

# A Comparison of Visual and Genetic Techniques for Identifying Japanese Marten Scats - Enabling Diet Examination in Relation to Seasonal Food Availability in a Sub-alpine Area of Japan

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We compared the reliability of visual diagnostic criteria to DNA diagnostic techniques, including newly designed primers, to discriminate Japanese marten (*Martes melampus*) feces from those of other sympatric carnivore species. Visual criteria proved > 95% reliable for fresh, odoriferous scats in good condition. Based upon this verification, we then examined if and how Japanese marten diet differs among seasons at high elevation study site (1500–2026 m). We also considered how intra-specific competition with the Japanese red fox (*Vulpes vulpes japonica*) may shape marten feeding ecology. From 120 Japanese marten fecal samples, high elevation diet comprised (frequency of occurrence) 30.6–66.0% mammals, 41.0–72.2% insects and 10.6–46.2% fruits, subject to seasonal variation, with a Shannon-Weaver index value of 2.77. These findings contrast substantially to seasonal marten diet reported in adjacent lowland regions (700–900 m), particularly in terms of fruit consumption, showing the trophic adaptability of the Japanese marten. We also noted a substantial dietary overlap with the red fox ( $n = 26$  scats) with a Shannon-Weaver index of 2.61, inferring little trophic niche mutual exclusion (trophic niche overlap: 0.95), although some specific seasonal prey selection differences were likely related to relative differences in body size between foxes and martens. This additional information on the feeding ecology of the Japanese marten enables a better assessment of the specific risks populations face in mountainous regions.

**Key words:** diet, fecal analysis, *Martes melampus*, niche overlap, PCR amplification, *Vulpes vulpes*

## INTRODUCTION

In order to fully understand the ecology and life-history strategies of species, it is essential to examine foraging behavior (Carr and Macdonald, 1986; Abrams, 1991; Geffen and Macdonald, 1993; Daan and Tinbergen, 1997), where the seasonal and spatial distribution of trophic resources can influence optimal foraging strategy, socio-spatial organization, and mating system (Gittleman, 1986; Roff et al.,

2002; Macdonald and Johnson, 2015). Here we expand on knowledge of the feeding ecology of the Japanese marten (*Martes melampus*) (e.g., Tatara and Doi, 1994; Nakamura et al., 2001; Arai et al., 2003; Tsuji et al., 2014; Adachi et al., 2016), which inhabits a wide range of elevations from lowland up to alpine forests (e.g., Hoshino et al., 2014; see also Zhou et al., 2011a) on the three main Japanese islands of Honshu, Shikoku and Kyushu (Proulx et al., 2005).

Fecal analysis is typically used to survey the diet of carnivores because it is easy to obtain large sample sizes without harming individuals, unlike the examination of stomach contents (e.g., Litvaitis, 2000). However, prerequisite is that feces can be attributed to the carnivore species depositing them without any ambiguity, or else misrepresentative conclusions may be drawn about their diet and foraging ecology. Marten feces are commonly misidentified due to their similar

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shape/texture to those of other sympatric carnivores. For example, Davison et al. (2002) report that 18% of feces visually identified by experts as 'marten' were actually from foxes. Similarly, Baines et al. (2013) found that 23% of marten/fox scats were misidentified by experienced observers, also with a bias towards assigning marten scats as fox. These studies illustrate how ability to discriminate the droppings of similar carnivores using visual and olfactory characteristics can be ambiguous, leading to unreliability (e.g., Davison et al., 2002; Birks et al., 2005). Because martens and foxes eat a similar, generalist, omnivorous diet (e.g., Goszczynski, 1986; Storch et al., 1990), inter-specific competition can arise, and so they can cross-influence each other's trophic niche. It is therefore crucial to establish the extent to which local diets overlap to include all of the constraints influencing marten diet.

Identifying the species depositing feces using DNA techniques is increasingly recognized as the most reliable and accurate method available (e.g., Kurose et al., 2005), although costly. DNA diagnostic techniques have been applied to marten species in other regions (for example, in Europe; see Balestrieri et al., 2010; Caryl et al., 2012), and so here we undertook DNA diagnostics to test and calibrate the accuracy of visual and olfactory diagnostic approaches. This required us to develop genetic markers specific to the Japanese marten (e.g., Foran et al., 1997; Farrell et al., 2000; Kurose et al., 2005), trialing PCR amplification techniques. Using this approach, we were able to assess the practical and financial trade-off between identifying feces directly, in situ, in the field, versus transporting samples to the laboratory for DNA extraction and sequencing.

Building on accurate scat identification, we examined whether the principal food types consumed by Japanese martens change with season in accord with changes in availability of each food type (e.g., Zhou et al., 2011b). We then tested whether niche overlap indices with the Japanese red fox exert any trophic niche constraint on Japanese marten diet; or if relative body-size differences cause these carnivores to select different primary prey, resulting in trophic niche segregation. We also examined whether Japanese marten diet differs between our high elevation study site (1500–2026 m) in the Mikuni mountain range (central Japan) and diets reported for lowland (700–900 m) populations studied in these same mountains (Kanto Regional Forest Office, 2012, 2013). Specifically, we posited that in upland areas martens would exhibit a lower reliance on fruits, because fruits are less abundant and are available for a shorter duration at higher elevation (see Kudo, 1993; Otani, 2002; Miyajima, 2007); noting that martens can compensate for changes in food availability through adaptive foraging (e.g., Zhou et al., 2011b).

## MATERIALS AND METHODS

### Study area

The study was conducted in the Joshinetsu-Kogen National Park near Mt. Sennokura (36°48'N, 138°50'E), in the Mikuni Mountain range, which extends into the Minami-Uonuma District (Niigata Prefecture). Surveys were conducted in the sub-alpine habitat zone, at an elevation ranging over 1500–2026 m (Fig. 1). These mountains receive weather systems off the Pacific Ocean and the Sea of Japan, leading to substantial winter snowfall. From

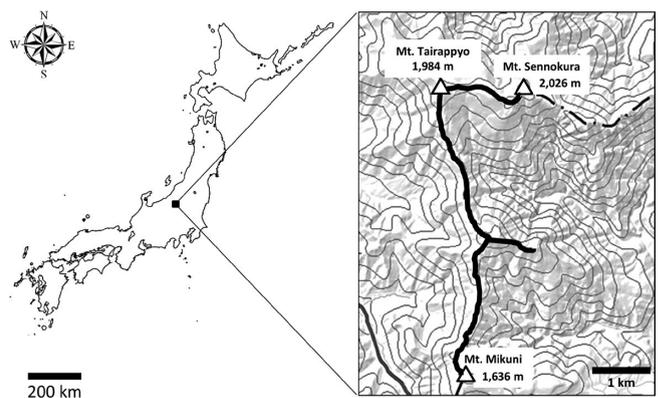
historic records obtained from the Yuzawa meteorological observatory (13 km northeast of research area; elevation 340 m), average temperature ranged from a minimum of  $-0.5^{\circ}\text{C}$  in January to a maximum of  $24.7^{\circ}\text{C}$  in August (average of 1981–2010). Annual rainfall between 1981 and 2010 averaged 2231 mm, with a maximum snow depth of 211 cm; higher up in this sub-alpine study area, snow cover remained for around seven months, between November and May, with a maximum depth of 350–500 cm. There were no residential homes, businesses or agriculture within the study area, with the nearest town, Minakami (Gunma Prefecture), lying approximately 15 km to the southeast.

The lowland broadleaf forests that comprise the study site were dominated by Japanese beech (*Fagus crenata*) and Mongolian oak (*Quercus crispula*) (Hoshino et al., 2014). Above 1600 m this transitioned into sub-alpine mixed woods composed of Maries' fir (*Abies mariesii*) and Erman's birch (*Betula ermanii*). Above ca. 1800 m, in the Pseudo-Alpine Zone (see Ohmori and Yanagimachi, 1991; Yasuda and Okitsu, 2007), there were shrub forests of Siberian dwarf pine (*Pinus pumila*), Japanese alpine cherry (*Prunus nipponica*), as-well-as dwarf bamboos (Bambusoideae spp.), and other high alpine vegetation. The ridge line of Mt. Tairappyo and Mt. Sennokura was a windswept grassland that consisted of narcissus anemone (*Anemone narcissiflora*), meadow buttercup (*Ranunculus acris* var. *nipponicus*), Japanese crowberry (*Empetrum nigrum* var. *japonicum*), and Japanese mountain heath (*Phyllodoce nipponica*).

### Collection of fecal samples

It was not possible to collect fecal samples during the winter (Nov–May) because they became obscured by snow; furthermore, snow depth of up to 500 cm, over steep terrain, made surveying unsafe and impractical. Consequently, fecal samples were collected between July and October in 2011, with some further supplementary collection from June to July in 2012. Samples were collected each month (three consecutive sampling days/month) along a 10 km transect following an existing mountain trekking route through the study area, which transcended habitat types effectively through the full elevation range (Fig. 1).

Upon detection, the location of each fecal sample was logged using a hand-held GPS device (Garmin). To enable subsequent DNA-based validation species depositing feces, all carnivore fecal samples were collected in plastic hermetic containers, preserved in 95% ethanol immediately and transported to the laboratory.



**Fig. 1.** The study area in central Japan. The dashed line shows the boundary between Gunma and Niigata prefectures, and the solid-bold line represents the fecal sampling route (based on the topographical map produced by the Geospatial Information Authority of Japan: <http://www.gsi.go.jp/ENGLISH/index.html>). Contours show 100 m intervals.

### Assigning feces to species in the field using visual and olfactory criteria

In situ, in the field, we assigned marten and fox feces to species using diagnostic criteria: diameter – marten feces are smaller (ca. 8–13 mm; see Tsuji et al., 2011a, b) than those of fox (> ca. 15 mm; see also Hisano et al., 2016); shape; odor – marten scats smell characteristically sweet (see Hisano et al., 2016) while those of fox smell musky and foul (see Vine et al., 2009). Corroborative support was established from nearby tracks and, in exceptional cases, the presence of marten hair (following the general methods of Zhou et al., 2011b). Note; we did assign any feces to other carnivores known to be sympatric in this region: masked palm civet (*Paguma larata*), ermine (*Mustela erminea*), Japanese weasel (*Mustela itatsi*), Raccoon dog (*Nyctereutes procyonoides*), Japanese badger (*Meles anakuma*), domestic cat (*Felis catus*) and domestic dog (*Canis familiaris*); all of which can easily be distinguished from marten feces by their appearance (e.g., color, shape and size), texture smell, or use of latrines (see Zhou et al., 2008a). Although feces of the Japanese weasel are morphologically similar to those of marten, they are smaller: 4.3–7.2 mm (Tsuji et al., 2011a, b), and so we ensured we did not include weasel scats by not collecting feces of less than 8.0 mm diameter.

### DNA-based validation of species depositing feces

We applied a genetic technique to approximately half of the total number of fecal samples collected, to test and verify whether our field-diagnostic criteria had identified Japanese marten feces accurately and reliably. That is, we sought to prove definitely that we had not mistakenly assigned any other superficially similar looking scats from the eight sympatric carnivore species among our putative set of marten scats. Total DNA was extracted from ca. 300 mg of each fecal sample using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions, and then 1.0 µl of the extract was used for PCR amplification. We trialed two methods of PCR amplification, general single PCR and multiplex PCR of partial sequences from the mitochondrial DNA (mtDNA) control region, which is the most variable part of mtDNA. We sourced mtDNA control region sequences from the DNA Data Bank of Japan (DDBJ), and aligned them to design PCR primers for: Japanese weasel (accession number of DDBJ: AB007327), Japanese marten (AB525722), Japanese badger (AJ563700), masked palm civet (EU910478), raccoon dog (EU642434), ermine (AB061213), red fox (AB292741), domestic dog (AB007398) and domestic cat (AJ441318 and EU864495). Species-specific sequences were selected to design the PCR primers, especially as to match the 3' end of the species' sequence (Table 1). In addition, we identified conservative sequences among these species, enabling us to design a universal reverse primer (UprR in Table 1). Target animals

were then distinguished by the molecular sizes of the PCR products. The Multiplex PCR method was able to discriminate definitively between all sympatric carnivore species by using the target-animal-specific primers as designed above. Two primers (spCat and spFox) for domestic cat and red fox, respectively, were referenced Nonaka et al. (2009). We used the Multiplex PCR Kit (Qiagen) on each primer set (primer set 1: for Japanese weasel, Japanese marten, masked palm civet and domestic cat; primer set 2: for Japanese badger, red fox, raccoon dog and domestic dog). The general single PCR was designed to identify specifically ermine DNA, using primer set ER-F1/R1, following Shimatani et al. (2008); this was used when no reactions occurred for the other species in the multiplex PCR program.

For both techniques, an aliquot of 9.0 µl of the PCR reaction product was mixed with 1.0 µl of loading buffer. Multiplex PCR products were electrophoresed on a 4% agarose gel and single PCR products on a 2% agarose gel. These PCR products were then stained with ethidium bromide and visualized under an ultraviolet illuminator.

### Dietary analysis

Following the methods of Jędrzejewska and Jędrzejewski (1998; modified by Zhou et al., 2011b), fecal samples assigned as marten or fox were first washed through a 0.5 mm mesh metal sieve, to remove larger digested remains, such as hair, bones, teeth, feathers, scales, exoskeleton, elytra, fruits cuticles, and seeds. These dietary remains were identified to prey category type using morphological criteria (Zhou et al., 2008a) under stereoscopic optical microscopy, with reference to collections of specimens (seeds, insects, reptiles and amphibians) from the study area. Mammal hairs were identified based on the features of the medulla, using the chart provided by Murai et al. (2011). The characteristic shape of spines and ribs allowed us to discriminate between amphibians and reptiles unambiguously (see Murai et al., 2011). Fecal contents were identified to at least the taxonomic level of Order, except for fish, birds and molluscs. In order to establish the percentage dry weight of each sample, and thus to calculate relative proportions of each dietary item with minimum weight of > 1 mg, samples were dried for 24 hours at 80°C using a circulation drier. Insects were identified to the level of taxonomic Order, with one entire insect assigned as the minimal contribution to the percentage dry weight. Food items were assigned to 10 categories: mammals, birds, reptiles, amphibians, insects, molluscs, fruits, fish, artificial materials and other plant material (that is, the "items" comprised "categories"). We used these methods in order to compare our findings with similarly-derived data from reports from lowland population in the same area (Kanto Regional Forest Office, 2012, 2013), as well as other literature on marten and red fox feed-

**Table 1.** Multiplex PCR primers for species identification and molecular characteristics.

Target species	Primer name	Primer sequence (5'–3')	Number of base-pair	$T_m$ value (°C)	Size of PCR products <sup>a</sup>	Maximum number of mismatches <sup>b</sup>	Conservation at 3' end <sup>c</sup>
Japanese marten	spMtn2	5'-TTCCTCTCCCCATGACTTAAT-3'	21	54.6	203	1	6
Japanese weasel	spJwl	5'-CAACATTTAATGTGCTTGCCC-3'	21	54.6	173	1	19
Masked palm civet	spMpc	5'-TATATCGTGCATTATACATTCA-3'	23	51.5	92	2	3
Raccoon dog	spRdg2	5'-GCCATGTGCGCACGTCCAC-3'	19	62.5	201	1	6
Japanese badger	spBgr	5'-AGCATTTTCTCAGCACACATT-3'	21	52.3	157	0	21
Domestic dog	spDog2	5'-AATGCATATCACTTAGTCCAAT-3'	22	51.1	59	1	9
Universal	UprR	5'-GCCTCGAGAAACCATCAATC-3'	20	56.3			

<sup>a</sup> Expected size of amplification products when PCR was performed with a corresponding forward primer and UprR.

<sup>b</sup> The maximum number of mismatched base pairs within target animal sequences.

<sup>c</sup> The number of consecutive base pairs which are conserved at the 3' end of the sequence within target animals.

ing ecology (e.g., Prigioni et al., 2008; Zhou et al., 2011a, b).

Marten diet composition was then calculated according to three indices: (1) The frequency of occurrence [FO (%): (the number of occurrences of each food item/ the total number of fecal items)  $\times$  100]; (2) The relative frequency of occurrence [RFO (%): (the number of occurrences of each food item/ the total number of food items occurring)  $\times$  100]; and (3) The proportion of dry matter [PDM (%): (dry weight of each fecal item/dry weight of each separate fecal deposit)  $\times$  100].

The six-month study period was divided into three functional seasons: 'Summer 2011' (July–August 2011), 'Autumn 2011' (September–October 2011), and 'Early-summer 2012' (June–July 2012). To assess whether marten diet differed between these seasons, Fisher's exact test of independence was applied to FO and RFO data. If a significant ( $\alpha = 0.05$ ), seasonal difference was detected, we then conducted *post-hoc* pairwise Fisher's exact tests with Bonferroni correction to determine in which season martens consumed food categories more/less frequently (see also Tsuji et al., 2014). In addition, FO and RFO for each food category were pooled across seasons and Fisher's exact test of independence was applied to compare of marten and fox diet.

The Shannon-Weaver Index ( $H'$ ) was also used to quantify the diversity of food types consumed:

$$H' = -\sum_{i=1}^S P_i \ln P_i$$

where  $S$  represents the number of food categories and  $P_i$  represents the ratio of the category  $i$  against total number of food categories. The RFO was used to investigate the trophic niche overlap (RO) between species for which samples were obtained:

$$R_o = \frac{\sum_i (x_i + y_i) \log(x_i + y_i) - \sum_i x_i \log x_i - \sum_i y_i \log y_i}{(x + y) \log(x + y) - x \log x + y \log y}$$

where  $x$  and  $y$  represent the sum of occurrence of each food category for each carnivore, and  $x_i$  and  $y_i$  give the  $i$ -th numbers of food items that occurred. We also contrast our data against dietary data reported by lowland studies (Kanto Regional Forest Office, 2012, 2013). All statistical analyses were conducted using R 3.2.4 (R Development Core Team, 2016).

## RESULTS

### Identification of fecal samples; species verification using DNA analysis

A total of 165 fecal samples was collected, aiming only to select marten and fox scats in the field. Seventy-eight of these were subjected to the DNA identification technique and 87 examined using only visual/olfactory identification criteria (due to costs and limited project resources). Among this total sample, a proportion of feces were broken ( $n = 23$ )/semi-broken ( $n = 22$ ), and we used these predominantly for the genetic technique ( $n = 20$  and 15, respectively) (Table 2), where the integrity of samples would make no difference.

From the genetic technique, 70 (89.7%) samples yielded

PCR products with expected molecular sizes, indicating reliability (Table 2). No PCR products were obtained from the other eight samples. Of these 70 samples, 54 samples were confirmed genetically to come from the Japanese marten, whereas 15 samples were identified as red fox. The remaining single sample was ambiguous, showing PCR amplifications for both the Japanese marten and red fox, and was excluded. We thus verified that we had fulfilled our objective of collecting only marten and fox scats from the field, and had not inadvertently collected scats from the other sympatric carnivores.

From this, we established that our non-genetic visual identification criteria were 97.1% relative to PCR diagnostics, but only when applied to the 34 intact/un-broken feces among the subset of 78 samples used to calibrate techniques. The reliability of visual identification decreased to 60.0% ( $n = 15$ ) for semi-broken feces and 45.0% ( $n = 20$ ) for broken feces. Consequently, we excluded those 10 samples that were not fully intact from our sub-sample examined only visually, and added the remaining intact 77 in this subset to the 69 species assignments assured by PCR (Table 2). This enabled us to extend > 95% confidence across 146 samples.

From these 146, we attributed 120 feces to marten, and these were then used in dietary analyses (21 from July 2011, 15 from August 2011, 21 from September 2011, 16 from October 2011, 27 from June 2012 and 20 from July 2012). The remaining 26 samples were attributed to fox (eight from July 2011, four from September 2011, three from October 2011, nine from June 2012 and two from July 2012; Table 3).

### Seasonal change in marten diet

The FO of food items detected from marten feces is provided in Table 3, and Fig. 2 illustrates the seasonal change in the RFO and the PDM. Martens consumed mammals, birds, reptiles, amphibians, insects, molluscs, fruits and other plants (i.e., eight categories); representing species observed in the environment while conducting surveys.

Mammal species comprised the main component of marten diet throughout the study (FO: 50.0%, range 13.3–75.0%; RFO: 23.6%, range 6.9–36.6%), although varying in extent. Mammal species consumed predominantly included rodents, such as *Apodemus* spp., Cricetidae spp., and Japanese hare (*Lepus brachyurus*). Additionally, Japanese serow (*Capricornis crispus*) occurred twice in the diet and wild boar (*Sus scrofa*) just once; presumably being eaten opportunistically, when the carcasses of dead animals were encountered (too large to be predated by martens) (Table 3). The FO of mammals differed significantly among seasons (Fisher's exact test for independence:  $\chi^2 = 10.3$ ,  $df = 2$ ,  $P = 0.006$ ), and was significantly lower in Summer 2011 (30.6%; *post-hoc* pairwise Fisher's exact tests:  $P = 0.006$  with

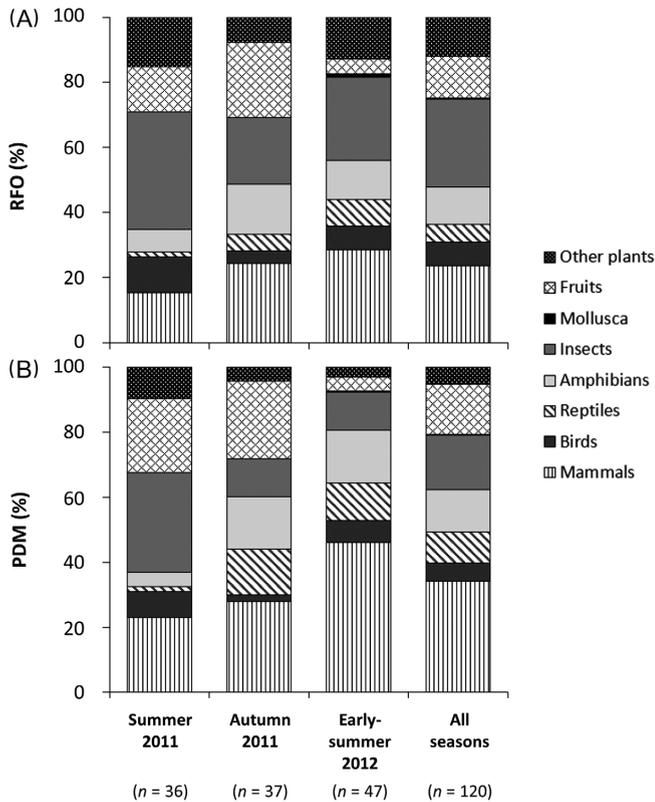
**Table 2.** The numbers of Japanese marten and red fox feces collected in the field and diagnosed by DNA and visual/olfactory diagnosis.

	Collected/diagnosed	Included for dietary analysis	Excluded from dietary analysis
DNA diagnostic techniques	78	Succeeded: 69*	Failed: 9
Visual/olfactory diagnostic techniques	87	Intact: 77	Broken/semi-broken: 10
Total	165	146	19

\* Comprised with intact ( $n = 34$ ), semi-broken ( $n = 15$ ) and broken ( $n = 20$ ) feces.

**Table 3.** The frequency of occurrence (FO; %) of food items occurring in the feces of Japanese martens from a sub-alpine area in central Japan between July–October 2011 and June and July 2012.

Food items	Japanese marten				Red fox
	Summer 2011 (Jul–Aug 2011) <i>n</i> = 36	Autumn 2011 (Sept–Oct 2011) <i>n</i> = 37	Early-summer 2012 (Jun–Jul 2012) <i>n</i> = 47	All seasons <i>n</i> = 120	All seasons <i>n</i> = 26
<b>Mammals</b>	<b>30.6</b>	<b>48.7</b>	<b>66.0</b>	<b>50.0</b>	<b>84.6</b>
Lagomorpha					
<i>Lepus brachyurus</i>	19.4	17.9	14.9	17.2	46.2
Artiodactyla					
<i>Capricornis crispus</i>	2.8	–	–	0.8	–
<i>Sus scrofa</i>	2.8	2.6	–	1.6	–
Rodentia					
<i>Apodemus</i> spp.	2.8	15.4	29.8	16.4	23.1
Cricetidae spp.	2.8	10.3	6.4	7.4	7.7
<i>Sciurus lis</i>	–	–	2.1	0.8	–
<i>Petaurista leucogenys</i>	–	2.6	–	0.8	3.8
Insectivora					
<i>Sorex shinto</i>	–	–	2.1	0.8	–
Soricidae spp.	–	–	–	–	–
<i>Urotrichus talpoides</i>	2.8	–	10.6	4.9	3.8
Unidentified	–	2.6	2.1	1.6	3.8
<b>Birds</b>	<b>22.2</b>	<b>7.7</b>	<b>17.0</b>	<b>15.6</b>	<b>11.5</b>
Unidentified adults	19.4	7.7	17.0	14.8	11.5
Unidentified eggs	5.6	–	–	1.6	–
<b>Reptiles</b>	<b>2.8</b>	<b>10.2</b>	<b>19.1</b>	<b>11.5</b>	<b>11.5</b>
Lacertilia spp.	2.8	10.2	12.8	9.0	3.8
Serpentes spp.	–	–	6.4	2.5	7.7
<b>Amphibians</b>					
Anura spp.	<b>13.4</b>	<b>30.8</b>	<b>27.7</b>	<b>24.6</b>	–
<b>Fish</b>					
Unidentified	–	–	–	–	<b>3.8</b>
<b>Insects</b>	<b>72.2</b>	<b>41.0</b>	<b>59.6</b>	<b>57.4</b>	<b>57.7</b>
Coleoptera spp.	13.9	2.6	48.9	23.8	34.6
Orthoptera spp.	11.1	15.4	4.3	9.8	30.8
Hymenoptera spp.	47.2	5.1	8.5	18.9	23.1
Diptera spp.	2.8	–	–	0.8	–
Hemiptera spp.	5.6	2.6	2.1	3.3	7.7
Unidentified larvae	2.8	–	4.3	2.5	–
Unidentified imagoes	13.9	18.0	–	9.8	3.8
<b>Mollusca</b>					
Pulmonata spp.	–	–	<b>2.1</b>	<b>0.8</b>	–
<b>Fruits</b>	<b>27.8</b>	<b>46.2</b>	<b>10.6</b>	<b>27.0</b>	<b>50.0</b>
Actinidiaceae	–	–	–	–	–
<i>Actinidia arguta</i>	–	17.9	–	5.7	26.9
<i>Actinidia polygama</i>	2.8	–	–	0.8	–
<i>Actinidia kolomikta</i>	–	–	–	–	3.8
Rosaceae	–	–	–	–	–
<i>Rubus</i> spp.	–	2.6	–	0.8	3.8
<i>Prunus</i> spp.	16.7	–	–	4.9	7.7
<i>Sorbus alnifolia</i>	–	12.8	–	4.1	–
<i>Sorbus commixta</i>	–	5.1	–	1.6	–
Vitiaceae	–	–	–	–	–
<i>Vitis coignetiae</i>	–	7.7	–	2.5	–
Cornaceae	–	–	–	–	–
<i>Swida controversa</i>	–	2.6	–	0.8	15.4
<i>Benthamidia japonica</i>	–	–	–	–	3.8
Ericaceae	–	–	–	–	–
<i>Gaultheria adenothrix</i>	–	–	2.1	0.8	7.7
<i>Vaccinium</i> spp.	5.6	–	6.4	4.1	–
Caprifoliaceae	–	–	–	–	–
<i>Viburnum furcatum</i>	–	5.1	1.6	1.6	–
Poaceae spp.	–	–	0.9	0.8	–
Unidentified	2.8	–	–	0.8	3.8
<b>Other plants</b>					
Twigs or grasses	<b>30.6</b>	<b>15.4</b>	<b>29.8</b>	<b>25.4</b>	<b>42.3</b>
<b>Artificial materials</b>					
Plastic envelope or raw garbage	–	–	–	–	<b>19.2</b>



**Fig. 2.** Seasonal changes in (A) the relative frequency of occurrence (RFO) and (B) proportion of dry matter (PDM) of food categories consumed by Japanese martens in a sub-alpine area of central Japan between 2011 and 2012.

Bonferroni correction) than Early-summer 2012 (66.0%; Table 3). The PDM of these food categories showed a similar tendency to the FO (Fig. 2).

Birds comprised 15.6% (FO, range 7.7–22.2%) and 7.4% (RFO, range 3.8–11.1%) of the Japanese marten's diet; feathers indicated the majority of these birds were mature adults, not chicks, although eggs (evidenced by thick and hard shell fragments, compared with reptiles) were also eaten. Both the FO and RFO consumed by birds did not show any significant differences among seasons.

Reptiles consumed included lizards (*Scincidae* spp.) (FO: 9.0%; range 1.4–12.7%) and snakes (*Serpentes* spp.) (FO: 2.5%, range 0.0–6.4%), which showed no significant seasonal variation. Amphibians were detected in feces in every season (FO: 24.6%, range 13.8–30.8%) with no significant seasonal difference. Additionally, one snail (*Gastropoda* sp.) was also eaten (FO: 0.8%; Table 3).

Insect species consumed were principally *Coleoptera* spp. (FO: 23.8%, range 13.9–48.9%), *Orthoptera* spp. (FO: 9.8%, range 4.3–15.4%) and *Hymenoptera* spp. (FO: 18.9%, range 5.1–47.2%; Table 3). The FO of insects differed significantly among seasons (Fisher's exact test for independence:  $\chi^2 = 7.6$ ,  $df = 2$ ,  $P = 0.022$ ), and these were consumed significantly more frequently in Summer 2011 (FO: 72.2%) than Autumn 2011 (FO: 41.0%; *post-hoc* pairwise Fisher's exact tests:  $P = 0.030$  with Bonferroni correction). Similar to FO, the PDM of insect prey also showed clear differences between these two seasons (Fig. 2).

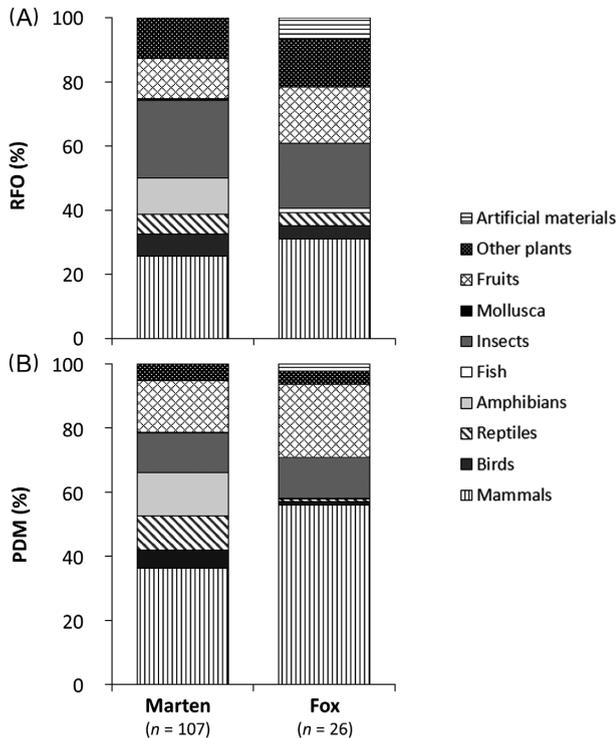
In terms of plant material, we detected a variety of seeds in marten feces, indicating that they consumed intact fruits, and a few fragments of other incidental plant material (categorized as other plants). Both the FO and RFO of fruits differed significantly among seasons (Fisher's exact test for independence:  $\chi^2 = 13.6$ ,  $df = 2$ ,  $P = 0.001$  for FO;  $\chi^2 = 14.1$ ,  $df = 2$ ,  $P < 0.001$  for RFO), and significantly smaller quantities of fruit were consumed in Early-summer 2012 (FO: 10.6%, RFO: 4.7%, with remarkable little fruit consumption in June 2012 ( $n = 1/27$ , FO: 3.7%, RFO: 1.5%)) compared to Autumn 2011 (FO: 46.2%, RFO: 23.1%; *post-hoc* pairwise Fisher's exact tests with Bonferroni correction:  $P = 0.001$  for FO;  $P < 0.001$  for RFO). The PDM for fruit followed the same trend as FO and RFO.

Focusing on month-to-month changes in the types of fruit species consumed, martens took advantage of whatever fruits were available in specific months, and no fruit species occurred over more than two consecutive months. Cherry (*Prunus* spp.) was eaten in July 2011 (FO: 28.6%); bilberry (*Vaccinium* spp.) was eaten in August 2011 (FO: 13.3%) and in July 2012 (FO: 15.0%); hardy kiwi (*Actinidia arguta*) and Korean whitebeam (*Sorbus alnifolia*) fruits were eaten from September (FO: 19.0% and 4.8%, respectively) to October 2011 (FO: 16.7%, 22.2%, respectively); and mountain ash (*Sorbus commixta*) was eaten in October 2011 (11.1%).

#### Dietary comparison between the marten and the sympatric fox

No fox feces were found in August 2011, and so we exclude August 2011 from our inter-specific comparison. The red fox had a broadly similar diet to the marten; the trophic niche overlap index (*RO*) between the two species was 0.95 and the trophic diversity indices (*H'*) of marten and fox was similar (2.77 and 2.61) when pooled across seasons. However, substantial inter-specific differences in the compositions of their diets were evident. Consumption of mammals differed significantly between the two species (Fisher's exact test for independence:  $\chi^2 = 9.8$ ,  $df = 1$ ,  $P = 0.001$  for FO). In particular, foxes consumed hares (FO: 46.2%, RFO: 16.2%) significantly more frequently than did martens (FO: 17.8%, RFO: 8.4%; Fisher's exact test for independence:  $\chi^2 = 9.4$ ,  $df = 1$ ,  $P = 0.004$  for FO;  $\chi^2 = 6.1$ ,  $df = 1$ ,  $P = 0.020$  for RFO). In terms of fruit consumption, the FO for foxes (50.0%) was significantly greater than for martens (27.1%) (Fisher's exact test for independence:  $\chi^2 = 5.1$ ,  $df = 1$ ,  $P = 0.034$ ). Unlike martens, foxes did not feed on amphibians (Fisher's exact test for independence for RFO:  $\chi^2 = 7.9$ ,  $df = 1$ ,  $P = 0.002$  for FO;  $\chi^2 = 7.8$ ,  $df = 1$ ,  $P = 0.002$ ), but did eat fish (FO: 3.8%) and artificial materials (scavenged human refuse) (FO: 19.2%;  $\chi^2 = 21.4$ ,  $df = 1$ ,  $P < 0.001$  for FO;  $\chi^2 = 18.6$ ,  $df = 1$ ,  $P < 0.001$  for RFO), which martens did not.

Figure 3 contrasts the RFO and the PDM of the food items eaten by martens (excluding data from August 2011) and foxes. RFO and the PDM results generally accorded with FO patterns, although RFO tended to weight insect prey more heavily than PDM for both species, whereas PDM weighted mammals more heavily for foxes and reptiles for martens (Fig. 3).



**Fig. 3.** Comparison of food categories consumed by Japanese martens and red foxes in a sub-alpine area of central Japan between 2011 and 2012: (A) the relative frequency of occurrence (RFO), and (B) the proportion of dry matter (PDM). Data for contrasting marten and fox diet for August 2011 were excluded because no fox feces were found in this month.

## DISCUSSION

### The application of genetic techniques to verify field diagnostic feces identification

Our genetic PCR techniques and newly designed primers worked well for identifying the species depositing feces unambiguously (see also Caryl et al., 2012). Several authors have questioned the reliability and veracity of relying visual and olfactory criteria (reviewed by Heinemeyer et al., 2008; for Japanese carnivores see Kaneko et al., 2009) and Heinemeyer et al. (2008) recommends that samples should be always be confirmed by DNA analysis. Nevertheless, the PCR technique we used proved that our visual and olfactory diagnostic techniques, when applied to fresh, odoriferous feces in good condition, were > 95% reliable for identifying marten and fox feces, against a back-ground of the seven other potential carnivore species that could have caused confusion. This refutes concerns on the ability to identify marten (and other small carnivore) scats correctly (e.g., Davison et al., 2002; Birks et al., 2005; Baines et al., 2013) and supports Caryl et al. (2012), who also describe that trained field surveyors can distinguish the feces of pine martens from those of red foxes correctly (obtaining an identification success > 90%). In part, we attribute the high reliability of identification in our study to the additional diagnostic value derived from considering the in-situ position of feces in the field, combined with the use of scent (for example, marten scats smell sweet (Hisano et al., 2016), whereas

fox droppings smell foul (Vine et al., 2009)). We acknowledge, however, that the reliability of visual identification declined to around 50:50 (random chance) when scats were broken. Additionally, samples are misidentified more often when target species are less abundant (Davison et al., 2002; Prugh and Ritland, 2005; Kelly et al., 2012). In our study area, the Japanese marten and red fox were the carnivores that occurred most commonly, further facilitating identification accuracy. Consequently, we were enabled to undertake our study of marten diet, and look at trophic interactions with the red fox, with confidence, restricted to fresh/intact fecal samples.

### Food habits and dietary switching of Japanese martens in sub-alpine habitat

Zhou et al. (2011a) reviewed the food habits of the genus *Martes* and found that they exhibit a preference for small mammals, invertebrates, and fruits. Our study was conducted at high elevation, where the productive summer season is short, and fruiting and peak insect abundance occur later in the summer than in lowland areas. Nevertheless, we found these same categories to be the most important; resonating with other studies from Japan (e.g., Tataru and Doi, 1994; Otani, 2002; Tsuji et al., 2014; Adachi et al., 2016).

While snow-cover and extreme mountain weather prevented us from collecting feces in the winter, we can be certain that during winter months no fruit was available for martens, and any insect and amphibian/reptile prey would be hibernating and thus difficult to acquire. This supports strong ecological inference that martens must feed almost exclusively on active small mammal (rodent/leporid) and bird prey throughout the winter.

Snowpack (up to 500 cm) does not melt until June in our elevated study area, which substantially retards leafing/fruitlet phenology. As a consequence, even by Early-summer 2012, we observed that with little fruits availability early in the year (FO: 10.6%, PDM: 4.3%) martens consumed predominantly mammals (FO: 66.0%, PDM: 46.1%). Our data show that this consumption of mammalian prey also persisted through the summer season (FO: 30.6%); however, similar to the dietary habits of yellow-throated martens (*M. flavigula*) in central China (Zhou et al., 2011b), we found that the Japanese martens switched to rely more substantially upon fruits and insects as they became available during the productive summer months. Although we did not collect formal data on environmental food availability, the specific time of ripening of local fruits was apparent to data recorders working in the field. This was reflected in the diet of the martens in our study, as they switched the fruit species they exploited most in accord with changes in fruit species availability (see Zhou et al., 2011b).

In accord with our predictions, we found that martens in our study area consistently consumed less fruit throughout the study period (RFO: 14.0% (July 2011); 13.7% (August 2011); 19.5% (September 2011); 27.0% (October 2011); 1.5% (June 2012); 5.9% (July 2012)) than the Kanto Regional Forest Office (2012, 2013) reported for lowland sites (36°40'N, 138°59'E) in the same region (RFO: ca. 50% (July 2011); ca. 30% (August 2011); ca. 70% (September 2011); ca. 70% (October 2011); ca. 15% (June 2012); ca. 24% (July 2012)); although the data summarized in these reports was

not presented in a format permitting robust statistical comparison. Furthermore, although martens consumed 17 fruit varieties in the foothills (478 fecal samples; Kanto Regional Forest Office, 2012, 2013) only 12 fruit species (120 fecal samples) were consumed at higher elevation. This is similar to the 11 fruit species from 141 fecal samples reported by Otani (2002) in a similar sub-alpine area in northern Japan. This accord with habitat differences in relation to elevation, where lowland broad-leaved forests (with a diversity of fruiting trees) transition into coniferous woodlands (with less fruiting trees) (Hoshino et al., 2014). We observed a substantial decrease in fruiting tree species with increasing elevation in our study area (see also Miyajima, 2007) due, in part, to the shorter period of primary productivity in highland mountain habitats (see Okitsu, 1984; Otani, 2002). Because of this, martens 'compensated' by eating more non-fruit food categories (see also Hisano et al., 2016). For example, in the mid-summer, they exploited insects to a greater extent (particularly in August; FO: 93.3%), as populations of each increased, due to annual breeding cycles, presumably enhancing the availability of these food types. These findings imply that these martens foraged fairly optimally, in accord with the marginal value of the food categories they consumed (*sensu* Charnov, 1976).

In terms of ecosystem function, by consuming fruit Japanese martens play a role in seed dispersal in both temperate (Tsuji et al., 2011a) and sub-alpine forests (Otani, 2002) in Japan; as do other *Martes* species in other regions (e.g., Schaumann and Heinken, 2002; Zhou et al., 2008b; Zhou et al., 2013). Due to differences in fruit consumption between lowland and upland areas, martens are likely to have a more important seed dispersal function at lower elevation, acting more as a carnivore/insectivore higher in the mountains (see Tsuji et al., 2011a).

### The effect of competition with foxes on marten diet

Even though martens and foxes showed quite high dietary niche overlap indices (0.95), there were some notable differences in food selection by the two species. Because foxes are larger (4.0–7.0 kg versus 1.0–1.5 kg; Komiya, 2002; Ohdachi et al., 2009) and faster across the ground than more arboreal martens, they seemed better able to predate upon hares, which comprised 46.2% (FO) of their annual diet (compared with just 17.8% in marten diet). This may be crucial to resource partitioning between the otherwise similar diets of foxes and martens in this region, permitting coexistence, but potentially restricting the extent to which martens can utilize the rodent prey available. Kondo (1980) found similar diet partitioning between martens and foxes in western Japan, and foxes in Italy (Prigioni et al., 2008), Spain (Padiál et al., 2002) and Poland (Goszczyński, 1986) also consumed more hares than did stone/pine martens (*M. foina/martes*). While smaller martens tend to show narrower mammalian prey niches than do larger foxes (e.g., Russell and Storch, 2004), they are able to climb trees to access fruit before it falls (see also Tsuji et al., 2011a).

Notably, we detected no fox feces in our study area in August 2011. In this month, no rodents occurred in marten feces and martens consumed hares less frequently (FO: 13.3%) compared to the previous (FO: 23.8%) and following months (FO: 19.0%). Martens appeared to compensate for

this lack of mammalian prey by consuming more insects, for which FO in August was the highest across the study period (93.3%). Foxes, however, are not this flexible and are more reliant upon mammalian prey. We thus speculate that foxes may have vacated the study area through August and headed down to lower elevations (larger foxes tend to have more extensive home-range than martens; e.g., Gittleman et al., 1982; Lindstedt et al., 1986); also explaining how they accessed human refuse in their diet, which is not present in the environment, and thus not consumed by martens, at higher elevation (see also Kondo, 1980; Ueuma et al., 2005).

### Conclusions

By establishing that visual/olfactory scat identification criteria were 97.1% effective compared with genetic techniques, our study demonstrates that Japanese marten scats can be identified accurately in the field by trained surveyors provided they are in good condition and fresh enough to have a characteristic odor. This is especially important with respect to distinguishing marten scats from similar red fox scats; feces of other sympatric carnivores can be distinguished much more easily and reliably. This is reassuring for ecological surveyors, to include academic, ecological consultants, government wildlife officers, because DNA diagnostic techniques are relatively expensive and beyond the scope of many surveys (see Masuda et al., 2009).

Based on this reliable identification of feces, we discovered that despite elevated habitat, martens seemed to thrive in our study site. Feces were abundant throughout the summer, and dietary switching appeared to function to maintain an optimal, predominance of the currently highest energy-ranked prey in the martens' diet (i.e., ratio of energy gained to costs incurred by prey acquisition; Charnov et al., 1976). We propose that the apparent ability of martens to avoid reliance upon sub-optimal prey types suggests that they are not in marginal habitat, compared to lowland populations (see Powell, 1979, 1981).

Knowledge of the ecology of the Japanese marten is lacking but important; as evidenced by the unanticipated predation of martens on a highly vulnerable population of Japanese ibis (*Nipponia nippon*) in Sado Island, when martens were introduced (The Asahi Shimbun, 2010). These diet data are informative for parameterizing future habitat suitability models for Japanese marten reintroductions (e.g., Schulz, 1992; Watt et al., 1996) and for assessing the possible ecosystem risks posed. For example, the commercial management of forests, altering tree species composition and age structure (Sano, 1997) has consequences for marten feeding ecology that must be better understood in order to assess the risks marten populations face in mountainous regions.

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## COMPETING INTERESTS

The authors have no competing interests to declare.

## AUTHOR CONTRIBUTIONS

LH, SK, RM and YK designed the research. LH and MH performed the fieldwork, and SK and RM performed the PCR and sequencing. LH, MH and YK analyzed the data. MH, CN, RM and YK wrote the paper. All of the authors read and approved the final manuscript.

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