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Detection of copy number variation of alpha amylase genes in domestic pigs (*Sus scrofa domesticus*) and wild boars (*Sus scrofa*)

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Abstract: Copy numbers of alpha amylase genes (*AMY*), which encode starch-digesting enzymes, are markedly increased in modern humans and domesticated dogs as an adaptive evolutionary mechanism in response to increased consumption of starch-rich foods acquired either by farming or domestication. In this study, we surveyed total *AMY* gene copy numbers in 150 domestic pigs (50 pigs of Berkshire breed, 50 of Landrace breed, and 50 of Large White breed) and 51 wild boars (30 *Sus scrofa leucomystax* and 21 *S. s. riukiuanus*) to identify whether the gene copy number has changed during the domestication of pigs. The relative copy number of *AMY* genes was measured using a quantitative polymerase chain reaction (qPCR) and it varied from 2.7 to 10.8 per haploid genome among individuals. However, in the four remaining populations, excluding *S. s. riukiuanus*, the average copy number was approximately six, and no significant differences were observed between the three selected pig breeds and *S. s. leucomystax* wild boar. Conversely, *S. s. riukiuanus* had an average of 7.2 copies. The results indicating six *AMY* copies per haploid genome were consistent with the porcine genome reference sequence (Sscrofa11.1). These results suggest that there has been no significant increase in the *AMY* gene copy number during the domestication process of pigs.

Keywords: *AMY* locus; domestication; porcine CNV; qPCR

Alpha amylase (AMY) is a major starch-digestive enzyme in mammals. In humans, two paralogous genes encode alpha amylase isozymes, salivary gland specific amylase (AMY1) and pancreatic amylase (AMY2) (Meisler et al. 1993). However, because the human *AMY1* locus is a hominid-specific paralog of the amylase gene, some animals, including domesticated dogs and pigs, do not have an *AMY1* locus (Pajic et al. 2019). Although *AMY2* is known as a pancreatic amylase based on the human gene function, alpha amylase was detected in the saliva of pigs and dogs (Boehlke et. al. 2015; Contreras-Aguilar et al. 2017).

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Copy number variation (CNV) of the *AMY* loci has been reported in various species (Perry et al. 2007; Axelsson et al. 2013; Janiak 2016; Abduriyim et al. 2019). In humans, ethnic groups with high starch diets have on average about 1.4 times more *AMY1* copies than those with low starch diets (Perry et al. 2007). It has also been reported that the copy number of human *AMY1* is positively correlated with the level of salivary amylase protein (Perry et al. 2007). In addition, Axelsson et al. (2013) reported that the copy number of *AMY2* in domestic dogs was significantly higher than that of wild wolves. These copy number gains of *AMY* are considered to be a result of positive selection for the ability to consume starch-rich diets (Perry et al. 2007; Axelsson et al. 2013).

Pigs were domesticated from boars (*Sus scrofa* L.) about 10 000 years ago (Ruvinsky and Rothschild 1998). During the domestication process of wild animals, their traits changed over successive generations in response to selection pressure under new environments imposed by the human community as well as artificial selection. Changes in diet are some of the largest differences animals had to adapt to during domestication. In the wild, the amount and type of available food resources fluctuate seasonally, and the wildlife, including boars, is constantly facing food inconsistencies. Conversely, domesticated animals are supplied with constant foodstuffs, including grains and agricultural waste. Human-supplied feed contains abundant carbohydrates, especially modern-day feed used for pig farming, which contains large amounts of maize and soybeans (Bach-Knudsen et al. 2013). Given this radical change in diet, domesticated pigs may have evolved to have an increased digestion efficiency of carbohydrates compared to wild boars.

The *AMY2* locus is located on pig chromosome (SSC) 4 in the current assembly of pig genome (Sscrofa11.1) and there are six *AMY*-like loci: LOC100521789, LOC100522672, LOC110260364, LOC110258046, LOC100153854, and LOC100153446. However, the last three loci are in unplaced genomic scaffold contigs. Although LOC110260364 was described as an *AMY*-like locus, it lacked most exons and showed no homology with the other loci. Paudel et al. (2013) analysed the whole genome sequence of 16 pigs, including wild boars, and they detected eight to 21 CNVs of amylase-like sequences per diploid genome. Therefore, *AMY* copy number polymorphisms are probably present in the porcine genome; however, there has been limited research focused on CNVs of the porcine *AMY* gene at population levels. In this study, we analysed the copy number of the *AMY* gene of 150 domestic pigs and 51 wild boars to determine the effect of domestication on the CNVs of the *AMY* gene in pigs.

MATERIAL AND METHODS

Pig DNA samples

We analysed DNA samples of 150 domestic pigs: 50 individuals of Berkshire breed, 50 of Landrace breed, and 50 of Large White breed. Tissue samples of Berkshire pigs were collected from pig farms that produce only Berkshire pork. Tissue samples of the other two breeds were collected at farms that produce heritage purebred pigs. Tissue samples were obtained during typical farm tasks, such as attaching ear tags for individual identification. We also analysed 30 Japanese wild boars (*S. s. leucomystax*) and 21 Ryukyu wild boars (*S. s. riukiuanus*). These wild boars were captured in the wild by local hunters. Collecting porcine tissue samples was approved by the institutional animal care and use committee of ZEN-NOH (research code number: 1501-766) and carried out according to animal experimentation regulations. Total DNA was extracted from muscle, tail tissue or ear chips using the QuickGene DNA Tissue Kit (Fujifilm Corporation, Tokyo, Japan).

Primer design and quantitative real-time PCR

For measuring the copy number of total *AMY* genes (including putative pseudogenes) a primer set was designed based on a well conserved sequence among porcine *AMY2* and the five *AMY2*-like loci (Sscrofa11.1). The PCR primer pair for the leptin (*LEP*) gene was used as an internal standard for a single-copy gene. The sequence information for these primers is listed in Table 1. The quantitative real-time PCRs (qPCR) were performed on a Thermal Cycler Dice® Real Time System II (Takara Bio Inc., Shiga, Japan) using THUNDERBIRD qPCR Mix (Toyobo Co., Ltd., Osaka, Japan), according to the manufacturer's instructions and cycling conditions. Each reaction contained 12.5 µl of THUNDERBIRD qPCR Mix, 0.75 µl of the forward and reverse prim-

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Table 1. Primer sequences used in this study

Gene		Primer sequences (5' to 3')	Amplicon size (bp)
Total *AMY*	F:	GCAGCTGCAGGAACGGG	118/119†
	R:	CCACTTGCAGTTTTACATTTACCA	
LEP	F:	AACAGAGGGTCACCGGTTTG	150
	R:	GGTTCTCCAGGTCATTCGATATT	

†Based on Sscrofa11.1, the amplification products of LOC100522672 and LOC100153446 are 118 bp, and the amplification products of *AMY2*, LOC100521789, LOC110258046, and LOC100153854 are 119 bp

ers (10 pmol/µl), 1 µl of genomic DNA (2 ng/µl) and 10 µl of distilled water. Each sample was run in duplicate. Thermal cycling conditions consisted of an initial denaturation step at 95 °C for 60 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing at 60 °C for 30 s (data collection). To confirm that the product from the PCR reaction was a unique amplification and that there was no contamination in the DNA negative control, amplification curves were generated using the Thermal Cycler Dice Real Time System II Software v4.02 (Takara Bio Inc., Shiga, Japan). All samples were run in duplicate, and average values were used to calculate the copy numbers of the total *AMY* gene.

Data analysis

The $2^{-\Delta Ct}$ method was used to analyse the data obtained from the qPCR. The copy numbers of the total *AMY* per haploid genome were measured as a relative ratio of amplification of the *AMY* to the *LEP* gene, a single-copy gene control, within each sample. Statistical analysis was performed using R software v3.6.1 (R Core Team, Vienna, Austria). Mann-Whitney *U*-tests with a Bonferroni correction were performed to test the differences between two independent populations. An original *P*-value of less than 0.005 (Bonferroni adjusted $\alpha < 0.05$) was considered to be statistically significant. Tukey-Kramer test was also performed to identify and separate significantly different means.

RESULTS

The relative copy number of the total *AMY* measured using qPCR varied from 2.7 to 10.8 per haploid genome among individuals (Figure 1). The range of the copy number of porcine *AMY* was generally consistent with previous studies (Paudel et al. 2013; Pajic et al. 2019). We identified that the copy number variation of the total *AMY* gene is present within a single breed of pig and even within a single wild boar subspecies.

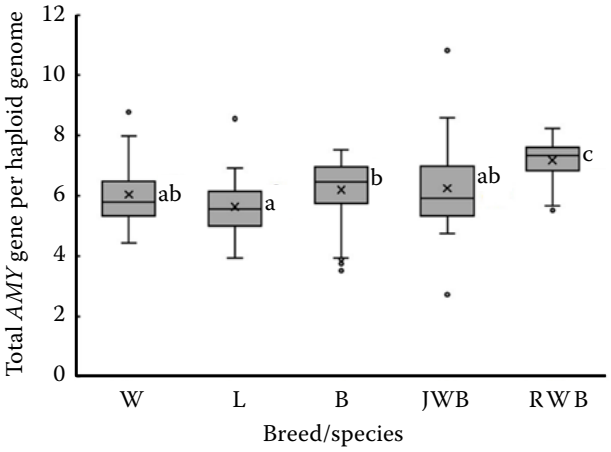

Figure 1. The boxplot of the *AMY* copy number per haploid genome in three pig breeds and two wild boar subspecies

B = Berkshire; JWB = Japanese wild boar; L = Landrace; RWB = Ryukyu wild boar; W = Large White

The boxes indicate the upper and lower quartiles. The horizontal lines at the centre of each box represent the median of the data. Daggers indicate the average value. Whiskers show the distance within the 1.5 times interquartile range. Dots beyond the whiskers are potential outliers. The letters a, b, and c indicate significant differences by the Mann-Whitney *U*-test with Bonferroni correction (original *P*-value < 0.005)

The average copy numbers of total *AMY* were not significantly different between the three pig breeds and Japanese wild boar: 6.2 ± 1.1 for Berkshire, 5.6 ± 0.9 for Landrace, 6.0 ± 0.9 for Large White, and 6.2 ± 1.5 for Japanese wild boar. However, significant differences were detected between the Berkshire and Landrace breeds by the Mann-Whitney *U*-tests ($P_{original} = 0.000\ 4$). Conversely, the total *AMY* copy number of *Sus scrofa riukiuanus* was 7.2 ± 0.7 on average, about one copy higher than that of the other populations ($P < 0.05$ by Tukey-Kramer, $P_{original} < 0.005$ by Mann-Whitney *U*-test). The results of six or seven *AMY* copies per haploid genome were consistent with the porcine reference genome sequence (Sscrofa11.1).

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DISCUSSION

The measurement of the copy number of *AMY* using qPCR is much simpler than the whole genome sequencing (Paudel et al. 2013). Therefore, we could survey a total of 201 individuals. By using a sufficient number of samples, we were able to analyse the distribution of *AMY* copy number variation in three pig breeds and two subspecies of wild boar. We demonstrated that copy number variants of *AMY* were widely distributed among domestic pigs and wild boars (Figure 1). The fact that the average and mode of *AMY* copy numbers in domestic pigs were consistent with the porcine reference genome suggested the accuracy of measurement in this study.

Unfortunately, we could not analyse European wild boar (*S. s. scrofa*) in this study. The Japanese wild boar is not a direct ancestor of European domestic pigs; however, it is positioned in a typical East Asian wild boar group that is closely associated with Asian pigs (Larson et al. 2005). The modern European pigs, including Landrace and Large White, had a hybrid origin between European native pigs and Asian pigs during the 18th and early 19th centuries (Giuffra et al. 2000). Therefore, Japanese wild boar can be considered to be a closely related population to the genetic source of modern pigs. On the other hand, Ryukyu wild boar does not contribute to the gene pool of modern European pigs (Watanobe et al. 1999; Larson et al. 2005). Thus, Ryukyu wild boar can be used as outgroup for domestic pigs. The average copy numbers of *AMY* were significantly different between Japanese wild boar and Ryukyu wild boar (Figure 1). Studies on copy number polymorphisms of the *AMY* gene in wild animals are few, but Abduriyim et al. (2019) reported copy number differences in Asian badger (*Meles leucurus*). The copy number difference in *AMY* between the two subspecies of wild boar might represent a genetic differentiation.

In domestic dogs, a drastic increase in copy numbers of *AMY*, about 7.4-fold larger than in wolves on average, was characterized as an adaptation to a starch-rich diet during domestication (Axelsson et al. 2013). However, unlike in the case of dogs, the average copy numbers of *AMY* of domestic pigs were not significantly higher than those of wild boars (Figure 1). Pajic et al. (2019) measured the copy number of the *AMY* gene in five boars and 11 pigs using droplet digital PCR and reported that there was no difference between wild boars and domesticated pigs. Our present results strongly supported the previous report that there was no significant increase in the copy number of the *AMY* gene during the domestication of pigs. A possible reason for this is that wild boars are omnivorous, thus they adapted themselves to the consumption of dietary starch prior to when pigs were domesticated as pointed out in a previous study (Pajic et al. 2019).

However, our measurements contain pseudogenes and thus they cannot show whether the detected copy number changes in total *AMY* gene affect amylase secretion or starch digestion. Further studies are needed on the effects of amylase gene duplication on pig traits.

CONCLUSION

In domestic dogs, a drastic increase in copy numbers of *AMY*, about 7.4-fold larger than in wolves on average, was characterized as an adaptation to a starch-rich diet during domestication (Axelsson et al. 2013). However, unlike in the case of dogs, the average copy numbers of *AMY* in domestic pigs were not significantly higher than those in wild boars. These results strongly suggest that there was no significant increase in the *AMY* copy number during the domestication of pigs.

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Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Abduriyim S, Nishita Y, Abramov AV, Solovyev VA, Saveljev AP, Kosintsev PA, Kryukov AP, Raichev E, Vainola R, Kaneko Y, Masuda R. Variation in pancreatic amylase

https://doi.org/10.17221/61/2020-CJAS

gene copy number among Eurasian badgers (Carnivora, Mustelidae, Meles) and its relationship to diet. J Zool. 2019 May;308(1):28-36.

Axelsson E, Ratnakumar A, Arendt ML, Maqbool K, Webster MT, Perloski M, Liberg O, Arnemo JM, Hedhammar A, Lindblad-Toh K. The genomic signature of dog domestication reveals adaptation to a starch-rich diet. Nature. 2013 Jan 23;495:360-4.

Bach-Knudsen KE, Laerke HN, Jorgensen H. Carbohydrates and carbohydrate utilization in swine. In: Chiba LI, editor. Sustainable swine nutrition. Hoboken (NJ): Wiley-Blackwell; 2013. p. 109-37.

Boehlke C, Zierau O, Hannig C. Salivary amylase – The enzyme of unspecialized euryphagous animals. Arch Oral Biol. 2015 Aug;60(8):1162-76.

Contreras-Aguilar MD, Tecles F, Martinez-Subiela S, Escribano D, Bernal LJ, Ceron JJ. Detection and measurement of alpha amylase in canine saliva and changes after an experimentally induced sympathetic activation. BMC Vet Res. 2017 Aug 22;13(1): [6 p.].

Giuffra E, Kijas JMH, Amarger V, Carlborg O, Jeon J-T, Andersson L. The origin of the domestic pig: Independent domestication and subsequent introgression. Genetics. 2000 Apr 1;154(4):1785-91.

Janiak MC. Digestive enzymes of human and nonhuman primates. Evol Anthropol. 2016 Sep-Oct;25(5):253-66.

Larson G, Dobney K, Albarella U, Fang M, Matisoo-Smith E, Robins J, Lowden S, Finlayson H, Brand T, Willerslev E, Rowley-Conwy P, Andersson L, Cooper A. Worldwide phylogeography of wild boar reveals multiple centers of pig domestication. Science. 2005 Mar 11;307(5715):1618-21.

Meisler MH, Ting CN. The remarkable evolutionary history of the human amylase genes. Crit Rev Oral Biol Med. 1993 Apr;4(3-4):503-9.

Pajic P, Pavlidis P, Dean K, Neznanova L, Romano RA, Garneau D, Daugherity E, Globig A, Ruhl S, Gokcumen O. Independent amylase gene copy number bursts correlate with dietary preferences in mammals. eLife. 2019 May 14;8: [22 p.].

Paudel Y, Madsen O, Megens HJ, Frantz LA, Bosse M, Bastiaansen JW, Crooijmans RP, Groenen MA. Evolutionary dynamics of copy number variation in pig genomes in the context of adaptation and domestication. BMC Genom. 2013 Jul 5;14: [13 p.].

Perry GH, Dominy NJ, Claw KG, Lee AS, Fiegler H, Redon R, Werner J, Villanea FA, Mountain JL, Misra R, Carter NP, Lee C, Stone AC. Diet and the evolution of human amylase gene copy number variation. Nat Genet. 2007 Sep 9;39(10):1256-60.

Ruvinsky A, Rothschild MF. The genetics of pig. In: Rothschild MF, Ruvinsky A, editors. Systematics and evolution of the pig. Wallingford (UK): CAB International; 1998. p. 1-16.

Watanobe T, Okumura N, Ishiguro N, Nakano M, Matsui A, Sahara M, Komatsu M. Genetic relationship and distribution of the Japanese wild boar (Sus scrofa leucomystax) and Ryukyu wild boar (Sus scrofa riukiuanus) analysed by mitochondrial DNA. Mol Ecol. 1999 Sep;8(9):1509-12.

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