Green, blue, and red lines represent RBR, BRR, and combined backcross mice, respectively. The horizontal dashed lines indicate significant and suggestive threshold LOD scores previously determined by 1,000 permutations in BRR mice (Suto 2015a). The short vertical lines at the bottom inside the frame of the plot area denote the chromosomal position of microsatellite markers. Marker symbols for *D7Mit76* and *D7Mit228* are especially provided because they are referred to in Table 1 and Figure 2.

distributions in the combined data; therefore, for further QTL mapping analyses to narrow the 95% CI for *Lsq1*, the data for each trait from the RBR and BRR mice were combined after standardization within each backcross. All subsequent QTL mapping analyses were performed using the nonparametric method as the standardized trait data did not follow a normal distribution. Threshold LOD scores from the combined backcross mice were redetermined by performing 1,000 permutations for each trait. The threshold LOD scores for significant and suggestive linkages were as follows: 2.5 and 1.3, 2.5 and 1.4, and 2.6 and 1.4 for TNB, NBA, and NSB, respectively. These threshold LOD scores did not substantially differ from those determined in BRR mice (Suto 2015a).

Nonparametric single-QTL scans (LOD score plots indicated by red lines in Fig. 1 (a)-(c)) showed that the highest LOD score peaks for TNB and NBA were identified in the vicinity of *Lsq1*, whereas other LOD score peaks were identified in the proximal part (Table 1). It was concluded that the proximal LOD score peak for TNB and NBA was a separate QTL because it was significant and located outside the 95% CI for the distal LOD score peak. Therefore, the proximal and distal LOD score peaks were termed *Lsq1-1* and *Lsq1-2*, respectively. The peak LOD score for NSB declined after the suggestive QTL was re-identified in the proximal part of chromosome 7.

4. Effects of QTL for NSB and the grandparental cross direction

The effects of *Lsq1-1* and *Lsq1-2* on TNB, NBA, and NSB were separately assessed for each backcross to examine the role of grandparental cross direction on the effects of QTL. Mice were partitioned according to the genotype of the markers nearest to the QTL, and trait values were statistically compared between BRR and RBR mice (Fig. 2). The effect of QTL on TNB and NBA was in the same direction, indicating that the R allele was significantly associated with increased litter size at *Lsq1-1* and *Lsq1-2*. In contrast, the effect of QTL on NSB depended on the grandparental cross direction, with the R allele significantly associated with increased stillbirths at *Lsq1-1* and *Lsq1-2* in RBR mice but not in BRR mice.

5. Identification of candidate genes and prioritization of nsSNVs using a publicly available dataset

Sixteen of the 537 genes located within the 95% CI of the QTL on chromosome 7 were selected as candidate genes based on the results of an MGI search. Whole-exome sequencing analyses of nsSNV/INDEL mutations in the coding regions identified ten nsSNVs in seven candidate genes (Table 2). Association tests were performed using

Table 1. Identification of quantitative trail loci (QTL) for litter size on chromosome 7 in combined backcross mice

QTL	Trait[a]	Location[b]	95% CI[c]	LOD[d]	Nearest marker	High allele[e]
Lsq1-1	TNB	13	3-22	4.1**	*D7Mit76*	B
	NBA	12	3-24	4.2**	*D7Mit76*	B
	NSB	9	NA	1.5*	*D7Mit76*	R
Lsq1-2	TNB	26	22-35	5.9**	*D7Mit228*	B
	NBA	26	24-34	6.0**	*D7Mit228*	B

[a] TNB, total number of pups born; NBA, number of pups born alive; NSB, number of stillbirths
[b] Location indicates the chromosomal position showing a peak logarithm of odds (LOD) score in cM.
[c] 95% confidence interval (CI) was defined as a 1.5-LOD support interval. NA: CI of *Lsq1-1* for NSB was unavailable because the peak LOD score was below 1.5.
[d] LOD score for suggestive QTL was accompanied by * and that for significant QTL by **.
[e] Allele associated with higher trait values

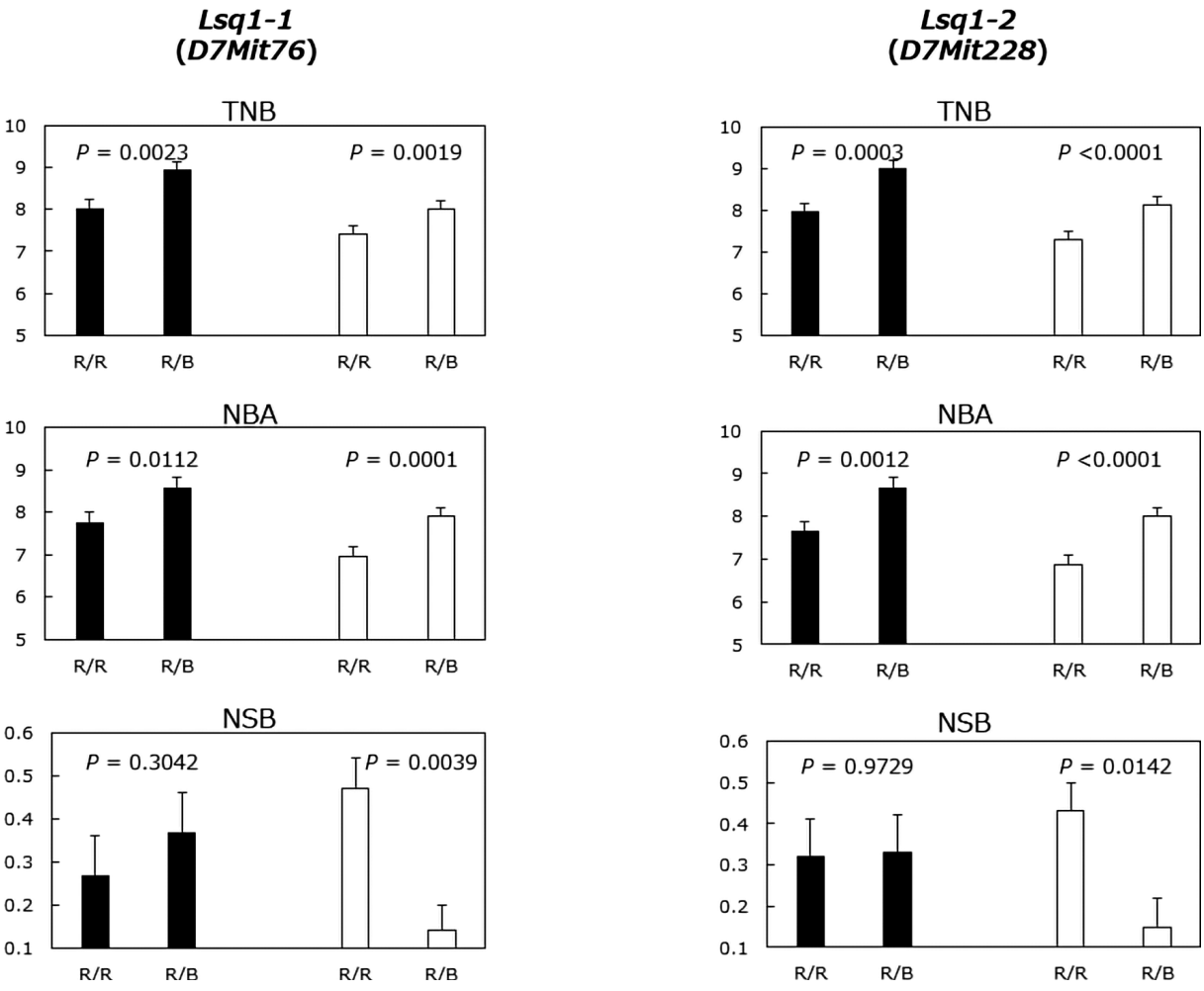

Fig. 2. Phenotypic effect of *Lsq1-1* and *Lsq1-2* on the total number of pups born (TNB), number of pups born alive (NBA), and number of stillbirths (NSB) in each backcross
The y-axis represents the number of pups. The number of mothers with an R/R genotype and R/B genotype at *Lsq1-1* was 124 and 131 in BRR mice and 96 and 132 in RBR mice, respectively. The number of mothers with an R/R genotype and R/B genotype at *Lsq1-2* was 124 and 131 in BRR mice and 102 and 126 in RBR mice, respectively. ■: BRR mice, □: RBR mice. Error bars, standard error

litter-size data and nsSNV data to further address whether the ten observed nsSNVs significantly impacted litter size in the murine population. The C/C genotype at rs13469893 in *Ppp5c* and the G/G genotype at rs13472312 in *Myod1* were significantly associated with decreased litter size (Fig. 3 (d), (g)). Intriguingly, the C allele at rs13469893 and G allele at rs13472312 were the same as the RR/Sgn strain-derived allele. Therefore, it is proposed that *Ppp5c* and *Myod1* are prioritized candidate genes for *Lsq1-1* and *Lsq1-2*, respectively.

Discussion

We analyzed litter-size data for female RBR mice in the present study because comparative analysis with data previously reported for female BRR mice (Suto 2015a) will make it possible to investigate putative mitochondrial effects on litter size. Indeed, mitochondrial genes are integral genetic components in determining litter size, as illustrated by studies in pigs and sheep (Chen et al. 2017, Pradhan et al. 2018, Tsai et al. 2016). The mitochondrial components of the RR/Sgn strain are rather different from those of other inbred mouse strains in terms of the restriction-fragment-length polymorphisms of the mitochondrial genes and some mitochondrial enzyme activities (Mikami et al. 1989a, b, Mikami et al. 1991). Therefore, we assume that the effect of grandparental cross direction is caused by the difference in mitochondrial components. However, we cannot rule out that the effect of grandparental cross direction could be caused by other genetic and epigenetic differences between BRR and RBR mice (Prows et al. 2009). Furthermore, although we determined litter size as the number of pups born from mothers, the BRR and RBR dams are sired by BRR and RBR mice, respectively.

Reed et al. (2008) identified a litter-size QTL (*Litsq1*) on chromosome 7 in a chromosome substitution mouse strain C57BL/6J-Chr7$^{129S1/SvImJ}$/Na. Intriguingly, the 129S1/SvImJ strain had the same nsSNVs as the RR/Sgn strain for all the candidate genes. Nevertheless, *Litsq1* and *Lsq1* are unlikely allelic because the 129S1/SvImJ-derived allele was associated with increased litter size compared with that of the B allele. Therefore, *Lsq1-1* and *Lsq1-2* are suggested to be novel litter-size QTL because there are no other coincidental QTL in mice (MGI).

Incidentally, FUT1 affects litter size in pigs (Distl 2007, Horák et al. 2005); however, a comparable fertile litter size was observed in *Fut1*-deficient and wild type mice (Domino et al. 2001). In mice, *Fut1* is located at 29.39 cM on chromosome 7, within the 95% CI for *Lsq1-2*. Nonetheless, the MGI search in this study did not retrieve *Fut1* as a candidate gene, and whole-exome sequencing analysis did not identify any nsSNVs in *Fut1* in RR/Sgn mice.

This study demonstrated that the effect of *Lsq1-1*

Table 2. Candidate genes

Gene	Pos (cM)	Location of nsSNVs	Nucleotide change	Amino acid change	SNP ID
Gp6	2.52	4370157	766A > T	Phe256Ile	rs38364678
		4385009	460A > G	Ser154Pro	rs32091711
Usp29	4.04	6962862	1703T > C	Leu568Ser	rs31993731
Aurkc	4.06	-	-	-	-
Trim28	7.73	-	-	-	-
Ppp5c	9.15	17027744	70T > C	Thr24Ala	rs13469893
Ceacam10	12.78	24780930	485C > G	Arg162Pro	rs32139009
Egln2	15.83	-	-	-	-
Dll3	16.67	-	-	-	-
Chst8	20.53	34748181	11C > T	Arg4Gln	rs31398221
Cebpa	21.02	-	-	-	-
Myod1	30.03	46376829	157A > G	Met53Val	rs13472312
		46376868	196C > T	Pro66Ser	rs13472315
		46377813	701C > T	Ala234Val	rs32790785
Gas2	32.87	-	-	-	-
Oca2	33.44	56429368	2380A > C	Lys794Gln	rs31090430
Ube3a	33.95	-	-	-	-
Magel2	34.37	-	-	-	-
Chrna7	34.47	-	-	-	-

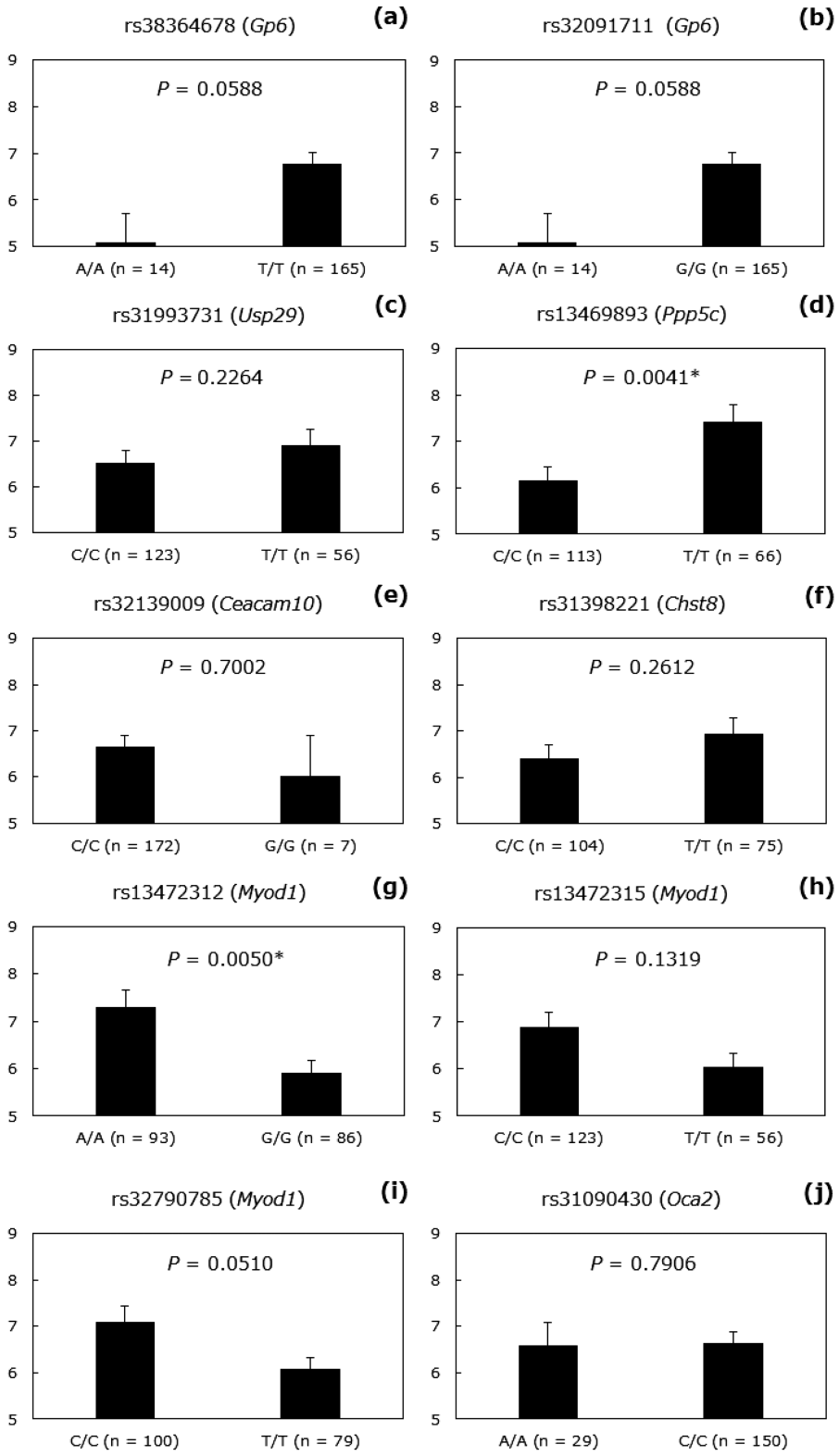

Fig. 3. Phenotypic effect of non-synonymous single nucleotide variants (nsSNVs) in a publicly available dataset (https://phenome.jax.org/measures/49401)

The y-axis represents the number of pups born in the second litter. The sample size is indicated in parenthesis immediately after the nsSNVs genotype. The single point threshold *P*-value for significance (0.05) was corrected to 0.05/10 = 0.0050 after taking the multiple testing into consideration. Statistically significant *P*-values are indicated by an asterisk. Error bars, standard error

and *Lsq1-2* on NSB depended on the grandparental cross direction. NSB is recognized as a heritable trait in pigs (Chen et al. 2010, Knol et al. 2002), and QTL for NSB has been identified (Cassady et al. 2001, Holl et al. 2004, Li et al. 2009); therefore, it is reasonable to conclude that NSB is also a heritable trait in mice. *Lsq1-1* and *Lsq1-2* should be regarded as having a genetic effect on stillbirths despite being not statistically significant at the genome-wide level. However, although the mechanism of increasing stillbirths remains unknown, determining whether *Lsq1-1* and *Lsq1-2* increase embryonic extinction or promote parturition failure is important. There is no evidence to support the idea of parturition failure, and increased stillbirths may be a consequence of poor maternal care immediately after delivery. This is because the maternal nurturing ability of the RR strain is impaired, and the suggestive QTL for inferior nurturing ability (defined by litter weight) is mapped onto chromosome 7 in BRR mice (Suto 2015b). It is crucial to investigate the number of live/dead embryos in utero to elucidate the mechanism underlying the increase in stillbirths.

In conclusion, the litter-size QTL (*Lsq1*) on chromosome 7 comprised two separate QTL (*Lsq1-1* and *Lsq1-2*), not one, as previously thought. *Lsq1-1* and *Lsq1-2* significantly affect TNB, NBA, and NSB, with their effect on NSB depending on the grandparental cross direction. Furthermore, *Ppp5c* and *Myod1* were prioritized candidate genes for *Lsq1-1* and *Lsq1-2*, respectively. Although the adequacy of these candidate genes requires further in-depth validation, the results provide insight into the molecular mechanisms underlying the genetic control of litter size in mice. This scenario is likely to repeat in other mammalian species, such as pigs.

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