

# eDNA metabarcoding for biodiversity assessment, generalist predators as sampling assistants

Louise Nørgaard<sup>1,2\*</sup>, Carsten Riis Olesen<sup>3</sup>, Kristian Trøjelsgaard<sup>1</sup>, Cino Pertoldi<sup>1,4</sup>, Jeppe Lund Nielsen<sup>1</sup>, Pierre Taberlet<sup>5,6</sup>, Aritz Ruiz-González<sup>8</sup>, Marta De Barba<sup>5°</sup> & Laura Iacolina<sup>1,4,9°</sup>

## Supplementary material

**Table 1.** Blocking oligonucleotides developed to prevent amplification of host DNA (European badger and red fox) with the vertebrate marker V5.

Target species	Primer name	Primer sequence 5'-3'
European badger	Meles_V5_B	ACTATGCTCAGCCCTAAACATAGATAATTCATAGAAC-C3
Red fox	Vulpes_V5_F	ACTATGCTTAGCCCTAAACATAAATAGTTCTATAACA-C3

**Table 2.** DNA concentration of taxa used to prepare the positive control DNA samples and number of positive controls where the taxon was detected (out of 10). Each DNA sample was diluted 1:100 in the positive control.

Taxa	Initial DNA concentration ng/ul	Number of positive controls where the taxon was detected
<b>Plants</b>		
<i>Carramboa trugillensis</i>	10.80	9
<i>Libanothamnus pluvulus</i>	5.14	9
<i>Passiflora crenata</i>	22.00	9
<b>Vertebrates</b>		
<i>Ursus arctos</i>	0.23	0
<i>Cephalophus monticola</i>	9.26	9
<i>Pagophyla eburnea</i>	3.75	0
<b>Invertebrates</b>		
<i>Coleoptera</i>	7.88	9
<i>Orthoptera</i>	8.38	0
<i>Mantodea</i>	3.75	4

## Supplementary note 1. eDNA metabarcoding

### *DNA extraction and amplification procedure:*

DNA extraction from collected subsamples was performed following the protocol described in Taberlet et al. (2012), using approximately 1-2 g of dried faeces and 5-10 mL of phosphate buffer ( $\text{Na}_2\text{HPO}_4$ ; 0.12 M; pH ~8) per sample, and recovering the DNA extracts in 100  $\mu\text{L}$ . Faecal DNA extractions were carried out in a room dedicated to processing eDNA samples. Ten extraction negative controls (containing only extraction reagents and no faecal material) were included to monitor contamination and were processed following the same protocol as used for the faecal samples. Diet analysis was performed by co-amplifying three universal markers, targeting short (<100 bp) and variable DNA fragments of components of the diet: (i) plants (on the P6 loop of the chloroplast trnL (UAA) intron), GH in<sup>1</sup> or Sper01 in<sup>2</sup>, (ii) vertebrate (marker V5, V5 loop of the mitochondrial 12S gene in<sup>3</sup> or Vert01 in<sup>2</sup>, and (iii) invertebrate (on the mitochondrial 16S gene, MAV in<sup>4</sup> close to Arth01 and Moll01 in<sup>2</sup>. For simplicity, throughout the paper, universal markers will be referred to as the target diet categories i.e. plants, invertebrates and vertebrates. A blocking oligonucleotide was designed and used to avoid amplification with the vertebrate marker of host DNA from each of the two omnivorous predators investigated (Supplementary Table 1). In addition, blocking oligonucleotides specific to human and mammalian sequences were included to prevent their amplification with the vertebrate and invertebrate universal markers, respectively, as suggested in De Barba *et al.*<sup>4</sup>.

Amplifications were carried out using the Qiagen Multiplex PCR kit (QIAGEN GmbH) and contained 1x Qiagen Master Mix, 0.5x Qiagen Q Solution, 0.1  $\mu\text{M}$  F/R universal markers, 1  $\mu\text{M}$  of each blocking marker and 2  $\mu\text{L}$  DNA extract, in 20  $\mu\text{L}$  reaction volume.

Thermocycling conditions had an initial denaturation step of 15 minutes at 95°C, followed by 45 cycles of 30 seconds at 94°C, 90 seconds at 55°C, 60 seconds at 72°C, and a final elongation of 10 minutes at 60°C. Following the protocol in De Barba *et al.* (2014), we used PCR negative (n=10) and positive (n=10) controls to monitor the performance of the amplification and sequencing, and to guide the selection of filtering parameters in the sequence analysis process. Positive controls were made by mixing known quantities of the DNA extract of nine taxa representing plants, vertebrates and invertebrates (Supplementary Table 2). Three PCR replicates were performed for all samples. Markers used in each PCR were uniquely modified by the addition of molecular identifier tags on the 5' end, to allow bioinformatic assignment of sequence reads to their source samples. Tags differed on both

ends of a PCR product and were composed by eight nucleotides, containing at least five differences among them<sup>5</sup>. Empty PCR wells, corresponding to unused tag combinations, were included in the experiment to control for potential tag-jumping events<sup>6</sup>. PCR products were purified using the MinElute PCR purification kit (QIAGEN GmbH), and finally mixed together in equivolume proportions. The sequencing was carried out on HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA), following the manufacturer's instructions, using a commercial service ([www.fasteris.com](http://www.fasteris.com)).

*Sequence analysis and filtering protocol (modified from De Barba et al. 2014):*

The sequence reads were first analysed using the OBITools package<sup>7</sup>. Direct and reverse reads corresponding to a single molecule were assembled and primers and tags were identified. The amplified regions, excluding primers and tags, were kept for further analysis. A separate dataset was created for each marker and strictly identical sequences were clustered together, keeping the information about their distributions among samples. Sequences shorter than 10 (plants, invertebrates) or 30 (vertebrates) bp, or with occurrence lower than 1000 were excluded. Each sequence within a PCR product was classified into the categories of 'head' (the most common sequence within a group of sequences differing by a single indel/substitution), 'internal' (sequences less frequent within the group of related sequences, *i.e.* corresponding to amplification/sequencing errors) or 'singleton' (a sequence with no other variant differing by a single indel/substitution). Taxon assignment was achieved by finding highly similar sequences to the query sequence in the reference databases and assigning a unique taxon to each sequence.

Taxonomically assigned sequences of each marker were then further filtered using R v. 3.3.3<sup>8</sup>. We used the positive and negative controls included in the experiment to set filtering parameters and to evaluate the performance of the experiment. Initially, we discarded sequences with a per sample frequency of occurrence below a given threshold, that was set separately based on each marker data (<0.01 for plants, <0.05 for vertebrates, and <0.02 for invertebrates). Sequences not identified as "head" in  $\geq 2$ , or "singleton" in 3 replicates in at least one sample were considered erroneous and therefore deleted. The sequence data of PCR replicates of every sample were then compared to evaluate the repeatability of results and identify unreliable amplifications. First, we compared PCR replicates of each sample based on sequence frequency of occurrence. A matrix of pairwise distances among sample replicates was created by computing *Rekonen Similarity Index*<sup>9</sup> based on sequence frequency data. Distance measures took a minimum value of 0 if the sets of relative abundances

compared were identical, and a maximum of 1 for completely distinct assemblages (no sequences in common). Only samples having at least 2 out of 3 replicates with distance among each other  $< 0.6$  (threshold set by comparing replicate similarity of PCR negative and positive controls, and faecal samples) were kept for further analysis. Secondly, we compared sequence presence/absence among sample replicates and requested a given sequence to be observed in at least 2 replicates of a sample. Sample replicates were finally combined to obtain a consensus sequence profile for each sample, by taking the mean of the non-zero sequence counts of sample replicates. PCR negative control consensus profiles were inspected and compared to those of positive controls and faecal samples to identify sequences deriving from contamination and low quantity DNA samples more likely to produce unreliable results. Finally, to increase accuracy of the automatic taxonomic assignation, we considered only sequences with identity  $\geq 90\%$  over the entire query sequence length with any reference sequence.

**Table 3.** Overview of species groups detected in the two study areas, Tofte Skov and Høstemark in Lille Vildmose by periodic field observations from 1929-2016. Generally, insects and spiders, as well as most vascular plants, were identified during the growing season (spring to fall; May - September), whereas mammals and birds were subject to more prolonged periods of study across all seasons.

Species group	Period of observation		Season of observation	Order	Families
	Tofte Skov	Høstemark			
<b>Plants</b>					
Vascular plants	2003-2009	2003-2009	Spring-Fall	Multiple	Multiple
Lichens	2016	1993-2000	Spring-Fall	Multiple	Multiple
Mosses	2001-2012	1994-2000	Spring-Fall	Multiple	Multiple
Mushrooms	2016	1992-1994	Spring-Fall	Multiple	Multiple
<b>Invertebrates</b>					
Beetles	2003-2010	1986-2000	Summer	Coleoptera	Multiple
Spiders	2003-2010		Summer	Araneae	Multiple
Dragon- and damselflies		1994-1998	Summer	Odonata	Multiple
Ants*		1994-2000	Summer	Hymenoptera	Formicidae
Butterflies		1922-2000	Summer	Lepidoptera	(Multiple
Buzz flies		1974-2000	Summer	Diptera	Syrphidae
Shield bugs		1988-2000	Summer	Hemiptera	(Multiple
<b>Vertebrates</b>					
Birds	1929-2010	1929-2010	All year	Multiple	Multiple
Mammals	1945-2010	1945-2010	All year	Multiple	Multiple
Reptiles and amphibians		1945-2010	All year	Multiple	Multiple

**Table 4.** Taxa identified in badger, fox and marten faecal samples using eDNA metabarcoding. Frequency of Occurrence (FO) is calculated for each sequence (badger (B), N = 96; fox (F), N = 56; martens (M), N = 13).

Sequence	Order	Family	Genus	Species	B	F	M
gh_00095	Apiales	Apiaceae	<i>Aegopodium</i>	<i>Aegopodium podagraria</i>			1.8
gh_00029	Apiales	Apiaceae	<i>Azorella</i>	<i>Azorella caespitosa</i>	5.2	3.6	7.7
gh_00029	Apiales	Apiaceae	<i>Azorella</i>	<i>Azorella caespitosa</i>			
gh_00029	Apiales	Apiaceae	<i>Azorella</i>	<i>Azorella caespitosa</i>			
gh_00064	Apiales	Apiaceae					1.8 7.7
gh_00307	Asparagales	Asparagaceae	<i>Maianthemum</i>		2.1		
gh_00163	Asparagales	Iridaceae	<i>Iris</i>				1.8
gh_00089	Asterales	Asteraceae	<i>Achillea</i>		5.2		
gh_00033	Asterales	Asteraceae					
gh_00033	Asterales	Asteraceae			2.1	5.4	
gh_00042	Asterales	Asteraceae			3.1	3.6	
gh_00042	Asterales	Asteraceae					
gh_00074	Asterales	Asteraceae			3.1		
gh_00085	Asterales	Asteraceae					5.4
gh_00097	Asterales	Asteraceae			2.1		
gh_00112	Asterales	Asteraceae			1.0		
gh_00041	Asterales	Asteraceae	<i>Scorzoneroides</i>				
gh_00041	Asterales	Asteraceae	<i>Scorzoneroides</i>		3.1	7.1	
gh_00006	Asterales				7.3	10.7	7.7
gh_00006	Asterales						
gh_00006	Asterales						
gh_00079	Asterales						1.8
gh_00301	Asterales						1.8
gh_00113	Boraginales	Hydrophyllaceae	<i>Phacelia</i>	<i>Phacelia tanacetifolia</i>			1.8
gh_00160	Brassicales	Brassicaceae			1.0		
gh_00122	Brassicales	Caricaceae			1.0		
gh_00086	Caryophyllales	Caryophyllaceae	<i>Cerastium</i>		1.0	5.4	
gh_00086	Caryophyllales	Caryophyllaceae	<i>Cerastium</i>				
gh_00044	Caryophyllales	Caryophyllaceae			3.1	3.6	
gh_00044	Caryophyllales	Caryophyllaceae					
gh_00055	Caryophyllales	Caryophyllaceae			5.2	7.1	
gh_00055	Caryophyllales	Caryophyllaceae					
gh_00073	Caryophyllales	Caryophyllaceae			2.1		
gh_00233	Caryophyllales	Caryophyllaceae	<i>Silene</i>		1.0		
gh_00194	Caryophyllales	Caryophyllaceae	<i>Stellaria</i>		1.0	3.6	
gh_00194	Caryophyllales	Caryophyllaceae	<i>Stellaria</i>				
gh_00004	Caryophyllales	Chenopodiaceae	<i>Beta</i>	<i>Beta vulgaris</i>			16.1
gh_00111	Caryophyllales	Polygonaceae			1.0	3.6	
gh_00111	Caryophyllales	Polygonaceae					
gh_00143	Caryophyllales	Polygonaceae					1.8
gh_00148	Caryophyllales	Polygonaceae			2.1		
gh_00030	Caryophyllales	Polygonaceae	<i>Rumex</i>		8.3	8.9	7.7
gh_00030	Caryophyllales	Polygonaceae	<i>Rumex</i>				
gh_00030	Caryophyllales	Polygonaceae	<i>Rumex</i>				

gh_00104	Cucurbitales	Cucurbitaceae			1.0	3.6	
gh_00104	Cucurbitales	Cucurbitaceae					
gh_00087	Cupressales	Cupressaceae					7.7
gh_00266	Dicranales	Dicranaceae	Dicranella	Dicranella heteromalla	1.0	3.6	
gh_00266	Dicranales	Dicranaceae	Dicranella	Dicranella heteromalla			
gh_00170	Dicranales	Dicranaceae				1.8	15.4
gh_00170	Dicranales	Dicranaceae					
gh_00245	Dipsacales	Adoxaceae	Viburnum		1.0		
gh_00082	Equisetales	Equisetaceae	Equisetum		2.1	1.8	7.7
gh_00082	Equisetales	Equisetaceae	Equisetum				
gh_00035	Ericales	Ericaceae	Calluna	Calluna vulgaris	2.1	8.9	7.7
gh_00035	Ericales	Ericaceae	Calluna	Calluna vulgaris			
gh_00035	Ericales	Ericaceae	Calluna	Calluna vulgaris			
gh_00110	Ericales	Ericaceae	Empetrum			1.8	7.7
gh_00106	Ericales	Ericaceae	Erica				
gh_00106	Ericales	Ericaceae	Erica		2.1	5.4	
gh_00146	Ericales	Ericaceae			3.1		
gh_00052	Ericales	Ericaceae	Rhododendron				3.6
gh_00090	Ericales	Ericaceae	Vaccinium		1.0	1.8	
gh_00047	Ericales	Ericaceae	Vaccinium	Vaccinium macrocarpon		1.8	7.7
gh_00168	Ericales	Ericaceae	Vaccinium	Vaccinium microcarpum	1.0		7.7
gh_00142	Ericales	Ericaceae	Vaccinium	Vaccinium vitis-idaea			1.8
gh_00050	Ericales	Primulaceae	Lysimachia		6.3		
gh_00031	Fabales	Fabaceae	Glycine				3.6
gh_00177	Fabales	Fabaceae	Lathyrus	Lathyrus pratensis	1.0		
gh_00043	Fabales	Fabaceae					1.8
gh_00028	Fabales	Fabaceae	Trifolium		1.0		
gh_00037	Fabales	Fabaceae	Trifolium		4.2	3.6	
gh_00037	Fabales	Fabaceae	Trifolium				
gh_00147	Fabales	Fabaceae	Vicia		2.1		
gh_00017	Fagales	Betulaceae	Alnus		20.8	3.6	7.7
gh_00017	Fagales	Betulaceae	Alnus				
gh_00017	Fagales	Betulaceae	Alnus				
gh_00008	Fagales	Betulaceae			20.8	16.1	7.7
gh_00008	Fagales	Betulaceae					
gh_00008	Fagales	Betulaceae					
gh_00023	Fagales	Betulaceae			1.0		
gh_00013	Fagales	Fagaceae	Fagus		16.7	17.9	23.1
gh_00013	Fagales	Fagaceae	Fagus				
gh_00013	Fagales	Fagaceae	Fagus				
gh_00020	Fagales	Fagaceae	Fagus			17.9	23.1
gh_00020	Fagales	Fagaceae	Fagus				
gh_00020	Fagales	Fagaceae	Fagus		16.7		
gh_00005	Fagales	Fagaceae					7.1
gh_00005	Fagales	Fagaceae			13.5		
gh_00007	Fagales	Fagaceae	Quercus		15.6	1.8	
gh_00007	Fagales	Fagaceae	Quercus				
gh_00129	Fagales	Juglandaceae			2.1	1.8	
gh_00129	Fagales	Juglandaceae					

gh_00034	Fagales	Myricaceae			5.4
gh_00141	Hypnales	Brachytheciaceae			1.0
gh_00132	Hypnales				4.2
gh_00193	Hypnales				1.0
gh_00102	Lamiales	Lamiaceae			1.0 1.8
gh_00025	Lamiales				1.0
gh_00039	Lamiales	Plantaginaceae	Plantago		5.2 5.4
gh_00039	Lamiales	Plantaginaceae	Plantago		
gh_00098	Malpighiales	Hypericaceae	Hypericum		1.8
gh_00003	Malpighiales	Passifloraceae	Passiflora		1.0
gh_00009	Malpighiales	Salicaceae			1.0 19.6
gh_00009	Malpighiales	Salicaceae			
gh_00057	Malpighiales	Salicaceae	Populus		3.6 7.7
gh_00057	Malpighiales	Salicaceae	Populus		
gh_00053	Malvales	Malvaceae	Tilia		1.0
gh_00267	Myrtales	Onagraceae	Chamerion	Chamerion angustifolium	1.0
gh_00296	Myrtales	Onagraceae			1.8
gh_00048	Oxalidales	Oxalidaceae	Oxalis		9.4 5.4
gh_00048	Oxalidales	Oxalidaceae	Oxalis		
gh_00253	Pinales	Pinaceae	Cedrus		1.8
gh_00128	Pinales	Pinaceae	NA		1.0
gh_00012	Pinales	Pinaceae	Picea		9.4 12.5 15.4
gh_00012	Pinales	Pinaceae	Picea		
gh_00012	Pinales	Pinaceae	Picea		
gh_00019	Pinales	Pinaceae	Picea		5.2 3.6 46.2
gh_00019	Pinales	Pinaceae	Picea		
gh_00019	Pinales	Pinaceae	Picea		
gh_00015	Pinales	Pinaceae	Pinus		
gh_00015	Pinales	Pinaceae	Pinus		2.1 7.1
gh_00154	Poales	Cyperaceae	Carex		1.0
gh_00228	Poales	Cyperaceae	Carex		1.0
gh_00040	Poales	Cyperaceae	Eriophorum		1.0 3.6
gh_00040	Poales	Cyperaceae	Eriophorum		
gh_00075	Poales	Juncaceae	Juncus		3.1 1.8
gh_00075	Poales	Juncaceae	Juncus		
gh_00184	Poales	Juncaceae	Luzula	Luzula pilosa	1.0
gh_00209	Poales	Juncaceae	Luzula		1.8
gh_00203	Poales	Poaceae	Calamagrostis		1.0 1.8
gh_00054	Poales	Poaceae	Dactylis	Dactylis glomerata	2.1
gh_00127	Poales	Poaceae	Holcus		1.0 1.8
gh_00002	Poales	Poaceae			18.8 7.1
gh_00002	Poales	Poaceae			
gh_00010	Poales	Poaceae			10.4 10.7 7.7
gh_00010	Poales	Poaceae			
gh_00010	Poales	Poaceae			
gh_00016	Poales	Poaceae			4.2 7.1
gh_00021	Poales	Poaceae			3.6
gh_00022	Poales	Poaceae			9.4 16.1
gh_00032	Poales	Poaceae			3.1 1.8



gh_00032	Poales	Poaceae				
gh_00036	Poales	Poaceae			1.0	1.8
gh_00049	Poales	Poaceae			1.0	
gh_00081	Poales	Poaceae			3.1	
gh_00116	Poales	Poaceae			1.0	3.6
gh_00116	Poales	Poaceae				
gh_00171	Poales	Poaceae			1.0	3.6
gh_00171	Poales	Poaceae				
gh_00280	Poales	Poaceae				1.8
gh_00070	Poales	Poaceae	Phragmites	Phragmites australis	2.1	1.8
gh_00070	Poales	Poaceae	Phragmites	Phragmites australis		
gh_00067	Poales	Poaceae	Poa		1.0	5.4
gh_00067	Poales	Poaceae	Poa			
gh_00108	Proteales	Platanaceae	Platanus	Platanus orientalis		3.6
gh_00014	Ranunculales	Ranunculaceae	Ranunculus			
gh_00014	Ranunculales	Ranunculaceae	Ranunculus		4.2	10.7
gh_00191	Ranunculales	Ranunculaceae	Ranunculus		1.0	
gh_00121	Rhizogoniales	Orthodontiaceae	Orthodontium	Orthodontium lineare		1.8
gh_00065	Rosales	Moraceae				1.8
gh_00065	Rosales	Moraceae			3.1	
gh_00065	Rosales	Moraceae				
gh_00051	Rosales	Rosaceae	Filipendula	Filipendula ulmaria	1.0	3.6
gh_00051	Rosales	Rosaceae	Filipendula	Filipendula ulmaria		
gh_00101	Rosales	Rosaceae	Filipendula	Filipendula ulmaria	1.0	
gh_00001	Rosales	Rosaceae			22.9	21.4
gh_00001	Rosales	Rosaceae				7.7
gh_00001	Rosales	Rosaceae				
gh_00018	Rosales	Rosaceae				3.6
gh_00046	Rosales	Rosaceae			2.1	3.6
gh_00186	Rosales	Rosaceae	Potentilla			1.8
gh_00011	Rosales	Rosaceae	Prunus		2.1	7.7
gh_00011	Rosales	Rosaceae	Prunus			
gh_00137	Rosales	Rosaceae	Pyrus			1.8
gh_00084	Rosales	Rosaceae	Rubus	Rubus idaeus	3.1	1.8
gh_00084	Rosales	Rosaceae	Rubus	Rubus idaeus		
gh_00109	Rosales	Ulmaceae				5.4
gh_00204	Sapindales	Aceraceae	Acer		1.0	
gh_00185	Saxifragales	Haloragaceae	Myriophyllum			1.8
gh_00066	Solanales	Solanaceae				1.8
gh_00288	Splachnales	Splachnaceae	Tetraplodon	Tetraplodon pallidus		1.8
gh_00024	Zingiberales	Musaceae			3.1	1.8
gh_00024	Zingiberales	Musaceae				7.7
mav_00067	Agriolimacidae	Agriolimacidae	Deroceras	Deroceras reticulatum	2.1	1.8
mav_00054	Arionidae	Arionidae	Arion	Arion circumscriptus	3.1	
mav_00182	Arionidae	Arionidae	Arion	Arion fuscus	1.0	
mav_00036	Arionidae	Arionidae	Arion	Arion silvaticus	5.2	
mav_00211	Arionidae	Arionidae	Arion		1.0	
mav_00031	Coleoptera	Cantharidae			1.0	
mav_00098	Coleoptera	Chrysomelidae	Aspidomorpha	Aspidomorpha miliaris	1.0	
mav_00007	Coleoptera	Chrysomelidae	Callosobruchus	Callosobruchus analis	6.3	3.6
						23.1

mav_00007	Coleoptera	Chrysomelidae	Callosobruchus	Callosobruchus analis		
mav_00007	Coleoptera	Chrysomelidae	Callosobruchus	Callosobruchus analis		
mav_00137	Coleoptera	Gyrinidae	Macrogyrus	Macrogyrus oblongus	2.1	
mav_00078	Coleoptera	Melyridae	Listrocerus	Listrocerus sp. 1186		7.7
mav_00008	Coleoptera				15.6	
mav_00123	Coleoptera					1.8
mav_00051	Coleoptera	Scarabaeidae				7.7
mav_00037	Coleoptera	Staphylinidae	Lasinus	Lasinus mikado		3.6
mav_00032	Coleoptera	Staphylinidae			2.1	
mav_00026	Diptera	Chironomidae	Paratanytarsus	Paratanytarsus grimmii	1.8	7.7
mav_00086	Diptera	Culicidae	Culex	Culex quinquefasciatus	3.6	7.7
mav_00086	Diptera	Culicidae	Culex	Culex quinquefasciatus		
mav_00092	Diptera	Culicidae	Culex	Culex quinquefasciatus	3.6	15.4
mav_00190	Diptera	Culicidae	Culex	Culex quinquefasciatus	1.8	
mav_00239	Diptera	Culicidae	Culex	Culex quinquefasciatus	1.8	
mav_00029	Diptera	Lauxaniidae	Pseudogriphoneura	Pseudogriphoneura sp. ACMJ-2016	2.1	
mav_00053	Diptera	Phoridae				1.8
mav_00060	Diptera	Sciaridae			2.1	
mav_00091	Glomerida	Glomeridae	Glomeris	Glomeris marginata	1.0	
mav_00096	Helocoidea	Helicidae	Arianta	Arianta arbustorum	1.0	
mav_00275	Helocoidea	Helicidae	Cepaea	Cepaea hortensis	1.0	
mav_00115	Helocoidea	Helicidae	Cepaea		1.0	
mav_00156	Helocoidea	Helicidae	Cepaea		2.1	
mav_00076	Hemiptera	Cicadellidae	Flexamia		1.0	
mav_00035	Hemiptera	Cicadellidae			1.0	
mav_00196	Hemiptera	Cicadellidae				7.7
mav_00284	Hemiptera	Cicadellidae			1.8	
mav_00056	Hemiptera					1.8
mav_00277	Hemiptera	Pentatomidae			1.0	
mav_00012	Hymenoptera	Apidae	Apis	Apis mellifera	5.2	
mav_00019	Hymenoptera	Apidae	Bombus	NA	2.1	7.7
mav_00019	Hymenoptera	Apidae	Bombus	NA		
mav_00165	Hymenoptera	Formicidae	Camponotus	Camponotus herculeanus	1.0	
mav_00304	Hymenoptera	Formicidae	Lasius			1.8
mav_00070	Hymenoptera				1.0	
mav_00129	Isopoda	Porcellionidae	Porcellio	Porcellio scaber	1.8	15.4
mav_00129	Isopoda	Porcellionidae	Porcellio	Porcellio scaber		
mav_00090	Ixodida	Nuttalliellidae	Nuttalliella	Nuttalliella namaqua	2.1	
mav_00099	Ixodida	Nuttalliellidae	Nuttalliella	Nuttalliella namaqua	1.0	
mav_00081	Julida	Blaniulidae	Proteroiulus	Proteroiulus fuscus		7.7
mav_00095	Lepidoptera	Hepialidae				7.7
mav_00015	Lepidoptera					
mav_00015	Lepidoptera				4.2	17.9
mav_00122	Lepidoptera					1.8
mav_00154	Lepidoptera					7.7
mav_00220	Lepidoptera					1.8
mav_00067	Limacoidea	Agriolimacidae	Deroceras	Deroceras reticulatum		
mav_00271	Nudibranchia	Dendrodorididae	Doriopsilla	Doriopsilla miniata		1.8

<b>mav_00050</b>	<i>Nudibranchia</i>				1.0	7.7	
<b>mav_00111</b>	<i>Ophiliones</i>	<i>Phalangiiidae</i>	<i>Phalangium</i>	<i>Phalangium opilio</i>	1.0		
<b>mav_00314</b>	<i>Psocoptera</i>	<i>Liposcelidae</i>	<i>Liposcelis</i>			1.8	
<b>mav_00017</b>	<i>Thysanoptera</i>	<i>Thripidae</i>	<i>Scolothrips</i>	<i>Scolothrips takahashii</i>		10.7	
<b>mav_00072</b>	<i>Thysanoptera</i>	<i>Thripidae</i>	<i>Thrips</i>	<i>Thrips flavidulus</i>			46.2
<b>v5_00056</b>	<i>Anseriformes</i>	<i>Anatidae</i>				1.8	
<b>v5_00012</b>	<i>Anura</i>	<i>Bufo</i>	<i>Bufo</i>	<i>Bufo bufo</i>	6.3	1.8	
<b>v5_00012</b>	<i>Anura</i>	<i>Bufo</i>	<i>Bufo</i>	<i>Bufo bufo</i>			
<b>v5_00011</b>	<i>Anura</i>	<i>Ranidae</i>	<i>Rana</i>		12.5		
<b>v5_00010</b>	<i>Anura</i>	<i>Ranidae</i>	<i>Rana</i>	<i>Rana temporaria</i>	7.3	7.7	
<b>v5_00010</b>	<i>Anura</i>	<i>Ranidae</i>	<i>Rana</i>	<i>Rana temporaria</i>			
<b>v5_00022</b>	<i>Artiodactyla</i>	<i>Bovidae</i>	<i>Bos</i>			1.8	
<b>v5_00028</b>	<i>Artiodactyla</i>	<i>Cervidae</i>	<i>Capreolus</i>	<i>Capreolus capreolus</i>		5.4	
<b>v5_00004</b>	<i>Artiodactyla</i>	<i>Cervidae</i>	<i>Cervus</i>	<i>Cervus elaphus</i>			
<b>v5_00004</b>	<i>Artiodactyla</i>	<i>Cervidae</i>	<i>Cervus</i>	<i>Cervus elaphus</i>	12.5	25.0	
<b>v5_00023</b>	<i>Artiodactyla</i>	<i>Cervidae</i>	<i>Dama</i>	<i>Dama dama</i>		10.7	
<b>v5_00006</b>	<i>Artiodactyla</i>	<i>Suidae</i>	<i>Sus</i>	<i>Sus scrofa</i>			
<b>v5_00006</b>	<i>Artiodactyla</i>	<i>Suidae</i>	<i>Sus</i>	<i>Sus scrofa</i>	3.1	39.3	
<b>v5_00005</b>	<i>Carnivora</i>	<i>Canidae</i>	<i>Nyctereutes</i>	<i>Nyctereutes procyonoides</i>		1.8	7.7
<b>v5_00008</b>	<i>Carnivora</i>	<i>Mustelidae</i>	<i>Martes</i>				107.7
<b>v5_00039</b>	<i>Columbiformes</i>	<i>Columbidae</i>	<i>Columba</i>				7.7
<b>v5_00024</b>	<i>Galliformes</i>	<i>Phasianidae</i>	<i>Phasianus</i>		1.0		
<b>v5_00064</b>	<i>Insectivora</i>	<i>Soricidae</i>	<i>Sorex</i>		1.0		
<b>v5_00020</b>	<i>Insectivora</i>	<i>Soricidae</i>	<i>Sorex</i>	<i>Sorex araneus</i>	1.0		
<b>v5_00042</b>	<i>Insectivora</i>	<i>Talpidae</i>	<i>Talpa</i>	<i>Talpa europaea</i>	1.0		
<b>v5_00149</b>	<i>Lagomorpha</i>	<i>Leporidae</i>	<i>Lepus</i>	<i>Lepus europaeus</i>	1.0		
<b>v5_00159</b>	<i>Passeriformes</i>	<i>Sturidae</i>				1.8	
<b>v5_00033</b>	<i>Passeriformes</i>	<i>Turdidae</i>	<i>Turdus</i>	<i>Turdus philomelos</i>	1.0		
<b>v5_00065</b>	<i>Pelecaniformes</i>	<i>Phalacrocoracidae</i>	<i>Phalacrocorax</i>	<i>Phalacrocorax carbo</i>		1.8	
<b>v5_00007</b>	<i>Rodentia</i>	<i>Cricetidae</i>	<i>Microtus</i>	<i>Microtus subterraneus</i>	13.5	19.6	30.8
<b>v5_00007</b>	<i>Rodentia</i>	<i>Cricetidae</i>	<i>Microtus</i>	<i>Microtus subterraneus</i>			
<b>v5_00007</b>	<i>Rodentia</i>	<i>Cricetidae</i>	<i>Microtus</i>	<i>Microtus subterraneus</i>			
<b>v5_00068</b>	<i>Rodentia</i>	<i>Cricetidae</i>	<i>Microtus</i>	<i>Microtus subterraneus</i>		1.8	
<b>v5_00026</b>	<i>Rodentia</i>	<i>Cricetidae</i>	<i>Myodes</i>	<i>Myodes glareolus</i>		1.8	
<b>v5_00015</b>	<i>Rodentia</i>	<i>Muridae</i>	<i>Apodemus</i>				
<b>v5_00015</b>	<i>Rodentia</i>	<i>Muridae</i>	<i>Apodemus</i>		1.0	3.6	
<b>v5_00034</b>	<i>Rodentia</i>	<i>Muridae</i>	<i>Micromys</i>	<i>Micromys minutus</i>		1.8	7.7
<b>v5_00074</b>	<i>Strigiformes</i>	<i>Strigidae</i>	<i>Asio</i>	<i>Asio otus</i>		1.8	

**Table 5.** Taxa identified in badger, fox and marten faecal samples using macroscopic diet analysis. Frequency of Occurrence (FO) is calculated for each taxonomic classification for all predators (badger, N = 96; fox, N = 56; marten, N = 13).

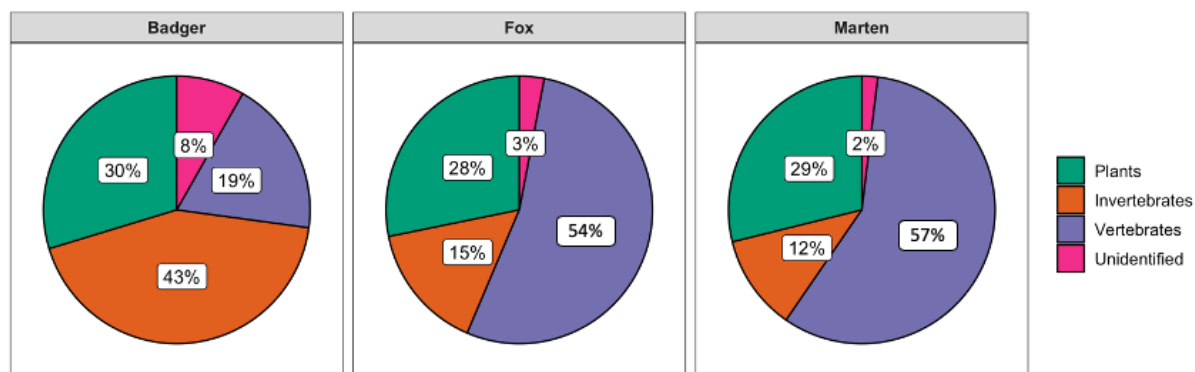
Order	Family	Genus	Species	Badger	Fox	Marten
Poales				16.7	10.7	15.4
Poales	Poaceae	Zea	Zea mays	22.9		
Rosales	Rosaceae	Malus		6.3	7.1	
Rosales	Rosaceae	Prunus		2.1	3.6	
Rosales	Rosaceae	Pyrus		1.0		
Rosales	Rosaceae	Rubus		2.1		
Rosales	Rosaceae	Sorbus	Sorbus aucuparia			7.7
Coleoptera	Carabidae	Carabua	Carabus granulatus	1.0		
Coleoptera	Carabidae			2.1		
Coleoptera	Chrysomelidae			1.0		
Coleoptera				84.4	25.0	30.8
Coleoptera	Scarabaeidae			2.1		
Coleoptera	Staphylinoidea			2.1		
Diptera	Muscidae	Musa	Musa domestica	1.0		
Diptera				1.0		
Hymenoptera	Formicidae			1.0		
Hymenoptera				32.3	7.1	
Isopoda				1.0	1.8	
Squamata	Lacertidae			3.1	5.4	
Squamata	Serpentes			3.1	3.6	
Squamata	Viperidae	Vipera	Vipera berus	2.1		
Anseriformes					3.6	
Anura				33.3	16.1	
Artiodactyla	Cervidae			3.1	51.8	
Artiodactyla	Suidae	Sus	Sus scrofa		1.8	7.7
Carnivora	Mustelidae	Martes	Martes foina		1.8	
Carnivora	Mustelidae	Martes	Martes martes			7.7
Carnivora	Mustelidae	Mustela	Mustela lutreola		1.8	15.4
Carnivora	Mustelidae	Mustela	Mustela nivalis		1.8	
Carnivora	Mustelidae				3.6	7.7
Columbiformes						7.7
Erinaceomorpha	Erinaceidae	Erinaceus			1.8	
Eulipotyphla				1.0		
Eulipotyphla	Soricidae			5.2		
Lagomorpha	Leporidae	Lepus	Lepus europaeus	1.0		
Passeriformes				3.1	3.6	7.7
Rodentia	Cricetidae	Arvicola	Arvicola amphibius	6.3		
Rodentia	Cricetidae	Arvicola	Arvicola amphibius		3.6	7.7
Rodentia	Cricetidae	Microtus	Microtus agrestis	2.1	14.3	
Rodentia	Cricetidae	Microtus	Microtus agrestis			30.8
Rodentia	Cricetidae	Microtus	Microtus arvalis			7.7
Rodentia	Cricetidae	Myodes	Myodes glareolus	2.1	1.8	
Rodentia	Cricetidae			4.2	17.9	23.1
Rodentia	Muridae	Mus	Mus musculus	1.0		
Rodentia	Muridae			1.0	3.6	
Rodentia	Muridae	Rattus		2.1		
Rodentia				2.1	3.6	

**Table 6.** Species and orders identified in the diet of red fox, European badger and martens using eDNA metabarcoding, but not observed by field observations. Origin and expected probability of presence in Denmark (based on national species databases).

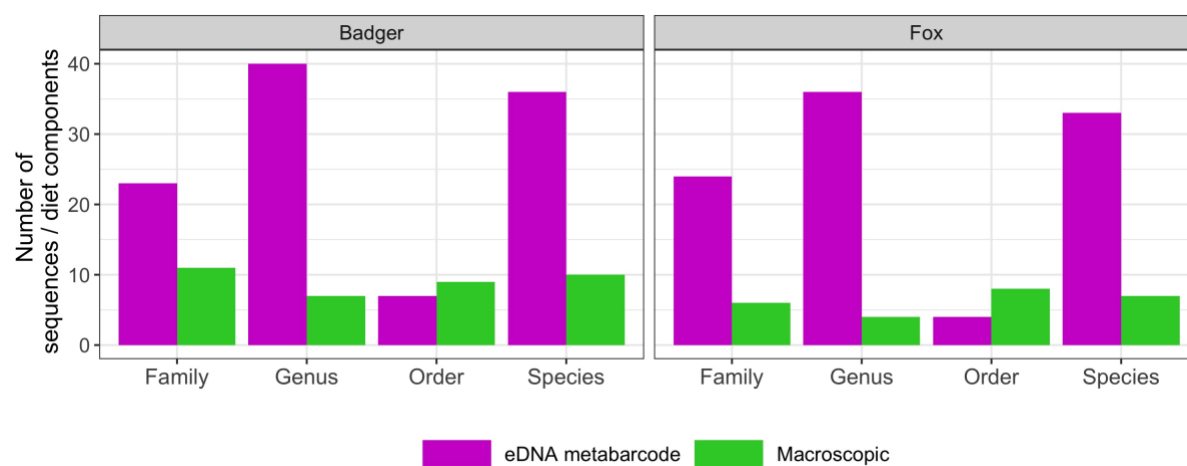
Order	Species	Distribution	In Denmark?
Acari	Ricinus	Common	Yes
Apiales	Azorella caespitosa	Fakland island (not Europe)	No (family, Apiaceae is in Denmark)
Arionidae	Arion circumscriptus	Common	Yes
Arionidae	Arion fuscus	Europe and Asia	Maybe
Arionidae	Arion silvaticus	Europe	Maybe
Artiodactyla	Dama dama	Common	Yes
Boraginales	Phacelia tanacetifolia	America	Maybe introduced
Carnivora	Mustela lutreola	Not present	No (genus Mustela, and Mustela putorius are found in Denmark)
Caryophyllales	Beta vulgaris	Southeast Europe	Maybe
Coleoptera	Aspidomorpha miliaris	Asia	No (genus, Aspidomorpha is in Denmark)
Coleoptera	Callosobruchus analis	Asia	Maybe introduced
Coleoptera	Lasinus mikado	Japan & Russia	Maybe introduced
Coleoptera	Listrocerus sp. 1186	Information limited	Limited information (Order, Coleoptera is in Denmark)
Coleoptera	Macrogyrus oblongus	Australia	No (family, Gyrinidae, is in Denmark)
Cucurbitales		Agriculturally grown	Yes
Cupressales		Common	Yes
Dicranales	Dicranella heteromalla	American	Yes
Diptera	Culex quinquefasciatus	Africa & south America	No (genus, Culex is in Denmark)
Diptera	Musa domestica	Common	Yes
Diptera	Paratanytarsus grimmii	America	Maybe introduced
Diptera	Pseudogriphoneura sp. ACMJ-2016	Europe	Maybe
Ericales	Vaccinium macrocarpon	America	Introduced
Ericales	Vaccinium microcarpum	Europe	Yes
Erinaceomorpha		Common	Yes
Glomerida	Glomeris marginata	Common	Yes
Helocoidea	Arianta arbustorum	Common	Yes
Helocoidea	Cepaea hortensis	Common	Yes
Hemiptera		Common	Yes
Hymenoptera	Apis mellifera	Common	Yes
Hypnales		Common	Yes
Isopoda	Porcellio scaber	Common	Yes
Ixodida	Nuttalliella namaqua	Africa	Maybe
Julida	Proteroiulus fuscus	Common	Yes
Lepidoptera		Common	Yes
Limacoidea	Deroceras reticulatum	Scandinavia	Yes

Myrtales	Chamerion angustifolium	Common	Yes
			No
Nudibranchia	Doriopsilla miniata	Tropical & subtropical	(family, Dendrodorididae is in Denmark)
Opiliones	Phalangium opilio	Common	Yes
Poales	Dactylis glomerata	Common	Yes
Poales	Zea mays	Common	Yes
Proteales	Platanus orientalis	Common	Yes
Psocoptera		Common	Yes
Rhizogoniales	Orthodontium lineare	Introduced	Introduced
Rodentia	Microtus arvalis	Common	Yes
Rodentia	Microtus subterraneus	Common	Yes
Rodentia	Myodes glareolus	Common	Yes
Splachnales	Tetraplodon pallidus	American	Maybe introduced
Thysanoptera	Scolothrips takahashii	Common	Yes
Thysanoptera	Thrips flavidulus	Africa, Europe	Maybe introduced
Zingiberales		Tropical	No (could be introduced or left by human*)

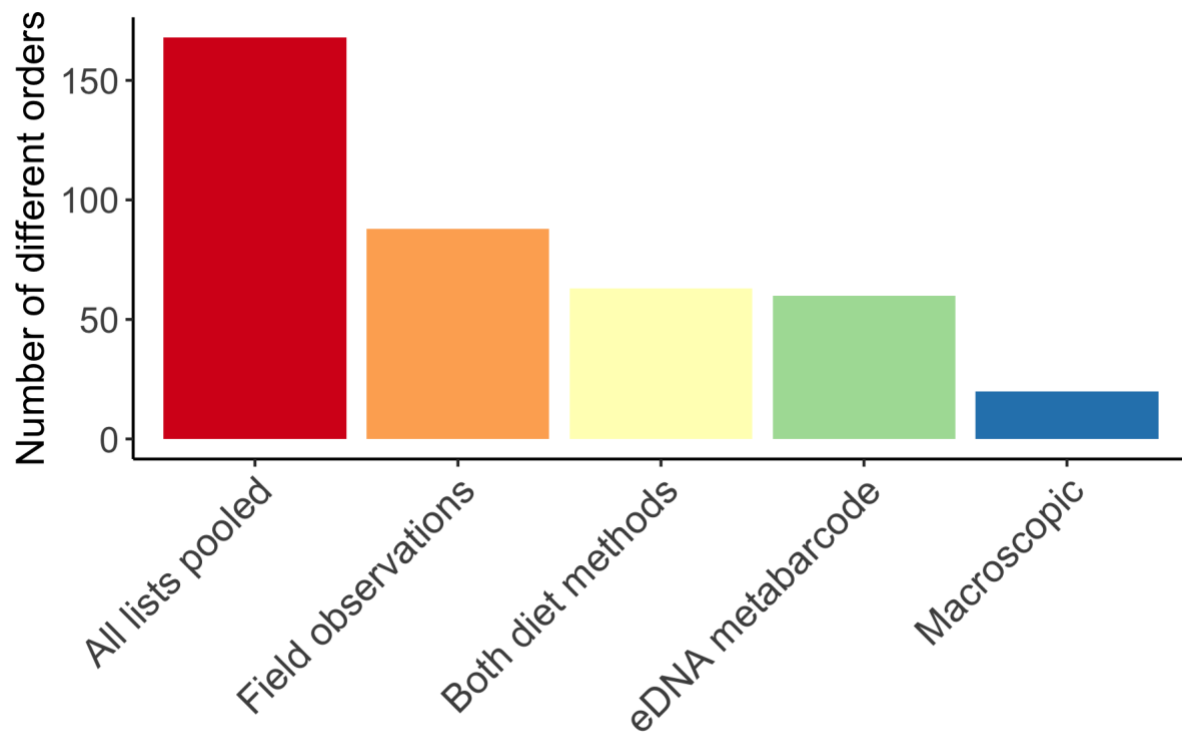
\* *Zingiberales* covers a series of tropical plants such as the ginger family, banana family etc., so this order could have been present due to human interventions.



**Figure S1.** Macroscopic diet composition on major dietary components in badger, fox and marten samples (plant, invertebrate, vertebrate and unidentified).



**Figure S2.** Taxonomic resolution achieved using eDNA metabarcoding and macroscopic diet analysis. Shown are number of different sequences (eDNA metabarcoding) and dietary components (macroscopic diet analysis) identified to either species, genus, family, order, or above order level (*i.e.* class or kingdom) in badger and fox samples.



**Figure S3.** Number of orders detected using the three approaches; all lists (field observations, eDNA metabarcoding and macroscopic diet analysis from badger, fox and marten) pooled, field observations only, both diet assessment methods pooled, and detections using either of the two dietary methods individually.



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