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Amylase activity is associated with *AMY2B* copy numbers in dog: implications for dog domestication, diet and diabetes

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Summary

High amylase activity in dogs is associated with a drastic increase in copy numbers of the gene coding for pancreatic amylase, *AMY2B*, that likely allowed dogs to thrive on a relatively starch-rich diet during early dog domestication. Although most dogs thus probably digest starch more efficiently than do wolves, *AMY2B* copy numbers vary widely within the dog population, and it is not clear how this variation affects the individual ability to handle starch nor how it affects dog health. In humans, copy numbers of the gene coding for salivary amylase, *AMY1*, correlate with both salivary amylase levels and enzyme activity, and high amylase activity is related to improved glycemic homeostasis and lower frequencies of metabolic syndrome. Here, we investigate the relationship between *AMY2B* copy numbers and serum amylase activity in dogs and show that amylase activity correlates with *AMY2B* copy numbers. We then describe how *AMY2B* copy numbers vary in individuals from 20 dog breeds and find strong breed-dependent patterns, indicating that the ability to digest starch varies both at the breed and individual level. Finally, to test whether *AMY2B* copy number is strongly associated with the risk of developing diabetes mellitus, we compare copy numbers in cases and controls as well as in breeds with varying diabetes susceptibility. Although we see no such association here, future studies using larger cohorts are needed before excluding a possible link between *AMY2B* and diabetes mellitus.

Keywords canine genetics, comparative genetics, starch digestion

Introduction

Adaptation to a new diet during dog domestication

A recent comparison of genome-wide patterns of genetic variation in a large panel of dogs and wolves identified genomic regions that were affected by directional selection during early dog domestication (Axelsson *et al.* 2013). Through functional characterization of genes residing in these domestication regions, new light was shed on characteristics of adaptive advantage to early dogs. These analyses identified several genes involved in digestion and energy metabolism, suggesting that the transition from wolf to dog was accompanied by a change in diet. Augmented by evidence from expression analyses and enzyme assays, it was concluded that changes in three consecutive steps in the pathway responsible for starch digestion and subsequent glucose absorption allowed dogs to rely on a diet rich in starch relative to the carnivorous wolf diet (Axelsson *et al.* 2013).

AMY2B and amylase activity

Pancreatic amylase (AMY2B) serves as the first step in the digestion of starch to glucose in the small intestine (Mocharla *et al.* 1990) by catalyzing the breakdown of starch to oligosaccharides maltose and maltriose. Axelsson *et al.* (2013) specifically demonstrated that selection had acted on a series of duplication events to favor the accumulation of additional copies of *AMY2B*, resulting in an average sevenfold copy number increase in dogs relative to in wolves, and that this increase corresponds to higher pancreatic *AMY2B* expression as well as higher serum amylase activity. Although these observations argue that dogs in general digest starch more efficiently than do

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wolves, considerable variation in *AMY2B* copy numbers within the dog population, with diploid copy numbers ranging from 4 to 30 ($n = 136$) (Axelsson *et al.* 2013), indicates that the ability to handle starch may vary significantly among dogs. In support of this idea, wide reference values for serum amylase activity in blood biochemistry panels indicate strong variability in amylase activity among dogs. Based on the simultaneous increase in *AMY2B* copy number and amylase activity in dogs relative to in wolves (Axelsson *et al.* 2013), it is reasonable to hypothesize that amylase activity is associated with *AMY2B* copy number within the dog population. Yet, a previous analysis within the dog population did not support this hypothesis (Axelsson *et al.* 2013).

AMY2B and breed variation

Although it is clear that *AMY2B* copy number varies considerably among dogs, it is not known how much of this variation is confined to breeds. Strong genetic drift associated with the shifting demographic histories of dog breeds is likely to have resulted in breed-specific patterns of copy abundance, but is also possible that regional dietary constraints may have resulted in differential selective regimes that may have shifted average copy numbers among breeds. Regardless of how, it is thus possible that the average ability to digest starch varies among dog breeds.

AMY2B and DM in dogs

In contrast to dogs, humans have acquired expression of amylase in saliva via an ancient gene duplication and a subsequent insertion of a retroviral promoter upstream of the duplicated gene copy (Ting *et al.* 1992). Parallel to the increase in *AMY2B* copy number during dog domestication, the copy number of the gene coding for salivary amylase, *AMY1,* has risen threefold in humans relative to in chimpanzees (Bank *et al.* 1992; Perry *et al.* 2007), and copy numbers are higher in human populations, such as American Europeans and Japanese, that traditionally relied on a diet that was relatively rich in starch (Perry *et al.* 2007), suggesting that dogs and humans have adapted to a similar change in diet. Both amylase activity and protein levels in human saliva correlate with *AMY1* copy number, arguing that efficient starch digestion in saliva is directly linked to copy number abundance (Perry *et al.* 2007; Mandel *et al.* 2010). In humans, high salivary amylase activity is furthermore associated with a rapid insulin response accompanied by a quick reduction in blood glucose levels following starch ingestion (Mandel & Breslin 2012), whereas low serum amylase activity is associated with an increased risk of cardiometabolic disorders (Lee *et al.* 2011; Nakajima *et al.* 2011a,b; Mandel & Breslin 2012; Muneyuki *et al.* 2012).

Although these associations are based on measures of amylase activity rather than *AMY1* copy number directly, it is feasible that genetically determined variation in amylase activity may predispose to conditions such as obesity and diabetes mellitus (DM) in humans (Lee *et al.* 2011; Nakajima *et al.* 2011a,b; Mandel & Breslin 2012; Muneyuki *et al.* 2012). DM is one of the most common metabolic disorders affecting both man and dog. In dogs, the exact etiology of DM is not known. Although dogs do not develop classical type 2 DM as do humans and cats (Catchpole *et al.* 2013), DM can occur secondary to gestation or diestrous in females due to hormonal changes. Other common secondary causes of diabetes are pancreatitis and Cushing's disease. It is estimated that more than 1 percent of all dogs will be affected by diabetes (Fall *et al.* 2007); however, incidence varies substantially among dog breeds, arguing that genetic components may contribute to this disease (Fall *et al.* 2007; Catchpole *et al.* 2013).

In this study, we estimated *AMY2B* copy numbers in a large number of dog samples to (i) test whether amylase activity is associated with *AMY2B* copy number in dogs, (ii) investigate how *AMY2B* copy number segregates within and among dog breeds and (iii) test for a potential link between *AMY2B* copy number and susceptibility for developing DM.

Materials and methods

Dog samples

To investigate the relationship between amylase activity and *AMY2B* copy numbers, EDTA blood and serum were obtained from leftover patient material at the Clinical Pathology service at the Swedish University of Agricultural Sciences in Uppsala, Sweden. Samples were collected randomly without any regard to age, sex or health status, with the exception of excluding all suspected pancreatitis cases from further analyses to avoid any potential confounding effects on amylase activity. In total, 55 samples from 35 different breeds (Table S1) were collected to have both amylase activity and *AMY2B* copy number measured (Fig. S1).

To study breed-specific patterns of *AMY2B* variation and to investigate the relationship between copy numbers and diabetes susceptibility, we collected eight dogs each from 19 different dog breeds (Table S2), as well as 19 Greenland Sledge dogs (total number of dogs, $n = 171$). All samples except the Greenland Sledge dogs, which were sampled on location in Greenland, were collected from the Canine Biobank at Uppsala University and the Swedish University of Agricultural Sciences. Breed selection was based on primarily the availability of breed-specific DM prevalence statistics (data available for 16 of the 20 breeds; Fall *et al.* 2007), whereas additional breeds were included as previous observations indicated deviant *AMY2B* copy numbers.

AMY2B copy numbers were compared in eight diabetic dogs and eight healthy controls (dogs ages >7 without diabetes) respectively in five different breeds (Samoyed,

Australian Terrier, Border Collie, Swedish Elkhound and Norwegian Elkhound), all of which have shown an increased risk of developing DM (Fall *et al.* 2007; Catchpole *et al.* 2013) (total number of dogs, $n = 80$). The 40 control dogs were also used in the breed-specific amylase copy analysis mentioned above. The Swedish and Norwegian Elkhounds as well as the Border Collies were all females and the cases were classified as hormone-dependent DM (gestational/diestral). The Samoyed and Australian Terrier populations were a mix of both females and males having adult-onset insulin-dependent DM without further classification.

In total, 266 dogs [55 + 171 + 40 (the 40 DM controls were included among the 171 dogs)] were assayed for *AMY2B* copy number in this study.

DNA extraction

DNA was extracted from EDTA blood using either manual salt extraction (Miller *et al.* 1988) or the QIASymphony DNA Midi kit (Qiagen) on the QIASymphony robot (Qiagen).

Amylase activity assay

Serum amylase activity was analyzed at the Clinical Pathology service (Swedish Agricultural University) using an Architect e400 instrument using the amylase reagents 7D58-21 (Abbott Laboratories).

Copy number assay

AMY2B copy number variation in dogs was previously studied using traditional qPCR (Axelsson *et al.* 2013). In this study, droplet digital PCR (ddPCR) was used, which allows for an absolute measure of DNA molecules partitioned into thousands of droplets. This enables a more precise estimation of DNA copy numbers, which in particular has the potential to overcome the limited capacity of qPCR to resolve high copy number gene duplications accurately (Hindson *et al.* 2011; Pinheiro *et al.* 2012). Probe and primers for the *AMY2B* target gene region and the *CCZ1B* (previous *c7orf28b)* reference gene were designed as described in Axelsson *et al.* (2013). Droplet digital PCR was performed using the QX100 third-generation droplet digital PCR system provided by Bio-Rad (Hindson *et al.* 2011). DNA was digested with DRAI (New England Biolabs) to separate individual amylase copies to allow for better partitioning. Raw copy number data were rounded to the nearest whole number.

Statistics

A mixed linear regression model was used to assess the association of *AMY2B* copy number with amylase activity in the 55 dogs. The STATA procedure 'xtmixed' was used for this purpose, and the variable dog breed (average 1.5 dogs per breed, range 1–6) was entered into the model as a random effect. Preliminary modeling showed non-normality of residuals; therefore, the dependent variable amylase activity was transformed to the natural logarithm scale before modeling.

A one-way ANOVA test was used to test whether mean *AMY2B* copy numbers differ among dog breeds and to establish how much of individual copy number variability could be ascribed to breed origin. We used Pearson's correlation coefficient to test for a correlation between mean copy number and DM incidence and two-way ANOVA tests to determine whether *AMY2B* copy numbers differed between DM cases and controls. These statistical analyses were performed using GRAPHPAD PRISM™ software.

Ethics

Dog samples were obtained with the owner's consent. The sampling conformed to the decision of the Swedish Animal Ethical Committee (no. C62/10) and the Swedish Animal Welfare Agency (no.31-1711/10).

Results

Serum amylase activity correlates with *AMY2B* copy number

AMY2B copy number and amylase activity were estimated in 55 dogs of 35 different breeds (Table S1 and Fig. S1). Amylase activity in serum varied widely throughout the samples. Median activity was 12.1 μkat/μl (IQR 8.6–17.3; range 4.9–34.5). We also observed considerable variation in *AMY2B* copy numbers among the 55 dogs. Mean diploid copy number was $2n = 10.3 \pm 2.5$ with individual values ranging from $2n = 4$ to $2n = 18$ (Table S1). Mixed linear regression modeling adjusting for breed showed a positive association of the number of *AMY2B* copies with ln amylase [$\beta = 0.05$ (95% CI, 0.1–0.9; P-value = 0.011)]. This corresponds to an increase of 5.4 percent in amylase activity for each extra copy. The copy number variation was estimated to explain 14.8% of the variance in amylase activity.

Amylase copy number variation within and among dog breeds

AMY2B copy numbers also varied considerably in a larger set of 171 dogs from 20 different breeds [eight dogs from each of 19 breeds (Table S2) and 19 Greenland Sledge dogs]. Mean diploid *AMY2B* copy number was $2n = 11.2 \pm 4.0$, and individual estimates ranged from $2n = 2$ to $2n = 21$. To investigate to what extent individual copy number variability depends on breed origin, we carried out a one-way ANOVA test. We found that copy numbers

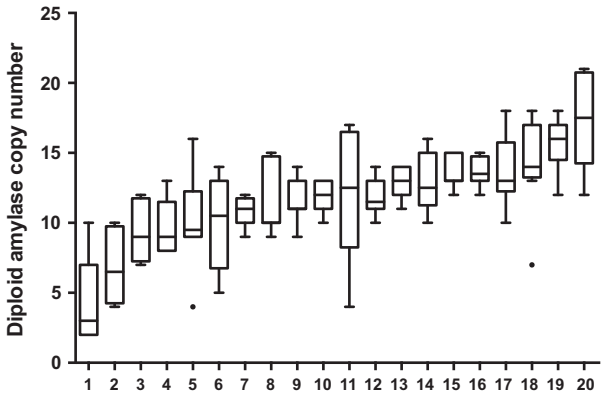

Figure 1 Tukey boxplot showing *AMY2B* copy number diversity in different dog breeds. Horizontal bars represent the median copy number within each breed. The 25th to 75th percentiles are boxed and the remaining distribution marked by a vertical line. Outside dots mark major outliers. 1 = Greenland Sledge Dog, 2 = Samoyed, 3 = Poodle, 4 = Shar Pei, 5 = Alaskan Malamute, 6 = Bearded Collie, 7 = PON, 8 = Drever, 9 = Boxer, 10 = Labrador, 11 = Beagle, 12 = Norwegian Elkhound, 13 = Border Collie, 14 = Golden Retriever, 15 = Rottweiler, 16 = Australian Shepherd, 17 = Swedish Elkhound, 18 = Duck Tolling Retriever, 19 = German Shepherd, 20 = English Springer Spaniel.

vary significantly among breeds and that nearly 70 percent of the individual variation can be attributed to breed ($P > 0.0001$, $R^2 = 0.67$) (Fig. 1 and Table S2). Among the 20 breeds analyzed here, *AMY2B* copy numbers were least abundant in Greenland Sledge dogs (mean diploid *AMY2B* copy number $2n = 4.3 \pm 2.7$), and we identified several Greenland Sledge dogs with only two *AMY2B* copies. To exclude a potential bias on the breed-based analysis due to this unusual copy number distribution, we excluded all Greenland Sledge dogs and reanalyzed the data. Mean copy numbers still varied significantly among breeds, and breed origin explained more than 50 percent of copy number variability ($P > 0.0001$, $R^2 = 0.51$). In addition to Greenland Sledge dogs, we also note that, in general, Samoyeds carry few *AMY2B* copies (mean diploid *AMY2B* copy number $2n = 6.9$, SD = 2.6), whereas German Shepherds (mean diploid *AMY2B* copy number $2n = 15.8$, SD = 1.9) and English Springer Spaniels (mean diploid *AMY2B* copy number $2n = 17.3$, SD = 3.4) consistently carry diploid copy numbers above 10. *AMY2B* copy number varied in all breeds studied (Table S2), and Beagles in particular are highly variable at this locus (mean diploid *AMY2B* copy number $2n = 11.9$, SD = 4.7), whereas Polish Lowland Sheepdogs (PON) are relatively homogenous (mean diploid *AMY2B* copy number 2n = 10.8, SD = 1.0).

Testing for an association between diabetes and *AMY2B*

Among the dogs analyzed above, reliable diabetes incidence rates have been estimated for 16 of 20 breeds (Fall *et al.* 2007; Table 1). To investigate a potential link between *AMY2B* copy number and susceptibility to DM, we first compared mean copy numbers and DM incidence across these breeds. We specifically note that incidence is high (ranked second) in Samoyeds, which generally carry few *AMY2B* copies; however, we see no general association between low copy numbers and high DM incidence when all breeds are analyzed jointly (Pearson's correlation test, one-tailed $P = 0.30$).

Table 1 Mean copy number and diabetes incidence per 10 000 dog years at risk (DYAR) for 16 dog breeds. Incidence values were published in Fall *et al.* (2007).

Breed	Mean *AMY2B* copy number	Diabetes incidence pr. 10 000 DYAR
Boxer	11.55	0
Bearded Collie	10.05	1
Golden Retriever	12.91	1
Poodle	9.451	2
Duck Tolling Retriever	14.25	3
German Shepherd	15.71	4
Labrador	11.86	13
English Springer Spaniel	17.28	13
Norwegian Elkhound	11.96	17
Rottweiler	13.66	23
Beagle	11.88	24
Drever	11.65	36
Border Collie	12.74	36
Swedish Elkhound	13.66	45
Samoyed	6.878	104
Australian Terrier	13.68	183

We then examined *AMY2B* copy numbers in eight DM cases and eight healthy controls from five different breeds (Samoyed, Australian Terrier, Border Collie, Swedish Elkhound and Norwegian Elkhound), all of which have shown an increased risk of developing DM (Fall *et al.* 2007; Catchpole *et al.* 2013). Although we note that *AMY2B* copy numbers on average tend to be lower in all cases ($2n = 11.4$, SD = 2.0, $n = 40$) than in all controls ($2n = 11.8$, SD = 2.853, $n = 40$, $P = 0.41$) and in four of five comparisons within breeds (Fig. 2, Table S3), no differences are significant. Furthermore, a two-way ANOVA analysis investigating the joint affects of breed origin and disease status indicates that breed origin accounts for 51.7 percent of the *AMY2B* copy number variation ($P < 0.0001$), whereas disease status is not related to copy number (proportion of variation explained = 0.45%, $P = 0.41$, Table S4).

Discussion

AMY2B copy number and serum amylase activity

Evidence for selection at the entire pathway responsible for starch digestion and glucose absorption indicates that efficient use of energy stored in starch was crucial to the survival and fitness of dogs during the domestication

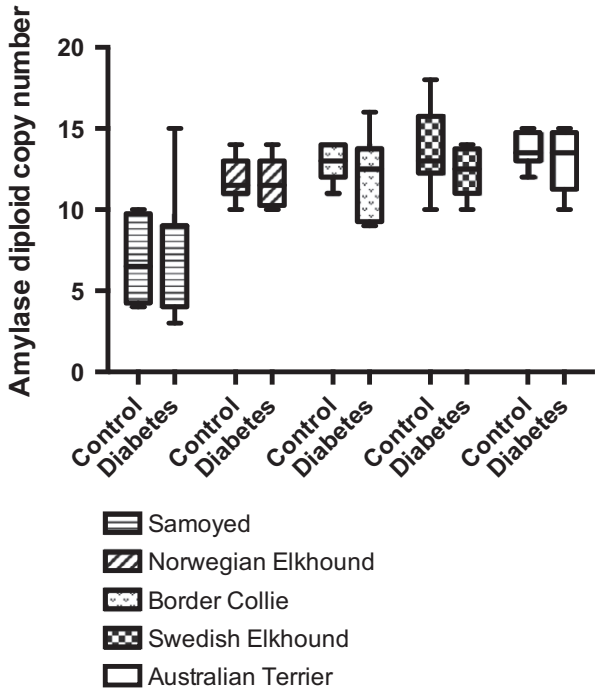

Figure 2 Tukey boxplot showing the distribution of *AMY2B* copy numbers in diabetic cases and healthy controls from 5 different high-risk dog breeds. The horizontal bar marks the median for each group. The 25th to 75th percentiles are boxed and the remaining distribution marked by a vertical line.

process (Axelsson *et al.* 2013). In the first step of this pathway, alpha-amylase initializes starch digestion by catalyzing the hydrolysis of starch to oligosaccharides maltose and maltriose. Increased amylase activity in dogs relative to wolves is associated with high *AMY2B* copy numbers in dogs, arguing that efficient starch digestion is linked to high copy numbers at this locus. However, this association has so far not been confirmed within the dog population (Axelsson *et al.* 2013). A lack of association may potentially question a causal link between copy number and amylase activity, or alternatively, reflect a combination of a limited sample size in the previous study, physiological effects on *AMY2B* activity (Swanson *et al.* 2000; Piccione *et al.* 2008; Nakajima *et al.* 2011a) and the limited precision of qPCR to accurately resolve high copy number gene duplications (Hindson *et al.* 2011). Independent of that, serum amylase activity measurements are currently of limited diagnostic value (Strombeck *et al.* 1981). To gain a better diagnostic use of this blood biochemistry analyte and to provide additional insight into the evolution of this trait during dog domestication, we measured *AMY2B* copy numbers and serum amylase activity in 55 dogs from a diverse set of breeds. We note that the variation in amylase activity throughout this sample (range 4.9–34.5 μkat/μl) largely recapitulates the wide reference values for serum amylase activity assays (the in-house laboratory normal reference values for the amylase test used were 5–25 μkat/μl). Similarly, in agreement with previous observations (Axelsson *et al.* 2013), it is also clear that *AMY2B* copy numbers vary substantially among individuals. By comparing amylase activity and *AMY2B* copy number, we then show that amylase activity increases linearly with copy number in this sample of dogs. Our result argue that starch digestion indeed is more efficient in dogs with many *AMY2B* copies compared with in individuals carrying few copies. This in turn strengthens the argument that selection for efficient starch digestion caused the increase in *AMY2B* copy numbers during dog domestication and that this change likely allowed dogs to thrive on a diet that was relatively rich in starch (Axelsson *et al.* 2013).

Although serum amylase activity thus depends on *AMY2B* copy number in dogs, only a relatively small proportion of the variation of serum amylase is explained by this variable ($R^2 = 14.8\%$), indicating that additional factors must be invoked to explain the majority of the variation. We can think of several such potential additional factors. First, the samples used in this study were taken from dogs that had blood samples taken for diagnostic purposes. Although no dogs that were suspected of having pancreatitis were included in this study, in principle, these dogs could be affected by other conditions that may affect serum amylase activity. Second, and probably more important, serum amylase activity is influenced by dietary habits, age and circadian rhythm, factors that have not been taken into account in this study (Piccione *et al.* 2008). Although serum amylase activity clearly is associated with *AMY2B* copy number in dogs, care should thus be taken at this point not to interpret activity measures as a direct indicator of inherited ability to handle starch. Future studies using larger cohorts under controlled settings may potentially allow us to establish copy-number-specific reference values for serum amylase activity that can be used to detect abnormal activity.

AMY2B copy number variability within and among breeds

In line with our previous observation, *AMY2B* copy numbers varied considerably among the 266 dogs analyzed here with individual measures ranging from $2n = 2$ to $2n = 21$. We note that the maximum copy number detected in this study ($2n = 21$) contrasts with our previous study in which the maximum *AMY2B* copy number was $2n = 30$. This discrepancy is likely due to the improved accuracy of ddPCR over traditional qPCR at resolving high copy number gene duplications, and $2n = 21$ likely represents the better estimate of the upper bound of the *AMY2B* copy number distribution in dogs.

AMY2B copy numbers vary significantly among breeds, and at least 50 percent of the individual copy number variability can be attributed to breed origin. This finding has

relevance for the use of serum amylase activity measures for diagnostic purposes, as it provides a first framework for interpreting activity measures based on a breed origin. A strikingly high amylase activity measure may be considered more unusual in, for instance, a Samoyed than an English Springer Spaniel. Differences in *AMY2B* copy numbers among breeds are expected based on the highly divergent demographic histories for several of the breeds studied here. Bottlenecks associated with the creation of breeds likely resulted in a random inclusion of small subsets of the entire range of *AMY2B* haplotypes into different breeds. This effect is likely reflected in our observations of restricted variation in *AMY2B* copy numbers in PON, whereas Beagles display a much wider copy number range. The PON breed was almost extinct around 1950 with only a few individuals serving as founders for the population we see today (Augustowska 2007), whereas the Beagle breed is based on a larger founder population and subdivided into working dogs and laboratory dogs, which adds to a larger expected diversity in this breed.

In addition to these demographic effects, based on the strong evidence for selection for increased *AMY2B* copy numbers during dog domestication, it is also tempting to speculate that selection has continued to mold copy number variation among dog breeds. Among the breeds analyzed here, we find two breeds, Greenland Sledge dogs and Samoyeds, with markedly lower copy numbers compared with other breeds. The Samoyed presumably represents an old breed that was developed among hunting and pastoralist populations in Siberia. Greenland Sledge dogs represent a breed that through many years has been isolated geographically and, more recently by laws, from other dog breeds. Both of these breeds have probably relied on a largely protein-based diet, including meat and fish. It is thus possible that the relatively low *AMY2B* copy numbers reflect a relaxation of the directional selections at this locus in these breeds. However, we cannot rule out that the observation of several Greenland Sledge dogs with a wolf-like haplotype in this study is the result of recent cross-breeding between wolves and sledge dogs, a practice that has been documented historically (Walker & Frison 1982). A more detailed analysis of dogs from traditionally protein-based vs. starch-based food cultures is needed to understand whether selection may have contributed to breed differences in average *AMY2B* copy numbers.

AMY2B copy number and DM

In humans, high salivary amylase activities have been associated with a rapid insulin response that in turn results in a rapid reduction in blood glucose levels (Mandel & Breslin 2012). This association may hint at a possible association between *AMY2B* copy numbers and risk of developing DM. In this study, however, we do not find any association between DM and *AMY2B* copy numbers in dogs, neither when comparing mean copy numbers in breeds with varying DM incidence nor when comparing cases and controls. Although these observations may rule out *AMY2B* copy number as a strong monogenic risk factor for DM in dogs, it is premature to rule out a link due to several factors. First, onset of DM is likely triggered by a multitude of factors including several environmental factors, indicating that the effect of individual factors, such as potentially *AMY2B* copy number, may be small. Furthermore, in regard to this multifactorial nature of DM susceptibility, it is very likely that the limited sample size of our case–control comparisons is insufficient to detect a risk allele with a weaker effect within a multigenic disease. Finally, incidence values used in this study represent all diagnosed cases of diabetes without distinguishing particular subtypes. Specific effects of *AMY2B* copy number on a particular diabetes subtype within individual breeds may hence go undetected in this comparison. A lack of association between DM incidence rates and mean *AMY2B* copy numbers among breeds could thus reflect the diversity of DM types involved in this comparison. Future studies including a larger collection of carefully characterized cases and controls should help disentangle these possibilities in more detail.

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Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1. *AMY2B* copy number variation relative to serum amylase activity. Scatter plot showing the relationship between *AMY2B* copy number and serum amylase activity.

Table S1. Breed, serum amylase activity and diploid *AMY2B* copy number for the 55 dogs used to test for an association between *AMY2B* copy number and serum amylase activity.

Table S2. Distribution of diploid *AMY2B* copy numbers in 20 different dog breeds

Table S3. Mean diploid *AMY2B* copy number in diabetic mellitus cases and controls respectively for five dog breeds with high risk of developing diabetes.

Table S4. Summary of the two-way ANOVA statistics analyzing how much of the variation in diploid *AMY2B* copy numbers is explained by diabetes and breed respectively.

