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Variation in pancreatic amylase gene copy number among Eurasian badgers (Carnivora, Mustelidae, *Meles*) and its relationship to diet

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amylase gene; Eurasian badger; copy number variation; diet; *Meles*; mustelids.

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Abstract

Occurrence of copy number variation (CNV) of gene loci is important in the adaptation of species and populations to their environments. High copy numbers of mammalian genes encoding the digestive enzyme amylase, which plays a role in starch digestion, have been associated with adaptation to high-starch foods. Here, we investigated CNV in the pancreatic amylase gene (*AMY2*) in Eurasian badgers (*Meles* species) and related taxa using real-time quantitative PCR. We found that *Meles anakuma* and *M. meles*, as well as the other mustelids *Mustela sibirica*, *Martes melampus* and *Martes zibellina*, had only one copy of the *AMY2* locus, whereas in *Meles leucurus* the copy number varied between one and four. The *AMY2* copy number also varied significantly among *M. leucurus* populations. This suggests that *M. leucurus* is better adapted to a diet rich in starch and/or glycogen than its congeners. As *Meles* spp. are basically omnivorous but favor a carnivoran diet, for example, earthworms and amphibians, we speculate that the CNV is related to the dearth of animal food in the range of *M. leucurus*, which enforces a more vegetal diet. This in turn would favor more efficient digestion of dietary starch, which could be achieved by increasing the copy number of *AMY2* .

Introduction

Aside from basic genetic variation in individual genes, such as single nucleotide polymorphisms (SNPs) and other allelic variation, differences in the number of repeated copies of individual genes, termed copy number variation (CNV) or copy number polymorphism (CNP), have emerged as an important component in the structural and functional variation of genomes. CNV was defined as losses or gains of a DNA segment of at least 50 base pairs (bp) or larger, compared with a reference genome (Alkan, Coe & Eichler, 2011; Mills *et al.*, 2011). CNV, which is a genomic region that differ in copy number between individuals within species due to duplication or deletion, has been recorded in a wide diversity of taxa (Schrider & Hahn, 2010). In a human study, CNV accounted for more than 17% of the total genetic variation in gene expression (Stranger *et al.*, 2007). Both recombination- and replication-based mechanisms can account for CNV formation. CNV can cause Mendelian or sporadic traits, or be associated with complex diseases, by various molecular mechanisms (Zhang *et al.*, 2009). CNV is also important in understanding the relationship between genotype and phenotype, and has been examined at the population-genetic level across diverse populations (Xu *et al.*, 2016).

Amylase catalyzes the hydrolysis of starch, breaking it into the oligosaccharides maltose and maltotriose in the oral cavity

and stomach. Starch digestion continues in the intestine to produce glucose for absorption into the bloodstream. Generally, two types of amylase occur in mammals: salivary amylase (*AMY1*) is produced abundantly orally in saliva (Noble, 2000), whereas pancreatic amylase (*AMY2*) is produced extensively in the pancreas and secreted into the small intestine (McGeachin & Akin, 1979, 1982). These enzymes are encoded by amylase genes located on chromosome 3 in cows (Laurent *et al.*, 1999), chromosome 13 in goats and chromosome 1 in humans (Dracopoli & Meisler, 1990; Schibler *et al.*, 1998). High CNV in *AMY1* genes has been reported in humans (Bank *et al.*, 1992; Samuelson, Phillips & Swanberg, 1996). Intriguingly, domestic dogs, which do not express amylase in the saliva (Simpson, Doxey & Brown, 1984), although Contreras-Aguilar *et al.* (2017) reported very low activities of alpha-amylase in a limited number of dogs, have multiple copies of *AMY2* (Axelsson *et al.*, 2013; Tonoike *et al.*, 2015). High copy numbers of amylase genes are associated with high levels of amylase expression and activity (Perry *et al.*, 2007; Axelsson *et al.*, 2013; Arendt *et al.*, 2014), and in dogs and humans CNV is presumed to be an adaptation to the starch-rich diet (Perry *et al.*, 2007; Arendt *et al.*, 2014; Tonoike *et al.*, 2015) resulting from the development of agriculture.

Eurasian badgers are mustelid carnivores in the genus *Meles*, which is distributed across most of the Palearctic, ranging from the British Isles to Japan. Both morphological and molecular studies recognize four distinct species, *M. meles*, *M. leucurus*, *M. canescens* and *M. anakuma* (Abramov & Puzachenko, 2013; Sato, 2016). The molecular evolution of functional genes among these species has so far been studied by means of the MHC complex involved in the immune system (Abduriyim *et al.*, 2017, 2018). While *Meles* is classified in the Carnivora, ecologically they are omnivores, feeding on both plants and animals (Table 1). Comparative studies have shown that omnivorous species tend to have higher copy numbers of amylase genes than do purely carnivorous taxa (Boehlke, Zierau & Hanning, 2015; German *et al.*, 2016; Kim *et al.*, 2016;). The vegetal component of the *Meles* diet includes bulbs, roots, tubers, nuts and fruits as well as seeds, cereals and maize (Roper & Michevicius, 1995; Remonti, Balestrieri & Prigioni, 2011; Li *et al.*, 2013), which tend to be rich in starches. The adaptation modulation hypothesis (Karasov, 1992), which derived from and is grounded in chemical reactor theory, postulates that the digestive enzyme activities should be modulated in response to intake of respective substrates included in their foods (Karasov & Douglas, 2013). Therefore, the aim of this study was to examine CNV in the *AMY2* amylase gene across several *Meles* species by analyzing a number of samples from each locality. To the best of our knowledge, no amylase gene studies were reported in mustelid species so far. Amylase gene studies were mainly focused on model species, including humans and domestic dogs, that have shown high copy number variation in amylase genes. The present study is the first attempt in wild mammal species to date. Domestic dogs were more likely not to express amylase in saliva. The same likely goes for other carnivoran species. Hence, we investigated

Table 1 Summary of occurance frequency (%) of different foods in the diet of four *Meles* species, according to Roper & Michevicius (1995), Kaneko *et al.* (2006), and Li *et al.* (2013).

Species	Food items											
	Earthworms	Mammals	Birds	Insects	Amphibian	Fishes	Reptiles	Fruits	Mollusks	Roots[a]	Cereals	Others
Meles meles	0–96.3	1.4–83.9	0–35.0	10.0–100	0–88.0	0–60.0	0–25.0	!–100	0–25.0	2.6–92.1	0–81.1[b]	!–3.5
M. canescens	!–7.0	3.0–36.0	0–38.0	27.0–100	0–98.0	!	0–70.0	!–27.0	!	!–24.0	!–19.0	!
M. leucurus	0–96.9	4.4–79.2	3.9–33.3	8.7–100	0–30.4	0–20.7	0–12.5	5.11–100	!–0.4	!–13.0	0–?	!
M. anakuma	10.0–85.7	0–14.3	0–28.6	4.5–84.2	0–4.8	!	!	5.0–68.4	0–15.8	9.1–89.5	!	5.0–47.6

[a]Includes grass, leaves, tubers and bulbs.
[b]Cereals were eaten by only populations in arable and agricultural regions (Remonti *et al.*, 2011).
! Indicates that it was not mentioned.

pancreatic amylase genes in several mustelid species, mainly focusing in Eurasian badgers, *Meles*. Knowledge of CNV in the amylase gene to some extent helps elucidate the food ecology and provides fundamental knowledge about adaptation of *Meles* species.

Materials and methods

Samples

In all, 86 skin or other tissue samples from the four species of Eurasian badgers were used for the analysis: *M. meles* ($N = 21$) from Bulgaria and Finland, *M. anakuma* ($N = 20$) from the Japanese Islands, *M. canescens* ($N = 2$) from Armenia and *M. leucurus* ($N = 43$) from Russia (Table 1). For comparison, we included three other mustelid species, *Mustela sibirica* ($N = 6$), *Martes melampus* ($N = 6$) and *Martes zibellina* ($N = 5$). Small pieces of tissue samples were obtained from incidental roadkills, while a few dried skin samples were obtained from museum specimens. All tissues were preserved in 99.5% ethanol at 4°C until DNA extraction.

Primer design

Primers for the amplification of the target *AMY2* sequences in *Meles* were initially designed from an alignment of carnivoran sequences available from GenBank, including *Panthera pardus* (accession number XM_019457671), *Panthera tigris* (XR_446458 and XM_015542658), *Mustela putorius* (XM_004780129), *Enhydra lutris* (XM_004780129), *Neomonachus schauinslandi* (XM_021690215), *Leptonychotes weddellii* (XM_021690215 and XM_006728164) and *Odobenus rosmarus* (XM_006728164). The primer sites corresponding to the most highly conserved segments in exon 4 allowed amplification of a 223-bp fragment (including the primers) spanning most of the exon. Forward primer MusAMYex4F-Long (TGT CGT CTG GTT GGT CTT CTT GAT CTT) and reverse primer MusAMYex4R (CCT CCT GGT AAA TGA AAG GTT TAC) were used for PCR amplification of genomic DNA from several mustelid species, and the amplicons were sequenced. From an alignment of sequences obtained from *Mustela*, *Martes* and *Meles* species, we then designed another forward primer, MusAMYex4F (GCA GGG TTC AGA ATT GAT GCT TCT AAG), for real time quantitative PCR (qPCR) reactions. The primer pair MusAMYex4F and MusAMYex4R amplifies 127 bp of qPCR product, fulfilling the requirement of primer specificity in qPCR (D'haene, Vandesompele & Hellemans, 2010).

As a reference gene, we used the albumin gene, which is a single-copy gene (Hawkins & Dugaiczyk, 1982; Fanali *et al.*, 2012). Based on the serum albumin gene sequences from two mustelid species, *Mustela putorius* (GenBank NW_004569262) and *Enhydra lutris* (NW_019154105), and canid and felid sequences, we designed the primer pair M.furoALB-F (forward; GCT GGC TGA CTG CTG TGA GAA ACA GG) and M-ALB-R (reverse; GCA GTG CAC ATG ACC TCA G), which amplifies a 120-bp region of the serum albumin gene. The specificity of the primer sets, both for the reference gene and the target gene, was assessed within mustelids, first by NCBI primer-blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and then further by experiments.

DNA extraction and qPCR profiling

Total DNA was isolated from samples using the DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's protocol. After measuring the concentration, total DNA was stored at 4°C in TE buffer until further use.

For qPCR, reactions were performed in 96-well plates sealed with a microplate seal in a Thermal Cycler Dice® Real Time System II (Takara, Japan). Reactions were conducted in 20 μL volumes, each containing 10 μL of KAPA SYBR® Fast qPCR MasterMix (2×) Universal, 1.5 pmol of each primer, and <30 ng of DNA (the same amount of DNA was used for reference and target genes of every single individual), with the rest of the volume made up with sterile distilled water. Thermal cycling conditions were 5 min at 95°C; 40 cycles of 5 s at 95°C and 30 s at 60°C (fluorescence data collected); and a final melting curve analysis comprising 15 s at 95°C, 30 s at 60°C and 15 s at 95°C (fluorescence data collected) for checking the primer specificity. Three replicate reactions for both the target and reference genes were performed for each sample.

Data processing and analyses

Raw data were processed with the software provided with qPCR Real Time System II (Takara) using the baseline subtraction and linear regression settings. To calculate the relative amylase gene copy number using the albumin gene as a reference, we applied the Pfaffl (2001) method, which considers the qPCR amplification efficiency (E). To calculate E, we established a 1:2 dilution calibration curve, which provides a rapid, simple, reproducible indication of E, the robustness of the assay, and the analytical sensitivity (Bustin *et al.*, 2009). E values between 90% and 110% were accepted for calculations (D'haene *et al.*, 2010). The threshold between copy numbers was determined to be their geometric mean (D'haene *et al.*, 2010). One-way analysis of variance (ANOVA) was used to test whether the mean *AMY2* copy number differed between the species studied and between geographic populations within a species. Statistical analyses were performed in R version 3.3.3 (R Core Team, 2017).

Results

The melting-curve analysis of qPCR products showed a single first-derivative melting peak, indicating high specificity for the primers. E values in qPCR reactions ranged from 91% to 106%. We presented the results of the analysis as haploid copy number (n) per individual. The copy number of the amylase gene in *Mustela sibirica*, *Martes melampus*, *Ma. zibellina*, *Meles meles* and *Meles anakuma* was uniformly one ($n = 1$), except for a few outliers in *M. meles* and *M. anakuma* (Table 2). By contrast, *Meles leucurus* showed from one to

Table 2 Profiles of samples investigated in this study

Species	Individual ID	Locality	HCN	Value	SD
Mustela sibirica	MSI-KT12	Japan	1	0.74	0.09
N = 6	MSI-364684	Russia	1	0.62	0.02
	MSI-rus25	Russia	1	0.71	0.05
	MSI-rus14	Russia	1	0.69	0.09
	MSI-rus23	Russia	1	0.66	0.02
	MSI-rus24	Russia	1	0.71	0.09
Martes melampus	MME-TSU61	Japan	1	0.68	0.07
N = 6	MME-G1	Japan	1	1.03	0.05
	MME-TSU7	Japan	1	0.84	0.05
	MME-K1	Japan	1	1.05	0.15
	MME-K2	Japan	1	0.93	0.08
	MME-K17	Japan	1	1.00	0.06
Martes zibellina	MZ14	Japan	1	0.92	0.12
N = 5	MZ16	Japan	1	0.89	0.05
	MZ20	Japan	1	0.95	0.16
	MZ55	Japan	1	0.66	0.03
	MZ74	Japan	1	0.34	0.03
Meles meles	MEL-2011-68	Bulgaria	2	1.45	0.28
N = 21	MEL-2011-101	Bulgaria	1	1.13	0.08
	MEL-2011-110	Bulgaria	1	0.83	0.04
	MEL-2011-112	Bulgaria	1	0.60	0.04
	MEL-2011-124	Bulgaria	2	1.86	0.08
	MEL-2011-138	Bulgaria	1	1.05	0.07
	MEL-2012-2	Bulgaria	2	1.62	0.15
	MEL-2012-7	Bulgaria	1	1.20	0.06
	MEL-2012-28	Bulgaria	1	1.33	0.14
	MEL-2012-35	Bulgaria	1	0.92	0.09
	MEL-2012-37	Bulgaria	1	0.91	0.06
	MEL-2012-39	Bulgaria	1	1.16	0.06
	MEL-2012-40	Bulgaria	1	1.20	0.03
	MEL-2012-53	Bulgaria	1	1.19	0.04
	MEL-2013-6	Bulgaria	1	0.95	0.05
	MEL-14-01	Bulgaria	1	1.00	0.05
	KS.KN46943	Finland	1	0.66	0.06
	KS.KN49014	Finland	1	1.37	0.16
	KS.KN49015	Finland	1	0.90	0.28
	KS.KN49016	Finland	1	0.84	0.02
	KS.KN49050	Finland	1	1.07	0.10
Meles anakuma	MEL-2	Japan	1	0.67	0.06
N = 20	HB990011	Japan	1	0.88	0.07
	MEL-MR-Liver	Japan	1	0.61	0.14
	TB06720	Japan	1	0.92	0.18
	HB99299	Japan	2	2.13	0.60
	HB99002	Japan	1	0.79	0.10
	HB05725	Japan	1	0.69	0.08
	HB05630	Japan	1	0.62	0.06
	H13-009	Japan	1	0.78	0.04
	H13-075	Japan	1	0.75	0.04
	H16-017	Japan	1	0.60	0.09
	H17-004	Japan	1	0.67	0.01
	H17-113	Japan	1	0.73	0.03
	H18-178	Japan	1	0.70	0.09
	MEL-K11	Japan	1	0.76	0.18
	MEL-K12	Japan	2	1.60	0.12
	MEL-K15	Japan	1	0.88	0.07
	MEL-K7	Japan	1	0.76	0.04
	MEL-K8	Japan	1	0.69	0.03
	MEL-K9	Japan	1	0.90	0.07

Table 2 Continued.

Species	Individual ID	Locality	HCN	Value	SD
Meles leucurus	Kir-70	Novosibirsk, Russia	2	1.48	0.33
N = 43	Kir-71	Novosibirsk, Russia	4	3.87	0.20
	Kir-72	Novosibirsk, Russia	2	1.80	0.46
	Kir-73	Novosibirsk, Russia	3	2.55	0.66
	Kir-74	Novosibirsk, Russia	2	2.21	0.32
	Kir-75	Novosibirsk, Russia	3	2.84	0.24
	Kir-76	Novosibirsk, Russia	2	1.97	0.24
	Kir-77	Novosibirsk, Russia	2	2.21	0.70
	Kir-78	Novosibirsk, Russia	2	2.07	0.49
	Kir-79	Novosibirsk, Russia	2	1.50	0.08
	452-478	Middle Urals, Russia	3	2.77	0.07
	452-634	Middle Urals, Russia	2	1.96	0.19
	416-829	Middle Urals, Russia	2	2.40	0.78
	364-617	Middle Urals, Russia	1	1.19	0.17
	364-618	Middle Urals, Russia	1	0.66	0.17
	364-619	Middle Urals, Russia	2	1.82	0.13
	364-620	Middle Urals, Russia	1	1.41	0.19
	364-621	Middle Urals, Russia	2	1.53	0.16
	452-479	Middle Urals, Russia	1	0.73	0.05
	IEPAE489-744	Kurgan, Russia	1	1.38	0.06
	IEPAE489-745	Kurgan, Russia	2	2.33	0.13
	IEPAE489-746	Kurgan, Russia	2	2.00	0.27
	IEPAE489-747	Kurgan, Russia	3	2.89	0.24
	IEPAE489-748	Kurgan, Russia	2	1.98	0.25
	IEPAE489-750	Kurgan, Russia	1	0.68	0.08
	IEPAE489-751	Kurgan, Russia	1	1.16	0.09
	IEPAE489-752	Kurgan, Russia	2	1.79	0.10
	IEPAE489-753	Kurgan, Russia	2	1.64	0.04
	IEPAE489-754	Kurgan, Russia	2	1.87	0.15
	IEPAE489-755	Kurgan, Russia	2	1.47	0.15
	1265	Primorsky Krai, Russia	4	4.26	0.11
	1652	Primorsky Krai, Russia	3	3.23	0.24
	1696	Primorsky Krai, Russia	3	2.67	0.12
	2029	Primorsky Krai, Russia	3	2.45	0.53
	2030	Primorsky Krai, Russia	3	2.99	0.17
	2031	Primorsky Krai, Russia	3	2.59	0.17
	2188	Primorsky Krai, Russia	3	2.87	0.09
	1833	Primorsky Krai, Russia	3	2.71	0.24
	1749	Primorsky Krai, Russia	4	3.62	0.32
	1833	Primorsky Krai, Russia	2	1.85	0.23
	1747	Primorsky Krai, Russia	3	3.19	0.13
	1748	Primorsky Krai, Russia	1	0.81	0.78
	2222	Primorsky Krai, Russia	4	4.41	0.15
Meles canescens	MEL-AR2	Armenia	2	1.94	0.23
	MEL-AR1	Armenia	3	3.12	0.12

HCN, haploid copy number (*n*) which was calculated by rounding off from value column; SD, standard deviation.

four copies among the individuals analyzed, and the two *Meles canescens* individuals had two and three copies, respectively (Table 2).

The ANOVA analysis (using HCN column values in Table 2) indicated that the amylase gene copy number in *Meles leucurus* was significantly different from the other species studied, with an average copy number of 2.28 (Fig. 1). The copy number also varied between geographic populations of *M. leucurus*: an average of 3.00 copies in the Primorsky Krai population, 2.40 in the Novosibirsk population, 1.82 in the Kurgan population, and 1.67 in the Middle Urals population (Fig. 2).

Discussion

The demonstration by Axelsson *et al.* (2013) and Arendt *et al.* (2014) that selection had targeted a duplication in the gene coding for pancreatic amylase (*AMY2*) in the ancestor of the

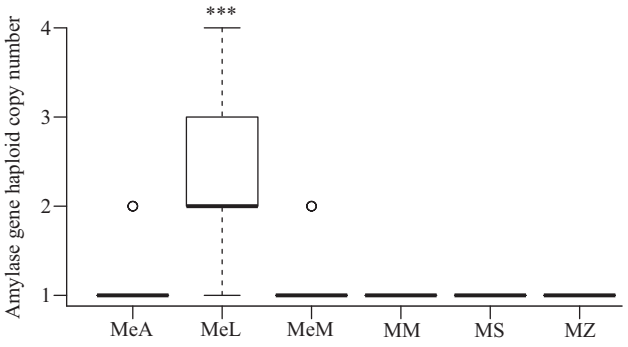

Figure 1 Tukey boxplot showing variation in copy number for the pancreatic amylase (*AMY2*) gene in Eurasian badgers, weasel, sable and marten. Horizontal bars indicate the median copy number; small circles indicate major outliers. ***Significance level $P < 0.001$. MeA, *Meles anakuma*; MeL, *Meles leucurus*; MeM, *Meles meles*; MM, *Martes melampus*; MS, *Mustela siberica* and MZ, *Martes zibellina*.

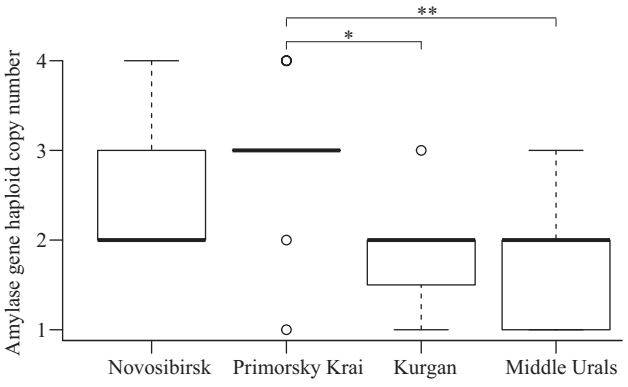

Figure 2 Tukey boxplot showing variation in copy number for the pancreatic amylase (*AMY2*) gene in Asian badger (*Meles leucurus*) populations. Horizontal bars indicate the median copy number; small circles indicate major outliers. *Significance level $P < 0.05$; **, $P < 0.01$.

modern dog, and that high copy number in the *AMY2* gene was an adaptation to a starch-rich diet during the common history of human and dogs, has provided an outstanding example of fast genomic adaptation to evolving dietary conditions. Our observations on amylase CNV among and within species of mustelids further support a relationship between gene copy number and diet.

From our qPCR data, the carnivorous mustelids *Mustela sibirica*, *Martes melampus* and *M. zibellina* have only one copy of *AMY2*. Likewise, genome-wide sequencing has shown that *AMY2* is a single-copy ($n = 1$) gene in hyper-carnivorous taxa such as the tiger (*Panthera tigris*), lion (*Panthera leo*), cheetah (*Acinonyx jubatus*), leopard (*Panthera pardus*) and cat (*Felis catus*) (Kim *et al.*, 2016), whose diets do not necessarily contain starch. Our results agree with the purported diet composition of *Mustela* and *Martes* species, where starch is not a major component (Heptner *et al.*, 1967; McDonald, Webbon & Harris, 2000; Sato, 2016).

For *Meles*, our results indicate a single copy of *AMY2* in the European *M. meles* and Japanese *M. anakuma*, but one to four copies in *M. leucurus* (Table 2, Fig. 1). This may be related to the feeding habits of these taxa. Diverse dietary compositions, geographically different between species and between populations, have previously been reported for *Meles* spp. The diet of *M. anakuma* includes plant matter such as roots, grasses, fruits, etc. that contain <10% starch (Remonti *et al.*, 2011), and the occurrence of these food items is seasonally variable, varying from 0% to 90% (Table 1; Kaneko, Maruyama & MacDonald, 2006). Similarly, the *M. meles* diet varies greatly among locations and habitat types (Heptner *et al.*, 1967; Roper & Michevicius, 1995; Remonti *et al.*, 2011; Li *et al.*, 2013). Diets including seeds, cereals, etc. with high starch content have been documented only in arable or agricultural areas (Table 1). One of the *M. meles* samples with a single copy of the *AMY2* locus in our study (Table 2, Fig. 1) was from Finland, where the proportion of plant material in the summer diet is low (Kauhala, Laukkanen & von Rége, 1998). While there are no data on diet of *M. meles* in the Bulgarian population, the single copy in most individuals suggests that vegetal foods rich in starch are uncommon or not much utilized. *Meles canescens* shows higher *AMY2* copy numbers (Table 2), but the sample size (two individuals) was low and additional sampling is needed to clarify the CNV in this species.

The Asian badger, *M. leucurus*, showed higher *AMY2* copy numbers than *M. anakuma* or *M. meles* (Table 2, Fig. 1). Elevated gene copy number seems to correlate with increased mRNA level and consequent elevation of amylase activities in many mammal taxa: humans (Perry *et al.*, 2007), mice (Sugino, 2007) and dogs (Axelsson *et al.*, 2013). In dogs, it has particularly been estimated that each additional copy of the amylase gene gives a 5.4% increase in serum amylase activity (Arendt *et al.*, 2014). Taking adaptive modulation hypothesis, a match (linear relationship) between digestive enzyme activities and dietary substrate intake (Karasov & Douglas, 2013), into consideration, these indicate that *M. leucurus* is to some extent adapted to a starchier diet than the other two species. In support of this conclusion, Roper & Michevicius (1995) found *M. luecurus* to consume cereals crops, fruits and roots containing starch. In Western Siberia, pine seeds and berries containing ≤5% starch comprised 100% of the diet of *M. leucurus* in spring and 13–83% in autumn, during the 4 years in which both seasons were studied (Zagainova & Markov, 2011), indicating these foods low in starch were regular dietary items in this species. Intriguingly, among *M. leucurus* populations, the Primorsky Krai population was found to have one *AMY2* copy more than the Kurgan and Middle Urals populations (Fig. 2), suggesting adaptation to starchy foods in this region. Though there are no direct data on the proportion of high-starch foods consumed by this population, two studies conducted in the Russian Far East, in which the Primorsky Krai population is located, report the utilization of cereal crops rich in starch (reviewed by Roper & Michevicius, 1995).

Global dietary analyses have shown that *Meles* species are generalist predators with opportunistic foraging behavior (Roper & Michevicius, 1995; Li *et al.*, 2013), consuming plant foods when animal prey is unavailable. Qualitative reports

have shown animal prey such as small mammals, birds, reptiles, amphibians and earthworms to be absent in the *M. leucrus* diet, especially in the Primorsky Krai population (Baranchecv, 1959; Kolosov, 1980; Roper & Michevicius, 1995). We hypothesize that the decline, unavailability or fluctuation of animal prey across the range of *M. leucurus* over a long period, perhaps even over evolutionary time, has necessitated an increase in the use of vegetal foods, including seeds and cereals rich in starch, and that an increase in *AMY2* copy number is an adaptation to this increased vegetal consumption. The available diet may differ from population to population, or vary through time, and this in turn could have resulted in differential *AMY2* copy number expansion among *Meles leucurus* populations.

Finally, compared to other *Meles* species, *M. leucurus*, with its distribution in Russia, lives under extreme conditions, including a long period of snow cover. Hence this species has the longest hibernation period. When animals leave their setts in the spring, they encounter a paucity of animal feed, with no availability of insects and earthworms, which are easily preyed upon. For one or two weeks, they must rely exclusively on vegetal foods. For example, in Tatarstan at the western edge of the distributional range, when the badgers leave their burrows, they actively search for and consume the tubers of the fumewort plant (*Cordyalis solida*), Papaveraceae, and other spring ephemerals (Gorshkov, 1997; Solovyev, Yuferev & Maltsev, 2007). Individuals or populations that have a diversified physiology, that is, the ability to digest vegetal foods, during this period of "ecological minimum" are likely better adapted to survive in this extreme (and most importantly, pre-breeding) period. This idea can be tested by examining the correlation between various ecological factors and amylase copy-number variation in Eurasian badgers across their entire ranges.

Amylase and amylase gene copy number certainly related to starchy diet in model species, nonetheless humans, mice and dogs, often share a range of livelihood and foods, are far related to *Meles* species. High amylase gene copy number cannot always well explain increased enzyme activity due to the action of a transcriptional control mechanism etc. that can autonomously suppress excess mRNA transcription, for example, salivary amylase in human (Carpenter, Mitchell & Armour, 2017) and amylase in fishes (German *et al.*, 2016). In addition, scarce knowledge of physiology is known in *Meles* species, and there is not a solid evidence how much volume of starchy food is consumed by *Meles* species, especially *M. leucurus* species. Thus, more detailed studies are necessary to understand evolutionary importance of the observed higher copy number and CNV in *Meles* species, *M. leucurus* in particular, and to test our speculation.

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References

Abduriyim, S., Nishita, Y., Kosintsev, P.A., Raichev, E., Väinölä, R., Kryukov, A.P., Abramov, A.V., Kaneko, Y. & Masuda, R. (2017). Diversity and evolution of MHC class II *DRB* gene in the Eurasian badger genus *Meles* (Mammalia: Mustelidae). *Biol. J. Linn. Soc.* **112**, 258.

Abduriyim, S., Nishita, Y., Kosintsev, P.A., Raichev, E., Väinölä, R., Kryukov, A.P., Abramov, A.V., Kaneko, Y. & Masuda, R. (2018). Evolution of MHC class I genes in Eurasian badgers, genus *Meles* (Carnivora, Mustelidae). *Heredity* [Epub ahead of print]. https://doi.org/10.1038/s41437-018-0100-3.

Abramov, A.V. & Puzachenko, A.Y. (2013). The taxonomic status of badgers (Mammalia, Mustelidae) from Southwest Asia based on cranial morphometrics, with the redescription of *Meles canescens*. *Zootaxa* **3681**, 44.

Alkan, C., Coe, B.P. & Eichler, E.E. (2011). Genome structural variation discovery and genotyping. *Nature Rev. Genet.* **12**, 363.

Arendt, M., Fall, T., Lindblad-Toh, K. & Axelsson, E. (2014). Amylase activity is associated with AMY2B copy numbers in dog: implications for dog domestication, diet and diabetes. *Anim. Genet.* **45**, 716.

Axelsson, E., Ratnakumar, A., Arendt, M.L., Maqbool, K., Webster, M.T., Perloski, M., Liberg, O., Arnemo, J.M., Hedhammar, Å. & Lindblad-Toh, K. (2013). The genomic signature of dog domestication reveals adaptation to a starch-rich diet. *Nature* **495**, 360.

Bank, R.A., Hettema, E.H., Muijs, M.A., Pals, G., Arwert, F., Boomsma, D.I. & Pronk, J.C. (1992). Variation in gene copy number and polymorphism of the human salivary amylase isoenzyme system in Caucasians. *Hum. Genet.* **89**, 213.

Baranchecv, L.M. (1959) The Badger. In *Prioroda Amurskoi Oblasti*: 232–233. Blagoveshchensk: Amurskoe Knizhnoe, Izdatelstvo.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. & Wittwer, C.T. (2009). The MIQE guidelines: minimum Information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**, 611.

Boehlke, C., Zierau, O. & Hanning, C. (2015). Salivary amylase - The enzyme of unspecialized euryphagous animals. *Arch. Oral Biol.* **60**, 1162.

Carpenter, D., Mitchell, L.M. & Armour, J.A.L. (2017). Copy number variation of human AMY1 is a minor contributor to variation in salivary amylase expression and activity. *Human Genomics* **11**, 2.

Contreras-Aguilar, M.D., Tecles, F., Martínez-Subiela, S., Escribano, D., Bernal, L.J. & Cerón, J.J. (2017). Detection and measurement of alphaamylase in canine saliva and changes after an experimentally induced sympathetic activation. *BMC Vet. Res.* **13**, 266.

D'haene, B., Vandesompele, J. & Hellemans, J. (2010). Accurate and objective copy number profiling using real-time quantitative PCR. *Methods* **50**, 262.

Dracopoli, N.C. & Meisler, M.H. (1990). Mapping the human amylase gene cluster on the proximal short arm of chromosome 1 using a highly informative (CA)nrepeat. *Genomics* **7**, 97.

Fanali, G., Di Masi, A., Trezza, V., Marino, M., Fasano, M. & Ascenzi, P. (2012). Human serum albumin: from bench to bedside. *Mol. Aspects Med.* **33**, 209.

German, D.P., Foti, D.M., Heras, J., Amerkhanian, H. & Lockwood, B.L. (2016). Elevated gene copy number does not always explain elevated amylase activities in fishes. *Physiol. Biochem. Zool.* **89**, 277.

Gorshkov, P.K. (1997). *Badger in the biocenoses of the Republic of Tatarstan*. Kazan: Tabigat Publications. [in Russian].

Hawkins, J.W. & Dugaiczyk, A. (1982). The human serum albumin gene: structure of a unique locus. *Gene* **19**, 55.

Heptner, V.G., Naumov, N.P., Yurgenson, P.B., Sludskiy, A.A., Chirkova, A.F. & Bannikov, A.G. (1967). *Mammals of Soviet Union, Vol. 2 (1) Sea cows and Carnivora*. Moscow: Vysshaya Shkola.

Kaneko, Y., Maruyama, N. & MacDonald, D.W. (2006). Food habits and habitat selection of suburban badgers (*Meles meles*) in Japan. *J. Zool.* **270**, 78.

Karasov, W.H. (1992). Test of the adaptive modulation hypothesis for dietary control of intestinal transport. *Am. J. Physiol.* **263**, R496–R502.

Karasov, W.H. & Douglas, A.E. (2013). Comparative digestive physiology. *Compr. Physiol.* **3**, 741–783.

Kauhala, K., Laukkanen, P. & von Rége, I. (1998). Summer food composition and food niche overlap of the raccoon dog, red fox and badger in Finland. *Ecography* **21**, 457.

Kim, S., Cho, Y.S., Kim, H.M., Chung, O., Kim, H., Jho, S., Seomun, H., *et al.* (2016). Comparison of carnivore, omnivore, and herbivore mammalian genomes with a new leopard assembly. *Genome Biol.* **17**, 1.

Kolosov, A.M. (1980). *Zoogeography of the Far East*. Moscow: Misl.

Laurent, P., Hayes, H., Eggen, A., Bishop, M. & Leveziel, H. (1999). Assignment of the amylase gene cluster to bovine chromosome 3 bands 3q23→q24 by in situ hybridization. *Cytogenet. Cell Genet.* **85**, 315.

Li, F., Luo, Z.H., Li, C.L., Li, C.W. & Jiang, Z.G. (2013). Biogeographical patterns of the diet of Palearctic badger: is badger an earthworm specialist predator? *Chinese Sci. Bull.* **58**, 2255.

McDonald, R.A., Webbon, C. & Harris, S. (2000). The diet of stoats (*Mustela erminea*) and weasels (*Mustela nivalis*) in Great Britain. *J. Zool.* **252**, 363.

McGeachin, R.L. & Akin, J.R. (1979). Amylase levels in the tissues and body fluids of the domestic cat (*Felis catus*). *Comp. Biochem. Physiol. Part B Biochem.* **63**, 437.

McGeachin, R.L. & Akin, J.R. (1982). Amylase levels in the tissues and body fluids of several primate species. *Comp. Biochem. Physiol. Part A Physiol.* **72**, 267.

Mills, R.E., Walter, K., Stewart, C., Handsaker, R.E., Chen, K., Alkan, C., Abyzov, A., *et al.* (2011). Mapping copy number variation by population-scale genome sequencing. *Nature* **470**, 59–65.

Noble, R.E. (2000). Salivary alpha-amylase and lysozyme levels: a non-invasive technique for measuring parotid vs submandibular/sublingual gland activity. *J. Oral Sci.* **42**, 83.

Perry, G.H., Dominy, N.J., Claw, K.G., Lee, A.S., Fiegler, H., Redon, R., Werner, J., Villanea, F.A., Mountain, J.L., Misra, R., Carter, N.P., Lee, C. & Stone, A.C. (2007). Diet and the evolution of human amylase gene copy number variation. *Nat. Genet.* **39**, 1256.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, 45e.

R Core Team (2017). *R: a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing. http://www.R-project.org/

Remonti, L., Balestrieri, A. & Prigioni, C. (2011). Percentage of protein, lipids, and carbohydrates in the diet of badger (*Meles meles*) populations across Europe. *Ecol. Res.* **26**, 487.

Roper, T.J. & Michevicius, E. (1995). Badger *Meles meles* diet: a review of literature from the former Soviet Union. *Mammal Rev.* **25**, 117.

Samuelson, L.C., Phillips, R.S. & Swanberg, L.J. (1996). Amylase gene structures in primates: retroposon insertions and promoter evolution. *Mol. Biol. Evol.* **13**, 767.

Sato, J.J. (2016). The systematics and taxonomy of the world's badger species – a review. In *Badgers: systematics, biology, conservation and research techniques: 1–30*. Proulx, G. & San, E.D.L. (Eds). Sherwood Park: Alpha Wildlife Publications.

Schibler, L., Vaiman, D., Oustry, A., Giraud-Delville, C. & Cribiu, E.P. (1998). Comparative gene mapping: a fine-scale survey of chromosome rearrangements between ruminants and humans. *Genome Res.* **8**, 901.

Schrider, D.R. & Hahn, M.W. (2010). Gene copy-number polymorphism in nature. *Proc. Biol. Sci.* **277**, 3213.

Simpson, J.W., Doxey, D.L. & Brown, R. (1984). Serum isoamylase values in normal dogs and dogs with exocrine pancreatic insufficiency. *Vet. Res. Commun.* **8**, 303.

Solovyev, V. A., Yuferev, G. I. and Maltsev, M. V. (2007). Food preferences and peculiarities of marking behaviour of

badger of the Volga-Kama Interfluve. In *Recent Problems of Nature Use, Game Biol. and Fur Farming. Proc. Int. Conf. VNIIOZ, Kirov: 410*.

Stranger, B.E., Forrest, M.S., Dunning, M., Ingle, C.E., Beazley, C., Thorne, N., Redon, R., Bird, C.P., de Grassi, A., Lee, C., Tyler-Smith, C., Carter, N., Scherer, S.W., Tavaré, S., Deloukas, P., Hurles, M.E. & Dermitzakis, E.T. (2007). Relative impact of nucleotide and copy number variation on gene expression phenotypes. *Science* **315**, 848.

Sugino, H. (2007). Comparative genomic analysis of the mouse and rat amylase multigene family. *FEBS Lett.* **581**, 355–360.

Tonoike, A., Hori, Y., Inoue-Murayama, M., Konno, A., Fujita, K., Miyado, M., Fukami, M., Nagasawa, M., Mogi, K. & Kikusui, T. (2015). Copy number variations in the amylase gene (AMY2B) in Japanese native dog breeds. *Anim. Genet.* **46**, 580.

Xu, L., Hou, Y., Bickhart, D.M., Zhou, Y., Hay, E.H.A., Song, J., Sonstegard, T.S., Van Tassell, C.P. & Liu, G.E. (2016). Population-genetic properties of differentiated copy number variations in cattle. *Sci. Rep.* **6**, 23161.

Zagainova, O.S. & Markov, N.I. (2011). The diet of Asian badger, *Meles leucurus* Hodgson, 1847, in Samarovskii Chugas Nature Park, Western Siberia. *Russ. J. Ecol.* **42**, 414.

Zhang, F., Gu, W., Hurles, M.E. & Lupski, J.R. (2009). Copy number variation in human health, disease, and evolution. *Annu. Rev. Genomics Hum. Genet.* **10**, 451.