

The A^y allele at the agouti locus reduces the size and alters the shape of the mandible in mice

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Abstract: To confirm my previous findings that the A^y allele at the agouti locus reduced the mandible size and therefore altered the mandible shape in a KK mouse strain background, I further investigated the effects of the A^y allele on mandible morphology on different strain backgrounds, DDD and B6. Principal component analysis revealed that the mandible was significantly smaller in A^y mice (DDD- A^y and B6- A^y) than in corresponding non- A^y mice (DDD and B6, respectively). Discriminant and canonical discriminant analyses revealed that most mice were classified correctly in their own strains, and misclassification was not observed between DDD (- A^y) and B6 (- A^y). The results confirmed that the A^y allele reduced the mandible size and altered the mandible shape regardless of the strain background. However, the difference in mandible morphology between A^y mice and the corresponding non- A^y mice within a strain was not as large as that which intrinsically underlay the two strains. Possible mechanisms of the A^y action are discussed.

Keywords: A^y allele, mandible size and shape, mouse, multivariate analysis

Introduction

The size and shape of the mandible are highly heritable quantitative traits that are controlled by multiple genes under the influence of environmental stimuli. Mandible morphology (when the size and shape are referred to simultaneously, they are called morphology in this paper) are sufficiently variable so that differences between inbred mouse strains can be identified.^{1),2)} Indeed, many studies have shown that strain identification in mice, rats, and rabbits can be accomplished reliably by means of multivariate analysis with use of mandible measurements.^{1)–8)} Because the mandible morphology differs greatly between KK/Ta Jcl (hereafter referred to as KK) and C57BL/6J (hereafter referred to as B6) mouse strains, I performed quantitative trait locus (QTL) analysis on the size and shape of the mandible in B6 \times KK- A^y /Ta Jcl (hereafter referred to as KK- A^y) F₂ mice.⁹⁾ The results suggested that the mandible morphology is controlled by multiple genes. Fur-

thermore, although the A^y allele at the agouti locus is known to increase the body weight and length of the trunk by constitutively impeding the action of α -melanocyte-stimulating-hormone at the melanocortin 4 receptor (MC4R),^{10),11)} the A^y allele reduced the mandible size in the KK strain background.⁹⁾ That is, KK- A^y was significantly larger than KK, but had a significantly smaller mandible than did KK. In addition, the A^y allele altered the mandible shape, because KK and KK- A^y were discriminated accurately each other based on the mandible morphology.

The aims of this study were as follows: [1] To address whether the effect of the A^y allele on the size and shape of the mandible was seen in other genetic backgrounds, B6 and DDD/Sgn (hereafter referred to as DDD) in the same way as in the KK background. For this purpose, a congenic strain for the A^y allele, DDD.Cg- A^y (hereafter referred to as DDD- A^y) was newly established and analyzed. If the effect of the A^y allele on the mandible morphology is confirmed in different strain background again, my previous findings will be further generalized. [2] To examine whether the A^y effect of reducing the size was limited to the mandible, I analyzed the spleen and testes weights. Spleen and testes are suitable for accurate weight measurements, because these organs

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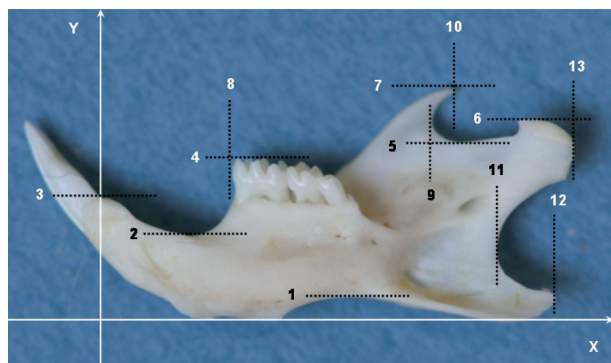


Fig. 1. Diagram of the 13 mandible measurement sites (X_1 - X_{13}) used in this study. Roughly, measurements X_1 - X_7 represent the height from the x-axis to the horizontal dotted line at each site, and X_8 - X_{13} represent the length measured from the y-axis to the vertical dotted line at each site.

are easy to remove without causing bleeding. If the A^y effect of reducing the size is observed in these organs, it will be possible to conclude that the A^y allele is not necessarily associated with increased size.

Materials and methods

Mice. The inbred mouse B6 strain was purchased from CLEA Japan (Tokyo). The congenic mouse B6.Cg- A^y /J (hereafter referred to as B6- A^y) strain was purchased from the Jackson Laboratory (Bar Harbor, ME). The inbred mouse DDD strain was maintained at the National Institute of Agrobiological Sciences (NIAS, Tsukuba, Japan). The DDD strain is one of the descendant strains of 'dd' mice. In 1928, the original colony of dd mice was introduced into the Kitasato Institute (Tokyo) from Germany; it was brought back to the Institute for Infectious Disease (Denken, Tokyo) by way of the Health Institute of Manchuria (China). Many inbred strains were established from dd mice of this stock [Mouse Genome Informatics (<http://www.informatics.jax.org>)].⁶⁾

The congenic mouse DDD- A^y strain was newly established by repetitive backcrossing of the A^y allele from the B6- A^y strain onto the DDD background for 12 generations. Because DDD had an albino coat color, congenic mice were further intercrossed between yellow (A^y) and agouti (A) littermates to eliminate the $Tyrc$ allele (the $Tyrc$ allele has not yet been thoroughly removed, and hence, albino mice were excluded from subsequent experiments).

DDD- A^y and DDD were produced from genetic crosses between $\text{♀DDD} \times \text{♂DDD-}A^y$, and B6- A^y and

B6 were crosses between $\text{♀B6} \times \text{♂B6-}A^y$. Three to five mice, regardless of whether they had the A^y allele or not, were housed together in each strain. In this paper, when DDD- A^y and B6- A^y are referred to together, they are called ' A^y mice'. Likewise, their control littermates, DDD and B6, are called 'non- A^y mice'. For statistical comparison, I defined four groups, each of which comprised A^y mice and corresponding non- A^y mice; that is, DDD- A^y males ($n = 12$) vs. DDD males ($n = 20$) was defined as group 'DM', DDD- A^y females ($n = 12$) vs. DDD females ($n = 13$) as 'DF', B6- A^y males ($n = 15$) vs. B6 males ($n = 15$) as 'BM', and B6- A^y females ($n = 13$) vs. B6 females ($n = 14$) as 'BF'.

All mice were maintained in a specific-pathogen-free facility with a regular light cycle and controlled temperature and humidity. Food [CRF-1 (Oriental Yeast Co. Ltd., Tokyo)] and water were freely available throughout the experimental period. All of the animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of NIAS.

Phenotypic measurements. At the age of 16 weeks, mice were weighed with an electric balance to the nearest 0.01 g. Then the mice were killed, and the spleen and testis on both sides (in males) were removed and placed in physiologic saline. After they were rinsed, excessive moisture was wiped with a wet chromatography paper, and the spleen and paired testes weights were determined to the nearest 1 mg.

Mandible bones were prepared by procedures used in an earlier study.⁹⁾ The carcasses were decapitated, and the heads were autoclaved for 5 min at 121 °C and skinned. The heads were soaked in 0.5% papain (MERCK KGaA, Darmstadt, Germany) solution and incubated at 37 degrees overnight. Then mandibles were separated and adhering soft tissues were carefully removed with a soft toothbrush in water and dried on a paper towel. Each mandible specimen (essentially the right half of the mandible was used, but the left one was used when the right one was unavailable) was photographed, and an enlarged photo (approximately ten times as large as the original mandible bone) was printed. On the photo, each parameter (indicated in Fig. 1) was measured with a ruler to the nearest 0.5 mm. A total of 13 measurements were taken on each right mandible (X_1 - X_{13} , Fig. 1). X_1 - X_7 were the distances from the X-axis and therefore considered to express the 'height' of the mandible, whereas X_8 - X_{13} were the

Table 1. Means for mandible measurement variables (mm) in each strain

Strain	Variables												
	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	X ₈	X ₉	X ₁₀	X ₁₁	X ₁₂	X ₁₃
DM													
DDD- <i>A</i> ^y	0.833	2.015	2.748	3.850	4.818	5.330	5.915	3.152	7.893	8.254	10.033	11.868	11.410
DDD	0.852	1.978	2.685	3.926	4.985	5.390	6.013	3.168	8.233	8.703	10.050	11.882	11.545
<i>p</i> -value (DDD- <i>A</i> ^y vs. DDD)	ns	0.045	0.026	0.0011	0.00067	ns	0.016	ns	0.0041	0.0052	ns	ns	0.0027
BM													
B6- <i>A</i> ^y	0.866	2.075	2.813	4.038	4.765	5.467	6.069	3.177	7.963	8.385	9.997	11.485	11.745
B6	0.806	2.030	2.765	4.051	4.781	5.453	6.103	3.174	8.001	8.521	9.973	11.535	11.823
<i>p</i> -value (B6- <i>A</i> ^y vs. B6)	0.017	0.010	ns	ns	ns	ns	ns	ns	ns	0.013	ns	ns	ns
DF													
DDD- <i>A</i> ^y	0.755	2.097	2.840	3.910	4.802	5.237	5.858	3.125	8.053	8.504	9.958	11.756	11.544
DDD	0.764	2.067	2.822	4.015	4.842	5.232	5.928	3.119	8.250	8.728	9.997	11.724	11.745
<i>p</i> -value (DDD- <i>A</i> ^y vs. DDD)	ns	0.031	ns	0.00073	ns	ns	ns	ns	0.000060	0.000013	ns	ns	0.00072
BF													
B6- <i>A</i> ^y	0.732	2.030	2.801	3.992	4.653	5.267	5.919	3.173	7.915	8.372	9.806	11.162	11.696
B6	0.697	2.021	2.774	4.024	4.706	5.271	5.940	3.194	8.131	8.671	9.909	11.253	11.980
<i>p</i> -value (B6- <i>A</i> ^y vs. B6)	ns	ns	ns	ns	ns	ns	ns	ns	0.000067	0.0000038	ns	ns	0.000030

ns: not significant

distances from the Y-axis and therefore considered to express the 'length'. Each measurement was thus considered as indicating the size of the mandible; therefore, the 13 measurements were first analyzed by regarding each of them as a conventional univariate character.

Multivariate analysis. Because of the volume of the data and the presence of a strong correlation between the variables, Festing²⁾ suggested that it was preferable to handle the vector of the 13 measurements for each individual as a single multivariate character. Therefore, the data were concurrently analyzed by multivariate analyses, including principal component analysis, discriminant analysis, and canonical discriminant analysis, all by use of SPSS for Windows (release 7.5.1J, SPSS Inc., Chicago, IL). In particular, canonical discriminant analysis (discriminant analysis with reduction of dimensionality) is a way to extract a few axes that clearly describe

the positions among groups on a two-dimensional plane. Coefficient vectors for the axes can be determined such that the ratio of the variance between the groups to that within the groups reaches a maximum. This axis is called the first canonical variate Z_1 , and it summarizes the most remarkable variation between groups. The second canonical variate Z_2 is extracted independently from Z_1 , and shows the second-best discrimination between groups.^{1),5)}

I analyzed the mandible size by performing principal component analysis between *A*^y mice and non-*A*^y mice within each group as defined above. The mandible shape was analyzed by means of principal component analysis, discriminant analysis, and canonical discriminant analysis.

Other statistics. Statistical analysis between *A*^y mice and non-*A*^y mice within each group was performed by use of Student's or Welch's *t*-test. Multivariate analyses were performed with SPSS

Table 2. Eigenvalue and its contribution to each PC

PC	Group	Eigenvalue	Cumulative contribution ratio (%)
1	DM	4.875	37.502
	BM	5.697	43.824
	DF	5.170	39.771
	BF	5.522	42.478
2	DM	2.668	58.025
	BM	2.455	62.706
	DF	2.635	60.038
	BF	2.436	61.219
3	DM	1.981	73.263
	BM	1.598	75.001
	DF	1.818	74.021
	BF	1.619	73.677
4	DM	1.149	82.100
	BM	1.031	82.935
	DF	1.409	84.858
	BF	1.033	81.622

software (SPSS for Windows Release 7.5.1J, SPSS Inc., Chicago, IL). $P < 0.05$ was considered to be statistically significant.

Results

Comparison of mandible measurements.

Mandible size was assessed by comparison of each of 13 measurements between A^y mice and non- A^y mice within a group. The means for the 13 measurements of the mandible (Fig. 1) of all mice are given in Table 1. Across the groups, a significant difference between A^y mice and non- A^y mice was detected in X_1 - X_5 , X_7 , X_9 , X_{10} , and X_{13} , and not in X_6 , X_8 , X_{11} , and X_{12} . In X_1 - X_3 , a significant difference was detected in five comparisons, and the measurements were always larger in A^y mice than in non- A^y mice. The A^y allele thus increased the anterior height. On the other hand, in the remaining measurements, a significant difference was detected in 14 comparisons, and the A^y mice invariably had smaller values than did non- A^y mice.

Multivariate analyses of mandible size and shape. Mandible size was assessed by means of principal component analysis by regarding 13 measurements as a single multivariate character. Table 2 gives the eigenvalue and its contribution with respect to the principal component (hereafter referred to as PC) in DM, BM, DF, and BF. Four PCs, in

which the eigenvalue was more than 1.0, were successfully extracted for each group. The first four PCs accounted for more than 80% of the variation in morphometric information. Table 3 gives the eigenvectors of the 13 variables classified according to PCs. In the case of PC1, all coefficients for the variables were essentially positive in four groups. In the case of PC2, all coefficients concerned with the mandible length (X_8 - X_{13}) were negative or small. In the case of PC3, three coefficients concerned with the posterior mandible height (X_5 - X_7) were negative or small, and three coefficients concerned with some of the length of posterior processes (X_9 , X_{10} , and X_{13}), were negative. In the case of PC4, one coefficient, X_7 , was negative or small.

The means \pm S.D. for PC scores are presented in Table 4. Essentially, A^y mice had a significantly smaller PC1 score than did the corresponding non- A^y mice in all groups. There were no significant differences in the PC2 score between A^y and non- A^y mice. Essentially, the PC3 score was significantly larger in A^y mice than in non- A^y . With regard to the PC4 score, although A^y mice had a larger score than did non- A^y mice in BM and BF, A^y mice had a smaller score than did non- A^y mice in DF.

A^y mice and non- A^y mice were mostly discriminated each other based on the mandible morphology. When classification analysis by means of the discriminant function was performed in the four groups separately, A^y mice and non- A^y mice were completely discriminated each other in DM, DF, and BF, except that one B6 male was misclassified into B6- A^y males (BM). Next, all mice were analyzed together. As a result, all DDD- A^y males and DDD males were classified correctly (Table 5). However, 1/15 B6- A^y males, 1/15 B6 males, 1/12 DDD- A^y females, 1/13 DDD females, 1/13 B6- A^y females, and 1/14 B6 females were incorrectly classified. With the exception that one B6- A^y male was identified as a B6- A^y female, misidentification occurred between an A^y mouse and a non- A^y mouse within each group. There were no cases of DDD (- A^y) being misclassified into B6 (- A^y), and vice versa.

I conducted canonical discriminant analysis to illustrate the relationships among all strains on a plane. Because up to the third canonical variates were adopted in this study; the results are shown in Fig. 2A (defined by the 1st and 2nd canonical variates) and 2B (defined by the 1st and 3rd canonical variates). The eigenvalue and its contribution are

Table 3. Eigenvector of each PC

Variable	PC							
	1				2			
	DM	BM	DF	BF	DM	BM	DF	BF
X ₁	0.171	0.094	0.140	-0.139	0.350	0.408	0.316	0.186
X ₂	0.068	0.153	-0.113	0.160	0.233	0.103	0.074	0.272
X ₃	0.075	0.275	0.044	0.096	0.068	-0.188	-0.069	0.199
X ₄	0.309	0.220	0.343	0.289	0.305	0.203	-0.020	0.267
X ₅	0.325	0.162	0.330	0.283	0.299	0.507	0.339	0.376
X ₆	0.151	0.187	0.222	0.213	0.383	0.424	0.476	0.435
X ₇	0.317	0.247	0.318	0.218	0.296	0.370	0.341	0.413
X ₈	0.237	0.326	0.150	0.297	-0.333	-0.267	-0.384	-0.222
X ₉	0.368	0.364	0.336	0.358	-0.298	-0.129	-0.340	-0.261
X ₁₀	0.356	0.321	0.245	0.279	-0.106	-0.070	-0.371	-0.319
X ₁₁	0.313	0.375	0.379	0.379	-0.321	-0.165	-0.078	-0.137
X ₁₂	0.257	0.331	0.318	0.344	-0.361	-0.230	0.043	-0.146
X ₁₃	0.392	0.360	0.385	0.365	-0.184	-0.015	-0.181	-0.143

Variable	3				4			
	DM	BM	DF	BF	DM	BM	DF	BF
	DM	BM	DF	BF	DM	BM	DF	BF
X ₁	0.085	0.338	-0.009	-0.181	0.311	-0.032	0.245	0.423
X ₂	0.606	0.661	0.668	0.478	-0.068	0.206	0.104	-0.062
X ₃	0.647	0.421	0.642	0.656	-0.179	0.129	0.297	-0.122
X ₄	0.143	0.251	0.065	0.178	0.017	-0.512	0.381	-0.098
X ₅	-0.263	-0.245	0.015	-0.283	-0.021	0.086	0.051	-0.092
X ₆	-0.202	-0.226	-0.024	-0.274	0.368	0.447	-0.121	0.119
X ₇	-0.092	-0.060	-0.044	-0.226	-0.028	-0.213	0.022	-0.068
X ₈	0.177	-0.037	0.223	0.043	0.313	-0.004	-0.438	0.527
X ₉	-0.114	-0.091	-0.110	-0.080	-0.461	-0.210	0.171	-0.194
X ₁₀	-0.110	-0.186	-0.191	-0.132	-0.491	-0.479	0.302	-0.387
X ₁₁	0.100	0.020	0.120	0.129	0.236	0.295	-0.295	0.270
X ₁₂	0.026	-0.133	0.146	0.083	0.341	0.195	-0.513	0.399
X ₁₃	-0.005	-0.180	-0.001	-0.146	0.073	0.165	0.120	-0.260

summarized in Table 6. As seen, the four strains belonging to DM and DF were localized closer to one another, and the remaining four strains belonging to BM and BF were localized closer to one another. The result of canonical discriminant analysis performed by incorporation of the data on KK- A^y and KK is shown in Fig. 3. In this case, each strain was plotted as a point. Roughly, the distance between A^y mice and non- A^y mice was again smaller than that between strains.

Effect of the A^y allele on body weight, testes weight, and spleen. As expected, the A^y allele significantly increased the body weight in both strain backgrounds (Table 7). Spleen and testes

weights were compared between A^y mice and non- A^y mice. Spleen weights did not differ significantly between A^y mice and non- A^y mice in DM, BM, and DF, but B6- A^y females had heavier spleens than did B6 females (BF). Unexpectedly, A^y mice had significantly lighter testes than did non- A^y mice in both DM and BM. It was thus shown that the A^y allele was not always associated with increased size and weight.

Discussion

This study showed that the A^y allele reduced the mandible size and altered the mandible shape in the DDD and B6 strain backgrounds. By means of uni-

Table 4. Means \pm S.D. for PC scores in each strain

Strain	PC scores			
	PC1	PC2	PC3	PC4
DM				
DDD- A^y	-0.667 ± 0.741	-0.204 ± 0.852	0.653 ± 0.921	0.162 ± 0.648
DDD	0.400 ± 0.930	0.123 ± 1.081	-0.392 ± 0.842	-0.097 ± 1.167
p -value (DDD- A^y vs. DDD)	0.0012	ns	0.0026	ns
BM				
B6- A^y	-0.073 ± 0.973	0.092 ± 1.005	0.569 ± 0.995	0.412 ± 0.964
B6	0.073 ± 1.055	-0.092 ± 1.021	-0.569 ± 0.622	-0.412 ± 0.883
p -value (B6- A^y vs. B6)	ns	ns	0.0010	0.021
DF				
DDD- A^y	-0.517 ± 0.774	0.300 ± 1.197	0.432 ± 0.877	-0.578 ± 1.023
DDD	0.477 ± 0.967	-0.277 ± 0.717	-0.399 ± 0.968	0.533 ± 0.631
p -value (DDD- A^y vs. DDD)	0.0091	ns	0.034	0.0031
BF				
B6- A^y	-0.490 ± 1.195	0.354 ± 0.751	0.301 ± 0.957	0.574 ± 0.705
B6	0.455 ± 0.467	-0.329 ± 1.112	-0.279 ± 0.990	-0.533 ± 0.951
p -value (B6- A^y vs. B6)	0.017	ns	ns	0.0020

ns: not significant

Table 5. Results of classification analysis by means of discriminant function (all of the strains were merged)

Strain	No. of cases classified in strain								Total (% of misclassification)
	DDD- A^y males	DDD males	B6- A^y males	B6 males	DDD- A^y females	DDD females	B6- A^y females	B6 females	
DDD- A^y males	12								12 (0)
DDD males		20							20 (0)
B6- A^y males			14				1*		15 (6.7)
B6 males			1*	14					15 (6.7)
DDD- A^y females					11	1*			12 (8.3)
DDD females					1*	12			13 (7.7)
B6- A^y females							12	1*	13 (7.7)
B6 females							1*	13	14 (7.1)

Blank means no incidence (0). *Incorrectly classified mandibles. In total, 6/114 was incorrectly classified.

variate analysis, although measurements X_1 - X_3 (representing anterior height) were larger in A^y mice than in non- A^y mice, measurements X_7 (representing total height) and X_{13} (representing overall length) were smaller in A^y mice than in non- A^y mice; it seemed that the A^y mice had a smaller mandible than did non- A^y mice. For further substantiation of this conclusion, the mandible morphology was ana-

lyzed by means of multivariate analyses. According to principal component analysis, PC1 was acceptable as a size factor. A^y mice had a significantly smaller PC1 score than did the corresponding non- A^y mice in all groups except for BM (Table 4). Even in BM, A^y mice tended to have a smaller PC1 than did non- A^y mice. These results suggested that the A^y allele reduced the mandible size, but its effect was slightly

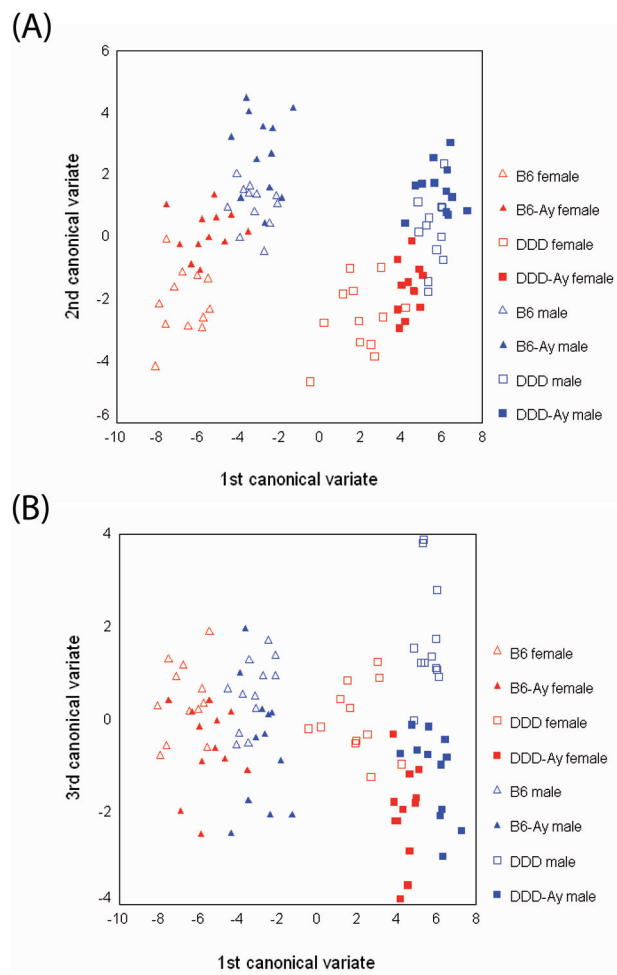


Fig. 2. Scatter diagram of 8 strains on a plane. (A) Plot of 1st canonical variate (x-axis) and 2nd canonical variate (y-axis). (B) Plot of 1st canonical variate (x-axis) and 3rd canonical variate (y-axis). Each point indicates the position of an individual mouse.

Table 6. Eigenvalue and its contribution

Canonical variate	Eigenvalue	Contribution ratio (%)
1st	24.985	79.9
2nd	3.251	10.4
3rd	1.554	5.0

dependent upon sex and genetic background. PC2 was recognized as a shape factor and represents the height of the mandible relative to its length. In other words, a mouse with a large PC2 value has a short mandible. However, there were no significant differences in the PC2 score between A^y and non- A^y

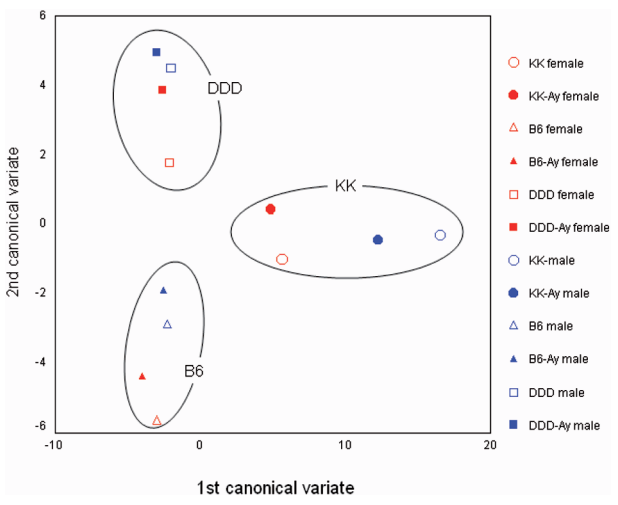


Fig. 3. Scatter diagram of 12 strains on plane by 1st (x-axis) and 2nd (y-axis) canonical variate. Canonical discriminant analysis was performed by incorporating the data on KK- A^y and KK. Each strain was plotted as a point. Lines are drawn around the strains to clarify the relationships, but these lines have no statistical meaning.

mice in the four groups. This suggested that the A^y allele did not reduce the mandible size by simply shortening the length relative to the height. PC3 was also considered to be a shape factor; a mouse with a larger PC3 value has a mandible with low posterior height and short posterior length, and therefore it has a mandible with an altered shape. The PC3 score was significantly larger in A^y mice than in non- A^y mice in all groups, except for BF. This means that the A^y mouse has a mandible with low posterior height (X_5 - X_7) and short posterior length (X_9 , X_{10} , and X_{13}), when compared to non- A^y mice. I could not characterize PC4 appropriately. However, one coefficient, X_7 , was negative or small in the four groups; therefore, PC4 may be related to the overall height of the mandible. Therefore, PC2, PC3, and PC4 should be regarded as shape factors.

On the basis of discriminant and canonical discriminant analyses, with the exception that one B6- A^y male was identified as a B6- A^y female, misidentification was limited to occur between an A^y mouse and a non- A^y mouse within each group. There were no incidences of DDD (- A^y) being misclassified into B6 (- A^y), and vice versa (Table 5 and Fig. 2A, B). The results suggested that the difference in mandible morphology between A^y mice and non- A^y mice within each group was not as large as that which intrinsi-

Table 7. Comparison of body weight, spleen weight, and testis weight (mean \pm S.D.)

Strain	Body weight (g)	Spleen weight (mg)	Testis weight (mg)
DM			
DDD- A^y	43.81 \pm 2.33	105.75 \pm 8.15	255.05 \pm 7.36
DDD	36.00 \pm 3.26	103.43 \pm 19.34	299.23 \pm 14.58
p -value (DDD- A^y vs. DDD)	2.94×10^{-9}	ns	1.15×10^{-7}
BM			
B6- A^y	42.63 \pm 2.06	85.69 \pm 8.53	192.47 \pm 9.44
B6	30.89 \pm 1.82	87.56 \pm 18.97	209.13 \pm 10.80
p -value (B6- A^y vs. B6)	1.97×10^{-15}	ns	0.00015
DF			
DDD- A^y	54.20 \pm 2.76	112.36 \pm 10.91	na
DDD	32.01 \pm 2.46	117.71 \pm 13.61	na
p -value (DDD- A^y vs. DDD)	1.28×10^{-16}	ns	
BF			
B6- A^y	38.53 \pm 3.69	104.03 \pm 12.37	na
B6	23.57 \pm 0.65	92.79 \pm 12.53	na
p -value (B6- A^y vs. B6)	5.89×10^{-14}	0.027	

ns: not significant; na: not applicable

cally was seen between the DDD and B6 strains. This was also true when I performed a canonical discriminant analysis by incorporating the data on KK- A^y and KK (Fig. 3). Because the KK- A^y had a significantly smaller mandible than did KK, and KK and KK- A^y were completely discriminated each other based on the mandible morphology, the A^y allele reduced mandible size and altered mandible shape in all three strain backgrounds examined so far (In the previous paper,⁹⁾ I only compared each of 13 measurements between KK- A^y and KK. However, a subsequent analysis based on principal component analysis confirmed this conclusion, because KK- A^y had a significantly smaller PC1 score than did KK in both sexes).

Like the A^y allele, a single-gene effect on the mandible morphology has been demonstrated previously. According to Goto *et al.*,¹²⁾ the NC and NC-*brp* mouse strains could be distinguished exactly based on the mandible morphology. The *brp* mutation (*brp* has subsequently been revealed as a mutation in the *Gdf5* gene; therefore, it is referred to hereafter as the *Gdf5^{brp}* allele)¹³⁾ arose spontaneously in the inbred NC strain. Therefore, NC-*Gdf5^{brp}* could

be regarded as a coisogenic strain (all of the genes except for the *Gdf5* are the same). Although NC-*Gdf5^{brp}/Gdf5^{brp}* mice were significantly lighter than NC-+/? mice, they tended to have a larger mandible.⁴⁾ This implies that the mechanism of action of the *Gdf5^{brp}* allele was different on the mandible than on the limb skeleton. In addition, knockout mouse models offered evidence that there are numerous genes that can modify the mandible morphology.^{14),15)}

The agouti gene is expressed only in the skin in normal mice; however, it is over-expressed ectopically in A^y mice.¹⁶⁾ This is because the A^y allele is accompanied by a large deletion, and its expression is controlled by an unrelated *Raly* gene promoter. Increased body weight and length are considered to be a consequence that agouti protein serves as a constitutive antagonist at the MC4R.¹⁰⁾ The expression of the MC4R mRNA was confirmed in the skull bone in rats;^{17),18)} therefore, the MC4R as well as melanocortin peptides appear to play roles in bone metabolism. Because the action of MC4R-melanocortin peptides is situated in the lower course of leptin signaling, and because leptin is reported to exert an effect on

bone metabolism,^{19),20)} knowledge about leptin- or leptin-receptor-deficient mice is highly suggestive. Yagasaki *et al.*²¹⁾ compared some craniofacial measurements between B6 and leptin-deficient B6-*Lep^{ob}/Lep^{ob}* mice, and they showed that the measurements of the total skull and four parts of the mandible (mandibular corpus length, mandibular ramus length, mandibular effective length, and angular process) were significantly smaller in B6-*Lep^{ob}/Lep^{ob}* than in B6 at the age of 11 weeks. Because the stature is by no means increased in B6-*Lep^{ob}/Lep^{ob}* mice,²⁰⁾ we cannot simply compare the skeletal phenotypes between B6-*Lep^{ob}/Lep^{ob}* mice and *A^y* mice. Nevertheless, as Dumont *et al.*¹⁸⁾ suggested that melanocortin peptides have a direct role in bone development and bone metabolism, it seems likely that such melanocortin peptides also influence the mandible bones in *A^y* mice.

With regard to the effect of the *A^y* allele on spleen and testes weights, Mountjoy *et al.*¹⁷⁾ reported that MC4R mRNA is expressed in the testis, but not in the spleen in rats, thus suggesting a possible role of melanocortin peptides in the testis. Results obtained for *Lep^{ob}/Lep^{ob}* mice are again suggestive, because they have been known to show hypogonadism. According to the results of Sainsbury *et al.*,²²⁾ the weights of the liver, kidneys, intestine, and pancreas were significantly higher in *Lep^{ob}/Lep^{ob}* than in *Lep^{ob}/+*, whereas the testis weight in *Lep^{ob}/Lep^{ob}* was significantly lower than in *Lep^{ob}/+*, even though the mice were on a mixed background between C57BL/6 and 129/SvJ. Thus, the effect of the *A^y* allele was different from one organ to another and was not necessarily associated with increased size. Therefore, it was suggested that the *A^y* allele exerts its multiple developmental effects rather regionally.

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