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NR6A1 Allelic Frequencies as an Index for both Miniaturizing and Increasing Pig Body Size

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Abstract. *Background/Aim: The number of vertebrae in swine varies from 19 to 23 and is associated with body size. Nuclear receptor subfamily 6 group A member 1 (NR6A1) is considered a strong candidate for affecting the number of vertebrae in swine. Wild boars, which uniformly have 19 vertebrae, have the wild type allele while multi-vertebrae European commercial pigs have the mutated allele. Our aim was to confirm the factor of the miniaturization. Materials and Methods: We examined vertebrae number and NR6A1 polymorphism in the Microminipig and three domestic breeds that vary in body size. Results: The Microminipig had 19 or less vertebrae and a wild type NR6A1 genotype. Three domestic breeds had more than 21 vertebrae while the largest vertebrae number was observed in multi-vertebrae-fixed Large White. Heterozygous genotypes were observed in the middle-sized indigenous pig while homozygous NR6A1 mutations were observed in European commercial breeds. Conclusion: NR6A1 could be a useful index for both miniaturizing and increasing pig body size.*

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Pigs have been domesticated from wild boars (*Sus scrofa*) and various traits, including growth rate, litter, and body size have been altered through breeding. European commercial pigs have long been selectively bred for body elongation to increase meat production and improve reproductive performance (1-3). As a result of selective breeding, European commercial pigs have a few more vertebrae compared to wild boar and indigenous breeds (3, 4). In contrast to stockbreeding, smaller body size is expected in experimental animals.

'Amami-Shimabuta' (indigenous middle black pig breed) in the Amami-Oshima Island, located approximately 400 km south of mainland Kagoshima Prefecture in southwest Japan, is raised as a regional culinary specialty. The body size is smaller compared to commercial crossbreed pigs derived from Europe.

Swine are considered to be increasingly attractive toxicological and pharmacological models because of their well-accepted physiological and anatomical similarities to humans (5-10). Microminipigs (brand name; registered with the Japanese Ministry of Agriculture, Forestry and Fisheries as a novel variety of swine; Fuji Micra Inc., Shizuoka, Japan) have emerged as an experimental animal model of non-clinical pharmacological and toxicological research (11-14). The body weight of a young mature Microminipig is <10 kg, enabling easy handling (5, 15, 16). Some human disease models (5, 6, 10, 13, 15, 17) and infectious disease models (18) of Microminipig have already been developed.

Mikawa *et al.* (1) have suggested that a missense substitution (p.Pro192Leu) in the nuclear receptor subfamily 6 group A member 1 (*NR6A1*) gene, mapped to porcine chromosome 1, is the causative mutation of a quantitative trait locus (QTL) affecting the number of vertebrae in pigs (19). Wild boars and some indigenous pig breeds have the wild type allele (C), while European commercial pigs are considered to be fixed for the mutated allele (T) (20-23).

Table I. *Information on the investigated pig breeds.*

Breeds name	Use and origin	Body size*	Producing region
Microminipig	Experimental minipig registered with the Japanese Ministry of Agriculture, Forestry and Fisheries as a novel variety of swine.	Very small	Fuji Micra Inc., Shizuoka, Japan
Amami-Shimabuta	Indigenous middle black pig raised as a regional culinary specialty. 'Akarinton' is one of the brands used in this research.	Middle	Amami-Oshima Island (Approximately 400 km south-west of mainland Kagoshima)
Kagoshima Berkshire	Commercial breed derived from Berkshire pig, branded as 'Kagoshima Kurobuta' and one of the most famous pork brands in Japan.	Middle	Various parts of Kagoshima prefecture, including remote islands
Multi-vertebrae-fixed Large White (mv-LW)	Commercial breed derived from Large White (LW). Its brand name is Meiko22.	Large	One farm in Kagoshima mainland (Southwest Japan)

*Classification of body size is defined by the range of general adult body weight as follows; Very small: 15-25 kg, Middle: 200-250 kg, Large: 300-380 kg.

NR6A1 has been studied in commercial and some indigenous pigs as well as in wild boar; however, limited studies have indicated a direct association between *NR6A1* polymorphism and vertebrae number (24-26). In addition, *NR6A1* polymorphism and vertebrae number have not been examined in Microminipigs. In the present study, we investigated vertebrae number and *NR6A1* gene variants in Microminipig and some domestic pig breeds of various size.

Materials and Methods

Animals. Eight Microminipigs were obtained from the breeder (Fuji Micra Inc., Shizuoka, Japan) and maintained in a dedicated room with filtered air laminar flow at Kagoshima University. Three domestic breeds: i) indigenous Amami-Shimabuta (n=15), ii) Kagoshima Berkshire (n=15), and iii) the multi-vertebrae-fixed Large White pig (mv-LW, n=102) were reared in commercial pig farms in the Kagoshima Prefecture (Table I). All protocols were approved by the Ethics Committees of Animal Care and Experimentation, Kagoshima University (MD19079). Finally, the research was performed according to the Institutional Guidelines for Animal Experiments and in compliance with the Japanese Act on Welfare and Management of Animals (Act No. 105 and Notification No. 6).

Vertebrae counting. The number of thoracic and lumbar vertebrae of Microminipigs were counted on X-ray images taken under anesthesia by an intramuscular injection of medetomidine (0.08 mg/kg) and midazolam (0.08 mg/kg). Three domestic breeds were examined by observation of carcass at slaughter. The rib number was counted as thoracic number. The number of analyzed animals of each breed is indicated in Table II. Thoracic and lumbar vertebrae of mv-LW pigs, except the number shown in Table II, were not distinguished because the investigation of them was done using carcass photos and the rib number could not be determined.

Table II. *Vertebrae number in four pig breeds.*

Breed (number of animals)	Number of vertebrae		
	TV+LV	TV	LV
Microminipig (8)	18.6±0.5^{a}	13.4±0.5^{a}	5.3±0.4^{a}
Amami-Shimabuta (9)	21.2±0.6^{b}	15.3±0.6^{bc}	5.9±0.3^{b}
Kagoshima Berkshire (15)	21.3±0.4^{b}	14.6±0.4^{b}	6.7±0.5^{c}
mv-LW (7)	22.4±0.5^{c}	15.7±0.5^{c}	6.7±0.5^{c}

TV: Thoracic vertebrae; LV: lumbar vertebrae; mv-LW: multi-vertebrae-fixed Large White breed. Values with the different letters show the significant difference. Data are shown as mean±SD.

Quantitative polymerase chain reaction (qPCR) for NR6A1 genotyping. DNA was extracted from hair root samples and used for genotyping. The number of analyzed animals of each breed is indicated in Table II. The extraction was performed using the Thermo Scientific GeneJET Genomic DNA Purification Kit (Thermo Fischer Scientific, Waltham, MA, USA). Quantification and qualification of total DNA was assessed using the NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific). The custom Taqman SNP c.575T>C *NR6A1* missense mutation genotyping assay was designed by Thermo Fisher Scientific (20, 23). Quantitative PCR was performed using a TaqPath ProAmp Master Mix kit and a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific). Thermal cycling was conducted according to the manufacturer's recommended program, and all experiments were performed in duplicate. In brief, 2.25 μl DNA (4.5 ng in total) was added to the reaction mixtures comprised of 2.5 μl TaqPath ProAmp Master Mix kit, 0.125 μl Taqman SNP genotyping assay (40 x) and 0.125 μl Nuclease free water. The quantitative PCR was performed under the genotyping experiments fast cycling conditions: i) pre-read step at 60˚C for 30 s, ii) initial denaturation/enzyme activation step at 95˚C for 5 min, iii) 40 cycles of denaturation step at 95˚C for 5 s and annealing/extension step at 60˚C for 30 s, and iv) pre-read step at 60˚C for 30 s.

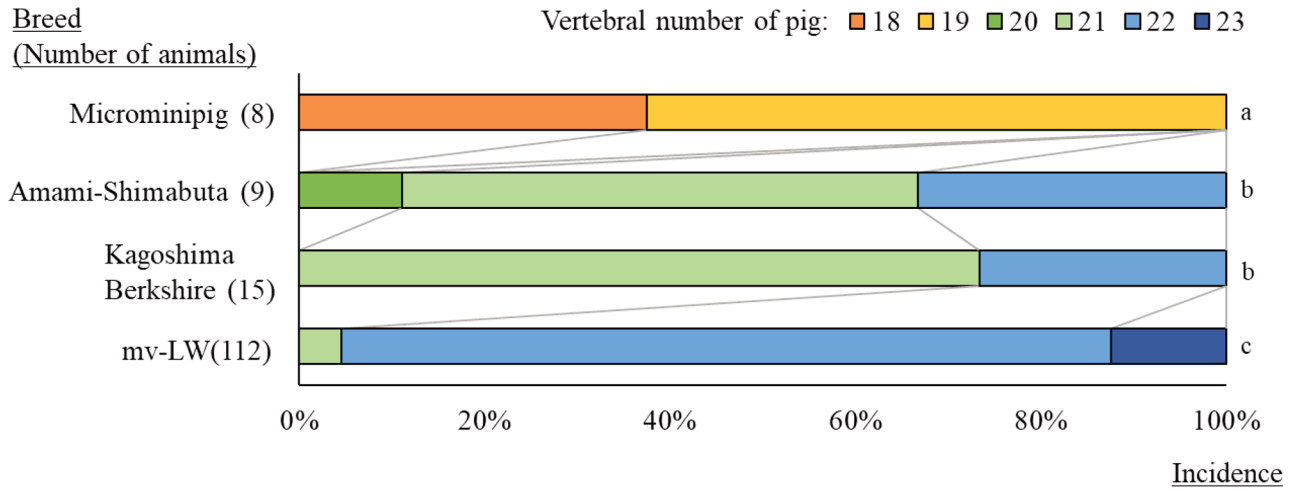

Figure 1. *Pattern of vertebrae number distribution in four pig breeds. a-c: Show the distribution ratio, where different letters indicate significant differences (p<0.01). mv-LW: Multi-vertebrae-fixed Large White breed.*

Table III. *Genotype and allele frequencies at the c.575T>C locus of NR6A1 in four pig breeds.*

Breed (number of animals)	Genotype frequency			Allele frequency	
	TT	TC	CC	T	C
Microminipig (8)	0.000	0.000	1.000	0.000	1.000
Amami-Shimabuta (9)	0.667	0.333	0.000	0.833	0.167
Kagoshima Berkshire (15)	1.000	0.000	0.000	1.000	0.000
mv-LW (34)	1.000	0.000	0.000	1.000	0.000

mv-LW: Multi-vertebrae-fixed Large White breed.

Statistical analysis. The number of vertebrae is expressed as mean±standard deviation. Statistical analysis of the differences between breeds was assessed by one-way ANOVA analysis of variance, followed by the Tukey-Kramer multiple comparison test and Fisher's exact test. All statistical analyses were performed using the IBM SPSS Statistics 25 software (IBM, Tokyo, Japan), and $p<0.05$ was considered statistically significant.

Results

Vertebrae number. The Microminipig had the lowest vertebrae number while mv-LW had the highest vertebrae number (Table II). The vertebrae numbers of the two middle-sized breeds were intermediate between those of the Microminipig and the mv-LW. The pattern of vertebrae number distribution of Microminipig significantly differed from the other three breeds and all Microminipigs had 19 or fewer vertebrae (Figure 1).

NR6A1 genotype and allele frequencies. Homozygous C/C genotypes were observed in Microminipigs, while homozygous T/T genotypes were observed in mv-LW and Kagoshima Berkshire pigs (Table III). Both homozygous T/T (n=6) and heterozygous C/T genotypes (n=3) were observed in Amami-Shimabuta pigs. The vertebrae numbers in Amami-Shimabuta pigs with homozygous T/T and heterozygous C/T genotypes were 21.0±1.0 and 21.3±0.5, respectively, and the difference was not significant.

Discussion

The *NR6A1* gene, encoding an orphan nuclear receptor, is located in a 300-kb region that is fixed in a variety of European commercial breeds (Landrace, LW, Yorkshire, Duroc, and Berkshire) (1). This gene has been focused on as a means of controlling economically important traits in the

swine market or as a tool to discriminate between wild boar and domestic pigs (19, 27, 28).

We found that the Microminipig was homozygous for the *NR6A1* C/C genotype, with a vertebrae number of lower than the 19 vertebrae uniformly found in wild boar (3, 23). The Microminipig is the smallest experimental minipig and represents a successful pig miniaturization. Our results indicate that the *NR6A1* C/C genotype could be an index of pig miniaturization. Other factors influencing decreased vertebrae numbers remain to be investigated further. To the best of our knowledge, this is first report associating *NR6A1* and vertebrae number in an experimental minipig.

In previous studies, the C allele of *NR6A1* has appeared in wild boars and in several indigenous pigs, especially Asian pigs (20, 22, 29). The T allele frequency in Amami-Shimabuta pig was higher compared to other indigenous Asian pigs (23, 29). This indicates that the domestication of Amami-Shimabuta is considered to be more advanced than that of the other indigenous Asian pigs examined on the body elongation to increase meat production.

In this study, the homozygous T/T genotype was observed in mv-LW pigs, as has been observed in commercial LW, Landrace, Duroc and other Western breeds (22, 23, 29). Most of the current commercial-crossbred pigs have 21 vertebrae (26), while most commercial LW pigs have 22 vertebrae, and 2.9% of them have 23 vertebrae (4). Our results show that 12.5% of mv-LW pigs have 23 vertebrae, indicating that multi-vertebrae traits have been successfully selected in mv-LW. Similarly, the Kagoshima Berkshire pigs were also homozygous for the T/T genotype, but the vertebrae number in this breed was lower than 22. Therefore, there are additional factors increasing vertebrae number that remain to be explored.

In conclusion, *NR6A1* polymorphism could be a useful index for both miniaturizing and increasing pig body size.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

MI and HK collected sample material. NM designed the primer/probe system targeting the *NR6A1*. MI, HK, NM and AT planned the study; YCL and MI performed the experiments and analyzed the data; MI, HK and AT drafted the manuscript; YF and TM revised the draft. All Authors read and approved the final manuscript.

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