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AMY2B Gene Copy-Number Variation Studied by Droplet Digital PCR (ddPCR) in Three Canids: Red Fox, Arctic Fox, and Chinese Raccoon Dog

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Short communication

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Copy-number variation (CNV) is an important source of genetic variation, and one that played a role in the process of domestication. The adaptation to a new diet is a characteristic feature of dog domestication. We therefore sought genomic signatures of this process. The pancreatic alpha-amylase gene (*AMY2B)*, expressed in the pancreas, exhibits a variable number of copies. It has been shown that the multiplication of this gene is associated with the adaptation of dogs to a starch-rich diet. To date, there has been no information made available on the copy-number variation of *AMY2B* in canid farm animals. The aim of the present study was to examine the *AMY2B* copy number in the red fox, the arctic fox, and in the Chinese raccoon dog. Droplet digital PCR (ddPCR) was used to count the gene copies in 152 animals (60 red foxes, 53 arctic foxes, and 39 Chinese raccoon dogs). We found that the majority (91%) of the animals had two copies of this gene. Of the red foxes and Chinese raccoon dogs, only 8% had three copies, while 32% of the arctic foxes had three copies. Our study showed that the multiplication of the *AMY2B* gene did not occur over several decades of breeding selection, which may reflect the low-starch feeding regime.

Key words: *AMY2B*, amylase, canid genetics, comparative genetics, starch digestion.

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The *Canidae* family contains 36 species, including three species that are bred for their fur – the red fox (*Vulpes vulpes*), the arctic fox (*Alopex lagopus*), and the Chinese raccoon dog (*Nyctereutes procyonoides procyonoides*) (SWITONSKI *et al.* 2003). The population size and breeding history of these species is distinct from the conditions met with in the history of dog domestication and selection. The earliest red fox farms started in Canada, approximately 120 years ago (STATHAM *et al.* 2011); they were followed by arctic fox farms in Scandinavia about 100 years ago (NOREN *et al.* 2005). Raccoon dog farms are the most recent, dating from 30 and 40 years ago in Poland and Finland, respectively (SLASKA *et al*. 2010). On the other hand, dogs (*Canis lupus familiaris*) have a long breeding history, having been domesticated about 15,000 years ago (OSTRANDER *et al*. 2017). The large number of dog breeds (approx. 400) show a vast diversity in terms of morphological and behavioral traits. There are many ancient dog breeds, such as the Japanese Akita and the Siberian Samoyed, which are closely related to their ancestor, the grey wolf (*Canis lupus*). There are also newer breeds, such as German Shepherds and Terriers (PARKER *et al*. 2004; PARKER *et al*. 2017). It is thought that majority of dog breeds arose during the last two centuries (VONHOLDT *et al*. 2010). On the other hand, there is evidence that additional copies of *AMY2B* appeared probably approximately 7000 years ago as reaction to the appearance of starch in dogs' diet (OLLIVIER *et al*. 2016).

The process of domestication involves changes in diet. It has been shown that one of the main contributors to dog domestication was the use of a different diet to that of wolves and other wild canids. Dogs' diet has been transformed to more closely resemble the omnivorous diet of humans (REITER *et al*. 2016). The

resequencing of the dog and wolf whole-genome has allowed for the identification of the genomic regions that were targeted for selection during dog domestication. These include regions harboring genes important for starch digestion and metabolizing fat (AXELSSON *et al*. 2013). One of the most interesting genes is pancreatic alpha amylase (*AMY2B*), which is responsible for the digestion of starch into maltose in the small intestine. It has been found that an increase in *AMY2B*'s copy number in dogs leads to increased enzyme activity and reflects the adaptation to increased starch consumption (ARENDT *et al*. 2014; AXELSSON *et al*. 2013). Copy-number variation (CNV) is a source of genetic variation which undergoes selection during domestication and environmental adaptation (AXELSSON *et al*. 2013). Human genome regions containing genes that encode different types of amylase enzymes are also highly polymorphic (SHWAN *et al*. 2017); for example, there are 2-15 copies of salivary amylase (*AMY1*) in humans (PERRY *et al*. 2007). It has been shown that a high number of copies of *AMY1* is negatively correlated with body mass index (BMI) (PINHO *et al*. 2018; VENKATAPOORNA *et al*. 2019), though not all studies confirmed this association (SHWAN and ARMOUR 2019).

The majority of studies of amylase genes in dogs deal with the *AMY2B* gene; a high variability in *AMY2B* copy number has been found across different breeds. Our study confirmed the copy number variability (9-18 copies) in dogs reported previously by ARENDT *et al*. (2014). Interestingly, it was shown that ancient breeds have fewer copies (2-12), while modern breeds have more than 20 (ARENDT *et al*. 2016; TONOIKE *et al*. 2015).

The *AMY2B* gene has been studied in relation to the predisposition of obesity in the Labrador Retriever breed, but no association between copy number variation and body mass or body condition score (BCS) was found (ANTKOWIAK *et al.* 2019). Moreover, variation in *AMY2B* copy number was also analyzed in relation to diabetes in dogs, but again no association was found (ARENDT *et al*. 2014).

To date, there has been no information on the copy-number variation of *AMY2B* in the canids kept on fur animal farms, under selection pressure for body size and fur quality. The aim of our study was thus to look for variation in *AMY2B* copy number in farmed red foxes, arctic foxes, and Chinese raccoon dogs.

Material and Methods

Biological samples were collected from 60 red foxes, 53 arctic foxes, and 39 Chinese raccoon dogs kept on a fur-bearing farm near Poznań. Samples were collected *postmortem* following the approval of the Local Ethical Commission for Investigations on Animals in Poznań (no. 19/2003; date of approval: 30 June 2003). DNA was isolated with a MasterPure DNA Purification Kit for Blood Version II (Epicenter) and a Genomic Mini kit (A&A Biotechnology) from blood and tissue, respectively. The concentration of DNA was measured by a Qubit 2.0 Fluorometer (Invitrogen).

AMY2B copy number was determined using droplet digital PCR (ddPCR). The PCR reaction was conducted using ddPCR Supermix for Probes (Bio-Rad) and, for better DNA separation into droplets, the restriction enzyme *Hae*III was added to the reaction mixture. The sequences for the studied gene and for the reference genes used in the assay design were taken from the dog genome assembly (CanFam 3.1); the tested gene was located on chromosome 6. The amplification of *AMY2B* employed the following primers: F: 5'CCAAACCTGGACGGACATCT, R: 5' TATCGTTCGCATTCAAGAGCAA, and TaqMan probe: 5' 6-Fam-TTTGAGTGGCGCTGGG-BHQ-1. The *HSD17B7* gene, in domesticated dogs located on chromosome 38, was used as a reference gene. The primers were as follows: F: ATGTCCACACAACTAGCCATAC, R: GTGTCTCGGTAGCGCATTT, TaqMan probe: Hex-CACGCCCAGTCCTAGTCATGCTT-BHQ-1. We carried out ddPCR in accordance with the manufacturer's instructions. The annealing temperature was 58°C. The QX200 droplet reader (Bio-Rad) was used for fluorescence detection and the results were analyzed using QuantaSoft software (v. 1.7.4.0917). The estimation of the *AMYB2* copy number was based on the known copy number of the reference gene (2 copies). For statistical analysis, the Kruskal-Wallis test and the Dunn's test (R software v. 3.4.0) was used.

Results and Discussion

Due to the high genome homology between canids, the CNV detecting assay for *AMY2B,* previously described (ANTKOWIAK *et al*. 2019; AXELSSON *et al.* 2013), was successfully used for amplification in foxes and Chinese raccoon dogs. We found that the median of diploid copy number of *AMY2B* for red foxes was 2.085 (with mean = 2.11 ± 0.29 SD). Only five animals (approx. 8%) had three copies of this gene. In Chinese raccoon dogs, the median copy number was 2.16 (with mean = 2.06 ± 0.4 SD) and three animals (approx. 8%) had three copies, while in two animals (approx. 5%) we observed only a single copy of *AMY2B*. The cohort of arctic foxes was the most polymorphic, with three copies of *AMY2B* being found in 17 animals (approx. 32%); the median was 2.38 (with mean = 2.47 ± 0.43 SD). Figure 1 presents exemplary results from the QuantaSoft software.

Comparison of the three groups of animals using the Kruskal-Wallis test showed that they differed statistically significantly with a p-value = 2.076×10^{-8}. Also, the comparison between particular groups showed

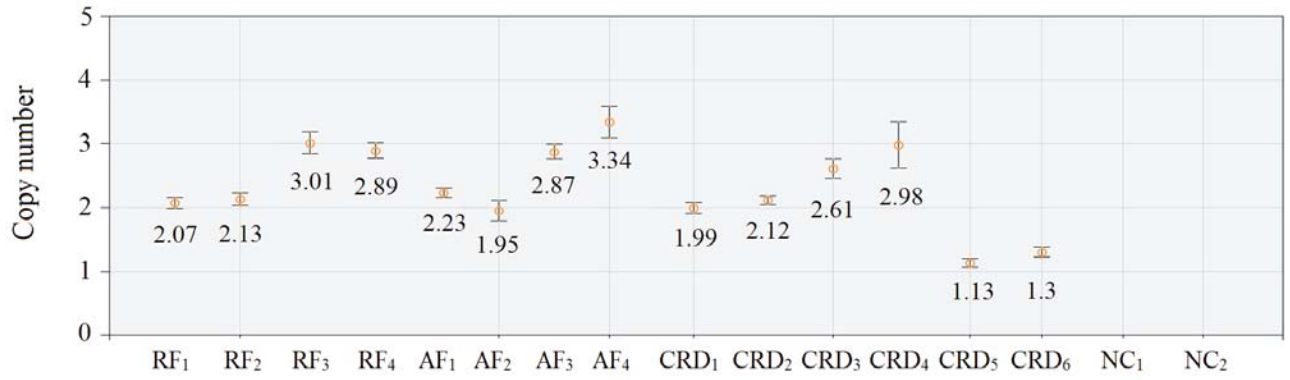

Fig. 1. Representative results of ddPCR; RF: red foxes; AF: arctic foxes; CRD: Chinese raccoon dogs; NC: negative controls.

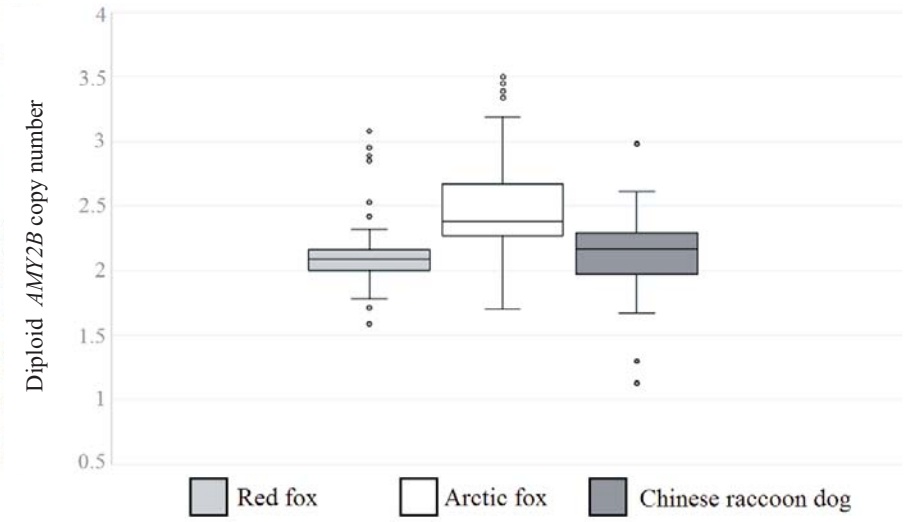

Fig. 2. Box-plot of the diploid number of *AMY2B* copies in the three studied canids. Upper and lower quartiles are marked in the upper and lower parts of the rectangles. Medians are marked by the lines crossing the boxes. The highest and lowest values are represented by the lines under and over the rectangles. The points under and over the boxes are outliers.

that the copy number in Arctic foxes was significantly different with a p-value <0.05. Figure 2 shows box-plots for the results of all three species. The results show that the copy number in most animals did not deviate from two copies, so the copy number polymorphism of *AMY2B* in the studied animals seems to be low.

Two copies of *AMY2B* have been observed in several carnivore species, including the grey wolf (*Canis lupus*) (FREEDMAN *et al.* 2014), the polar bear (*Ursus maritimus*) (RINKER *et al.* 2019), species of the Mustelidae family (ABDURIYIM *et al.* 2019), and the domestic cat (PAJIC *et al.* 2019). The situation was similar in our observations, as most of the red foxes and Chinese raccoon dogs also had two copies. It is worth mentioning that the diet of farmed canid species includes plant-derived ingredients (GUGOŁEK *et al.* 2014). We can assume that a similar diet was fed to the canids studied here, although, only the arctic foxes presented a significant level of *AMY2B* polymorphism. Interestingly, it was shown that farmed arctic foxes present a higher digestibility of starch in comparison with wild arctic foxes (AHLSTRØM *et al.* 2003). This could explain the observed higher incidence of three copies of *AMY2B* in the studied cohort. Other predators that consume plants in their diet, such as the Asian badger (*Meles leucurus*) and the brown bear (*Ursus arctos*), as well as the American black bear (*Ursus americanus*), had up to four or three copies, respectively (ABDURIYIM *et al.* 2019; RINKER *et al.* 2019). It should be mentioned that the two bear species also show polymorphism in their copy number of *AMY1B*, another gene associated with pancreatic amylase; 3-4 copies of *AMY1B* were detected in American black bears and 4-8 copies in brown bears (RINKER *et al.* 2019).

In two Chinese raccoon dogs a single copy of *AMY2B* was found. This is not an exceptional observation, since it was sporadically also found in dogs and wolves (ARENDT *et al.* 2016), panthers (KIM *et al.* 2016), and polar bears (RINKER *et al*. 2019).

In this study, we have described the *AMY2B* copy number of three canid species farmed for fur for the first time. We found that the copy number variation for this gene was low. This can be explained by the short breeding history of these species, accompanied by the introduction of plant ingredients to their diets. We can assume that this period is probably too brief to introduce high variability on the genomic level, especially when compared to the long history of dog domestication and the increasing amounts of starch in their diet.

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Author Contributions

Research concept and design: M.S.; Collection and/or assembly of data: M.S., M.Sz.; Data analysis and interpretation: M.A., J.N.-W., I.S.; Writing the article: M.A., J.N.-W., I.S.; Critical revision of the article: J.N.-W., I.S., M.S.; Final approval of article: M.A., J.N.-W., I.S., M.S., M.Sz.

Conflict of Interest

The authors declare no conflict of interest.

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