RESEARCH ARTICLE

Molecular characterization of the microbial communities in the subcaudal gland secretion of the European badger (*Meles meles*)

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Abstract

Many mammals possess specialized scent glands, which convey information about the marking individual. As the chemical profile of scent marks is likely to be affected by bacteria metabolizing the primary gland products, the variation in bacterial communities between different individuals has been proposed to underpin olfactory communication. However, few studies have investigated the dependency of microbiota residing in the scent organs on the host's individual-specific parameters. Here, we used terminal restriction fragment length polymorphism analysis of the PCR-amplified 16S rRNA gene and clone library construction to investigate the microbial communities in the subcaudal gland secretion of the European badger (*Meles meles*). As the secretion has been shown to encode individual-specific information, we investigated the correlation of the microbiota with different individual-specific parameters (age, sex, body condition, reproductive status, and season). We discovered a high number of bacterial species (56 operational taxonomic units from four phyla: Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes), dominated by Actinobacteria (76.0%). The bacterial communities of cubs and adults differed significantly. Cubs possessed considerably more diverse communities dominated by Firmicutes, while in adults the communities were less diverse and dominated by Actinobacteria, suggesting that the acquisition of a 'mature bacterial community' is an ontogenetic process related to physiological changes during maturation.

Introduction

Scent marking is common in mammals, and urine, feces, or secretions from specialized scent glands can convey information about the marking individual (Brown & Macdonald, 1985). Scent glands typically contain sebaceous and apocrine glands that secrete fatty and/or organic substances into scent pouches or dense hair tufts on the skin surface (Albone & Shirley, 1984; Brown & Macdonald, 1985; Thody & Shuster, 1989). These places are generally warm, moist, nutrient rich, and ideal for the proliferation of symbiotic bacteria, which are proposed to play an important role in scent marking (Albone *et al.*, 1974; Albone & Perry, 1975) as they are likely to metabolize the primary scent gland products. The fermentation hypothesis for chemical recognition (Albone *et al.*, 1974; Albone & Perry, 1975; Gorman, 1976) proposes that symbiotic bacteria living in scent glands produce volatile odorants that contribute to cues used for individual recognition and that these cues vary with the composition of the bacterial community. Mammalian scent glands often harbor diverse bacterial communities (reviewed in Archie & Theis, 2011), which include fermentative anaerobic bacteria that produce short-chain fatty acids (SCFAs) among other odorants. Depending on pH, available primary gland products, and other factors, bacterial communities may vary with individual-specific parameters, such as the age, sex, individual identity, reproductive status, health status, diet, and competitive ability of their hosts.

Although odor generation and olfactory communication in mammals generally involve microbiota, few studies have investigated the microbial communities residing

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in scent organs or their potential dependency on individual-specific parameters. Here, we investigated the bacterial communities in the subcaudal gland of the European badger (*Meles meles*). As a nocturnal carnivore with a well-developed sense of smell, *M. meles* constitutes a good model species to study olfactory communication (Buesching & Macdonald, 2001). Badgers possess a unique skin gland, the subcaudal gland, which consists of a well-developed subcaudal pouch and is lined with sebaceous and apocrine tissues secreting paste-like secretion into a common lumen (Stubbe, 1971). The pouch opens into the environment through a 20–80 mm wide horizontal slit (Buesching *et al.*, preparation), situated between the base of the tail and the anus. The secretion is transferred from the pouch onto the substrate by a quick squatting motion during which the pouch opening is pressed briefly against objects (Buesching & Macdonald, 2004) or conspecifics (Buesching *et al.*, 2003).

The subcaudal gland secretion has been shown to encode information about fitness-related parameters in addition to group membership and individuality (Gorman *et al.*, 1984; Buesching *et al.*, 2002b). The lower molecular weight components of the odor are thought to be byproducts from bacteria metabolizing the primary gland products (Kruuk *et al.*, 1984), and the time-related changes in the chemical composition of the secretion are believed to be the results of chemical and microbial degradation (Buesching *et al.*, 2002c). The microbial communities inhabiting the subcaudal pouch of *M. meles* is thus likely to play an important role in generating the factual odor of the subcaudal gland secretion. Therefore, we hypothesized that the bacterial pouch communities should vary with individual-specific parameters such as age, sex, body condition, and reproductive status. Badger cubs, however, do not start to produce subcaudal gland secretion until approximately 4 months of age (Buesching *et al.*, 2003; Fell *et al.*, 2006), and increase in secretion during their first year is age related (Buesching *et al.*, 2003). The slow raise in the amount of secretion stored in the subcaudal pouch should thus boost bacterial colonization (Nordstrom & Noble, 1984), which is likely to happen as a succession of communities (Favier *et al.*, 2002). Hence, we anticipated the bacterial communities of cubs and adults to differ.

The objective of this study was to investigate the microbial community using a culture-independent method: terminal restriction fragment length polymorphism (T-RFLP; Liu *et al.*, 1997) in combination with cloning and sequencing of PCR-amplified 16S rRNA genes. In the past, most studies on the microbial communities of mammalian scent organs used traditional culture-dependent techniques to isolate and identify bacteria from cultures. However, only a very small portion of bacteria (< 1%) from the environment can be cultivated (Amann *et al.*, 1995; Pace, 1997; Hugenholtz *et al.*, 1998), whereas culture-independent molecular methods, such as T-RFLP, can overcome the limitations of the traditional cultivation approach. Although all fingerprinting techniques have limitations, such as not detecting rare species (Bent *et al.*, 2007; Orcutt *et al.*, 2009), T-RFLP provides a convenient way to determine the diversity of bacterial communities. More importantly, it also allows comparison of the relative proportions of bacteria among sampled communities, which would be impossible with culture techniques. In this study, we aimed to (1) characterize the microbial community of the subcaudal gland secretion and (2) investigate the correlation of the microbiota with different individual-specific parameters (age, sex, season, body condition, and reproductive status). According to the fermentation hypothesis, we predicted bacterial communities to show differences between some, if not all, of the factors, especially between age categories.

Materials and methods

Study animals and sample collection

Subcaudal gland secretion samples were collected in spring (27 May–9 June) and summer (6–18 September) of 2010 from a population of *M. meles* in Wytham Woods, Oxfordshire, UK (global positioning system reference 51:46:26N, 1:19:19W; trapping and handling was subject to the University of Oxford's ethical review and performed under government licenses). As part of an ongoing long-term study, this badger population has been trapped 3–5 times per year since 1987 (for details see Macdonald & Newman, 2002). A unique number, tattooed in the left inguinal region at first capture (usually as cubs), permits individual identification and determination of age. After capture, badgers were sedated with 0.2 mL of ketamine hydrochloride per kg body weight. Tattoo, sex, and body condition (categorized as 1 [= emaciated]−3 [= very good condition]; following Buesching *et al.*, 2009; Macdonald *et al.*, 2009) were recorded. Length and diameter of teats were measured to establish recent lactation in females (Dugdale *et al.*, 2011a), and the degree to which the testes were descended into the scrotum was examined in males (Buesching *et al.*, 2009). As badgers are induced ovulators (Yamaguchi *et al.*, 2006), it is unlikely that the microbial community would mirror such short-term endocrinological changes, and thus, no attempt was made to correlate pouch bacteria with female estrus.

Thirty-nine secretion samples were collected in spring and 40 in summer (Table 1). Eighteen animals (8 males and 10 females) were sampled in both trapping events.

Table 1. Details of analyzed *Meles meles* subcaudal gland secretion samples

	Season		
	Spring	Summer	Total
Cubs			
Male	1	2	3
Female	1	2	3
Adults			
Male	10	16	26
Female	27	20	47
Total	39	40	79

Subcaudal gland secretion was scooped out of the subcaudal pouch using a rounded stainless steel spatula (Buesching *et al.*, 2002b) and stored inside a microcentrifuge tube. Samples were frozen immediately and stored at −20 °C until DNA extraction. Between sampling, the spatula was wiped clean and sterilized with absolute ethanol and a flame to avoid contamination of samples. Contact of the spatula with body parts other than the inside of the subcaudal pouch was strictly avoided.

Only 16.7% (total number = 42) of the cubs had detectable amounts of secretion in their subcaudal pouches in spring, and 57.9% (total number = 19) in summer. However, the volume was usually too small to collect for further analyses, and thus, only six samples were derived from cubs in this study.

DNA extraction

Pretreatment for Gram-positive bacteria was carried out to lyse the bacterial cell walls before DNA purification. Secretions were transferred to microcentrifuge tubes, resuspended in 180 μL of enzymatic lysis buffer [20 mM Tris·Cl (pH 8.0), 2 mM EDTA, 1.2% Triton X-100] containing 20 mg mL^{-1} of lysozyme, and incubated at 37 °C for at least 1 h in a shaking incubator. Samples were then incubated with proteinase K at 56 °C for at least 1 h, followed by the addition of 200 μL of ethanol. DNA isolation was continued with pipetting the mixture into the spin column according to the manufacturer's instructions for animal tissue samples using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany).

T-RFLP analysis

Internal fragments of 16S rRNA genes were amplified from the isolated DNA using universal bacterial primers 341f (5′-CCTACGGGAGGCAGCAG-3′; Muyzer *et al.*, 1993) and 926r (5′- CCGTCAATTCMTTTRAGTTT-3′; Muyzer *et al.*, 1995). Primer 341f was labeled at its 5′ end with the dye 6-carboxyfluorescein (6-FAM) and 926r labeled with hexachloro-6-carboxyfluorescein (HEX). PCR mixtures comprised 1× PCR buffer, 1 mM $MgCl_2$, each dNTP at a concentration of 0.2 mM, and each primer at a concentration of 0.6 μM and 0.5 U Taq polymerase (Bioline). Amplification was carried out in two separate 25-μL reaction mixtures under conditions: 95 °C for 3 min, amplified for 35 cycles of 95 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min 15 s, and a final extension at 72 °C for 10 min. For each sample, duplicate fluorescently labeled PCR products were pooled after PCR. PCR product sizes were confirmed by agarose gel electrophoresis using 100-bp DNA ladders as size markers and by staining with ethidium bromide. The amplified products (around 586 bp) were purified using the QIAquick gel extraction kit (Qiagen).

Fluorescently labeled PCR products were digested with MspI restriction enzymes [Applied Biosystems (ABI), Foster City, CA] for 6 h at 37 °C, followed by treatment for 20 min at 80 °C for enzyme inactivation. The digested fragments were separated on the ABI 3730 Genetic Analyzer (ABI). The sizes of the fluorescently labeled terminal restriction fragments (TRFs) were determined by comparison with the GeneScan 500 ROX size standard (ABI). Before injection, 0.5 μL of the DNA sample was denatured in the presence of 9.5 μL of Hi-Di formamide and 0.05 μL of GS 500 ROX size standard (ABI) at 95 °C for 5 min. Injection was performed electrokinetically at 2 kV for 10 s, and electrophoresis was run at 15 kV for 30 min. After electrophoresis, T-RFLP electropherograms were imaged using the GENEMAPPER software (v. 3.7; ABI). The lengths of TRFs were determined by comparison with the internal standard, within the lower threshold at 50 bp and upper threshold at 500 bp. Only peaks with heights exceeding 50 fluorescence units were evaluated.

T-RFLP profiles were aligned using T-Align (Smith *et al.*, 2005), which identified all fragments unequivocally with ±0.5 bp in all profiles generated, and determined the presence and absence of TRFs and their relative fluorescence in all samples. log $(n + 1)$ transformation was performed to normalize the data.

Cloning and sequencing

The identities of bacterial species corresponding to the TRFs were confirmed by cloning and sequencing. The 16S rRNA genes were amplified from genomic DNA using the unlabeled primers 341f and 926r using the cycling conditions as described previously. DNA samples from nine individuals, which comprised > 80% of the TRFs were selected. Duplicate PCR products from each individual were pooled after PCR and the around 586-bp-amplified products were purified using the QIAquick gel extraction kit (Qiagen). Purified PCR fragments were ligated into a

TA cloning vector (pGEM-T Easy Vector Systems; Promega, Madison, WI). Transformation, growth and blue–white selection were carried out in *Escherichia coli* competent cells (JM109). PCRs were performed on 384 positive clones using M13 forward and reverse primers (Messing, 1983). The PCR products were digested with MspI (ABI) as above and restriction fragments were separated in a 2.5% agarose gel. Unique RFLP patterns were identified and a total of 96 corresponding PCR products were purified with ExoSAP-IT (GE Healthcare, Buckinghamshire, UK) and sequenced.

All amplified fragments were sequenced with M13 primers using dideoxynucleotide chain termination and loaded on an automated DNA Sequencer (48-well capillary ABI3730; ABI). Nucleotide sequences were analyzed using CodonCode Aligner 3.7.1 (CodonCode Corporation, Dedham, MA) and were compared with GenBank sequences using the NCBI BLAST program (Altschul *et al.*, 1990). The DNA sequences from this study were assigned GenBank accession numbers JN806160–JN806215.

Phylogenetic tree reconstruction and statistical analyses

Phylogenetic analyses were performed on the consensus alignments of the sequenced 16S rRNA sequences. Bayesian phylogenetic inference was performed using MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003). The web-based application FINDMODEL (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) was employed to find the best-fit model of nucleotide substitution. The general time-reversible model with gamma-distributed rate variation (GTR + Γ) was determined to be the best fit for the dataset. A Markov chain Monte Carlo search was initiated with random trees and run for 3 000 000 generations, with a sampling frequency of every 100 generations. The standard deviation of split frequencies converged to a value < 0.01 for the 16S rRNA gene analyses. After convergence was checked by plotting the likelihood scores against generation, the first 25% of the generations were discarded as the burn-in of the chain (Ronquist & Huelsenbeck, 2003).

Discriminant function analysis (DFA) was used to test the hypothesis that the relative abundance of bacteria groups in the secretion was different between different classes of individuals. The samples were categorized by age, sex, body condition, reproductive status of the individual, and season of sampling. Nineteen males (with fully descended testes) were classified as in breeding condition, while only one male (with completely retracted testes) was classified as not in breeding condition and six males were in an intermediate state so that the reproductive activity could not be determined unambiguously.

DFA generates discriminant functions that provide the best discrimination between the defined categories. Here, the statistical significance of category differences was tested using Wilks' λ and chi-squared. The effectiveness of the discriminant function to correctly assign cases to categories was tested using classification statistics. The 'leave-one-out' cross-validation analysis was reported because it produced a more reliable function as the case to be classified was not used to develop the discriminant function.

An analysis of similarity (ANOSIM; Clarke, 1993) was conducted to verify the significance of the grouping and examine the degree of similarity between bacterial communities. A Bray–Curtis similarity matrix computed from the relative abundance of all TRFs was used to generate one-way ANOSIM statistics with 999 permutations. It calculated a test statistic (R) that is equal to 1 if all individuals within a category were more similar to each other than to any individual in another category and 0 if there was no difference between categories. The similarity percentage (SIMPER; Clarke & Gorley, 2001) procedure was used to determine the percentage contribution of each TRF to the overall difference between categories.

The bacterial complexity was described using two diversity indices. Richness was defined as the total number of TRF (bacterial groups) in the TRF profile. The Shannon index (H′) took into account the evenness in addition to the species number and was thus higher if the diversity is more even. The diversity indices were compared using Kruskal–Wallis test.

DFA and Kruskal–Wallis tests were performed using PASW 18.0 (SPSS, Chicago, IL). The ANOSIM and SIMPER analyses were performed using PRIMER 6 (Primer-E Ltd; Clarke, 1993).

Results

T-RFLP analysis

The structure of bacterial communities associated with the subcaudal gland secretion of European badgers was determined by T-RFLP analysis of the 16S rRNA gene. This approach enabled us to determine differences in relative abundances of some phyla. Analysis of the T-RFLP data revealed a total of 50 TRFs in the subcaudal gland secretion of the 79 sampled badgers. The mean number of TRFs found in an individual was 5.58 (range 2–15, SD ± 1.58). None of the TRFs were recorded in all individuals. The most common TRF was 140.4 (Supporting Information, Tables S1 and S2), which was present in 91.1% (72/79) of the individuals. The corresponding reverse primer TRFs (62.2 and 368) were present in 19.0% and 97.5% of the individuals, respectively. This

TRF (140.4) accounted for an average of 72.9% (SD ± 16.3%) of the total fluorescence area of the T-RFLP profiles from all badgers.

Analysis of clone libraries

To provide information regarding the identity of the bacterial species, 16S rRNA gene clone libraries were constructed from DNA isolated from the subcaudal gland secretion. Clone libraries were generated from nine badgers, and a total of 384 clones were screened for their individual RFLP types. All unique and redundant RFLP types were selected for sequencing.

Of the 96 clones sequenced, 56 operational taxonomic units (OTUs) were found. We assigned a species to an OTU with $\geq$ 97% identity, while those with $\geq$ 95% identity were assigned to the same genus (Schloss & Handelsman, 2005; Santelli *et al.*, 2008). The OTUs clustered into four bacterial phyla: Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes (Fig. 1). The Proteobacteria present belonged to the alpha, beta, and gamma classes, with the most OTUs (eight OTUs) belonging to the Alphaproteobacteria (Fig. 1), while members of Gammaproteobacteria were not abundant [relative abundance (i.e. abundance of corresponding TRFs within T-RFLP profiles) = 0.06%]. Members of the Actinobacteria were the most abundant (relative abundance = 76.0%), with the majority corresponding to the order Actinomycetales (SGS-17, 19, 20, 21, 22, 24, 25 and 26). The phylum Firmicutes was the most diverse group with 23 OTUs identified, which were closely related to *Enterococcus*, *Aerococcaceae*, *Lactobacillus*, *Streptococcus*, *Eubacterium*, *Clostridium*, and *Negativicoccus*. Only four OTUs of Bacteroidetes were found, which were not abundant and diverse compared with other phyla (relative abundance = 0.6%).

Identification of TRFs from 16S rRNA-sequenced genes

The nucleotide sequence data obtained were used to identify the corresponding TRFs based on the predicted sizes for the TRFs generated following restriction digestion (Table S1). Twenty-eight per cent of TRFs were identified, which contributed to 93.9% of the total peak area in all individuals. However, for many of the TRFs, more than one species/genus could be matched with the particular fragment size. Differences existed between the

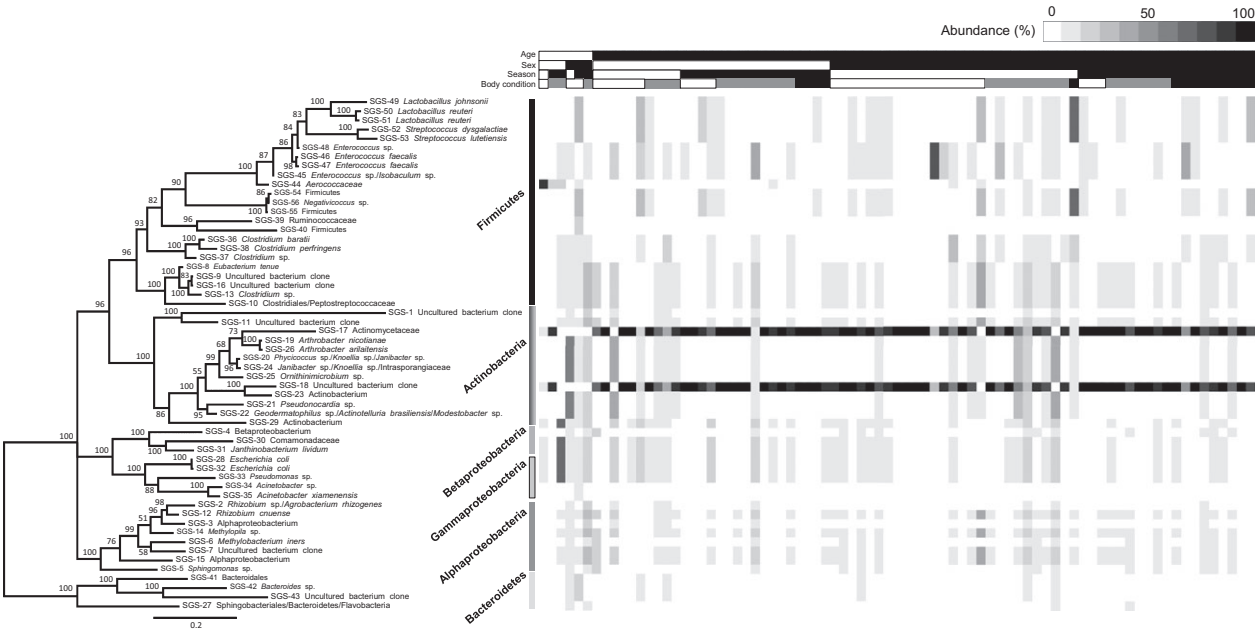

Fig. 1. Phylogenetic tree showing different OTUs found in the subcaudal gland secretions of nine *Meles meles* based on 16S rRNA gene sequences. Bayesian posterior probabilities above 50% are shown above branches. Sequences were assigned to the same species with $\geq$ 97% identity, whereas sequences with $\geq$ 95% identity were assigned the genus name followed by 'sp.'. The right side of the figure shows the relative abundance of forward TRFs per sample in gray scale values (white: 0% present; black: 90–100%). Bars above the abundance graph indicate age (white = cubs; black = adults), sex (white = male; black = females), season of sampling (white = spring; black = summer), and body condition (white = emaciated; black = very good condition) of each individual sampled. Attention should be paid that some OTUs correspond to the same TRFs (see Table S1 for details) and thus have the same abundance patterns.

observed and predicted TRFs, which have been reported frequently in other studies (Kaplan *et al.*, 2001).

By linkage of TRFs to the sequence data, we found that the abundance of TRFs corresponding to Actinobacteria represented more than 76% of all bacterial communities, followed by Firmicutes and Proteobacteria with relative abundances of more than 9% and 3%, respectively. The most dominant TRF (140.4), found in the majority of the individuals, were in phylum Actinobacteria (Fig. 1). Approximately, 6.1% of the T-RFLP abundance data, mainly TRFs with low relative abundances, could not be correlated with any sequence data (Fig. 2).

Effects of age, sex, season, body condition, and reproductive status

The bacterial communities of cubs and adults differed significantly. DFA based on age revealed a discriminant function that differentiated the T-RFLP profile between cubs and adults (Wilks' $\lambda = 0.003$, $P < 0.001$, Table 2). The function explained 99.7% of the variance in the grouping variable. Using the computed discriminant function, 98.7% of secretions were assigned to the correct category (Table 2), with 100% of cubs (6/6) and 98.6% of adults (72/73) classified correctly. The ANOSIM analysis also illustrated a significant overall difference (Global R statistic = 0.71, $P = 0.001$, Table 2) of the bacterial community in cubs and adults. This was not attributable to pseudoreplication as there was also a significant difference when only one sample per individual was included (Table 2). When age was categorized as cubs (age 0; $n = 6$), yearlings (age = 1; $n = 12$), young adults (age 2–5; $n = 33$), and old adults (age > 5; $n = 17$), only cubs were significantly different from other age categories (Wilks' $\lambda = 0.0$, $P < 0.001$, Fig. 3).

The T-RFLP profile of adults of different body condition was significantly different in spring, shown by DFA (Wilks' $\lambda = 0.205$, $P = 0.044$, Table 2), but not ANOSIM. However, this difference between body conditions was not apparent in summer. We found no significant differences between T-RFLP profiles of males and females nor among females of different reproductive status, and bacterial communities did not differ with seasons (Table 2). DFA, but not ANOSIM, showed a significant difference between males in breeding condition ($n = 19$) and males in intermediate reproductive condition ($n = 6$) when all samples were included. However, if only one sample per male was included, analyses showed no significant difference (data not shown), indicating that the significant result might have been caused by pseudoreplication.

The diversity of the microbiota was estimated based on the T-RFLP data using the Shannon index of diversity and richness. On average, there were 8.67 (SD ± 1.69) TRFs in cub and 5.33 (SD ± 1.51) TRFs in adult subcaudal gland secretions. The bacterial community in cub secretions was significantly more diverse ($P = 0.014$) and rich ($P = 0.019$) than in the secretions of adults (Fig. 4). In addition, the diversity ($P = 0.014$) and richness ($P = 0.023$) of recently breeding and nonbreeding females differed significantly (Fig. 4), although no other life-history parameters appeared to affect the composition of the subcaudal gland microbiota.

The proportions of different bacterial phyla varied significantly between the bacterial pouch communities of adults and cubs (Fig. 2; see Fig. 1 for inter-individual differences). SIMPER analysis showed that TRF 140.4, 212.0, and 160.6 bp contributed the most to the differences between bacterial communities of cubs and adults (16.4%, 11.5%, and 8.79% respectively, Table 3). TRF 140.4 bp, which was identified to be in phylum Actinobacteria (SGS-17, 18), was more common in adults (present in 95.9% of individuals) than in cubs (present in 33.3% of individuals) and also had higher relative abundance in adults (average: 77.7%, SD ± 16.5%) than in cubs (average: 14.0%, SD ± 13.8%). Conversely, TRF 212.0 bp, which was identified as a member of the

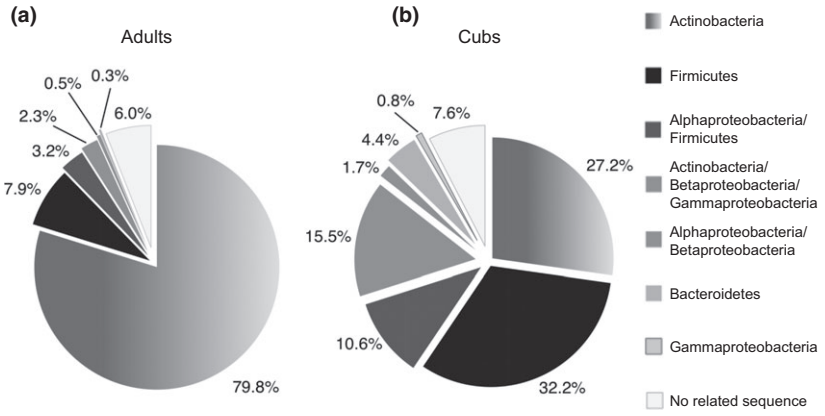

Fig. 2. Pie chart showing the relative abundance of different bacterial phyla/classes found in subcaudal gland secretion of *Meles meles* (a) adults ($n = 73$) and (b) cubs ($n = 6$). The proportions are based on T-RFLP data. TRFs with no matches in the clone libraries were classified as no related sequence (white area).

Table 2. DFA and ANOSIM comparing T-RFLP profiles of different categories (age, sex, body condition, reproductive status, and season of sampling) of *Meles meles* subcaudal gland secretion samples

Test	Data set	λ	χ^2	d.f.	*P*	% Cross-validation	ANOSIM Global R	ANOSIM *P*
Sex	All data	0.44	47.03	41	0.24		0.00	0.43
	One sample per ID	0.38	40.58	35	0.24		−0.02	0.61
	All adults	0.47	40.14	36	0.29		−0.01	0.49
	Spring, adult only	0.37	23.56	23	0.43		0.08	0.21
	Summer, adult only	0.34	23.10	25	0.57		−0.01	0.66
Age	All data	**0.003**	**327.44**	**47**	**< 0.001**	**98.7**	**0.71**	**0.001**
	One sample per ID	**0.001**	**271.53**	**35**	**< 0.001**	**96.7**	**0.71**	**0.001**
Season	All data	0.45	45.59	41	0.29		0.02	0.03
	One sample per ID	0.44	33.62	36	0.58		0.01	0.15
	All adult	0.48	38.58	36	0.35		0.02	0.07
Reproductive status	All adult females*	0.24	39.47	29	0.09		0.20	0.01
	One sample per female	0.27	27.18	24	0.30		0.10	0.07
Body condition	All data							
	Function 1	0.18	95.59	82	0.15		−0.03	0.80
	Function 2	0.47	42.80	40	0.35			
	One sample per ID							
	Function 1	0.12	85.72	72	0.13		−0.03	0.80
	Function 2	0.39	37.79	35	0.34			
	All adults							
	Function 1	0.20	84.37	72	0.15		−0.01	0.54
	Function 2	0.48	38.42	35	0.32			
	Spring, adult only†	**0.21**	**35.71**	**23**	**0.044**	**80.6**	0.12	0.07
	Summer, adult only‡							
	Function 1	0.06	60.85	50	0.14		0.01	0.36
	Function 2	0.37	20.92	24	0.64			

Analyses were performed on different data sets, that is, either all or only one sample per individual was included. Results of functions 1 and 2 from the DFA were shown for analyses with three factor levels.

We report classification results only for significant analyses. Significant DFA analyses and significant ANOSIM with Global $R > 0.5$ are in bold.

d.f., degrees of freedom.

*Nonbreeding females: $n = 14$; breeding females: $n = 30$.

†Only body condition 1 ($n = 24$) and 2 ($n = 13$) are included, as body condition 3 occurred only once.

‡Body condition 1: $n = 7$; body condition 2: $n = 16$; body condition 3: $n = 13$.

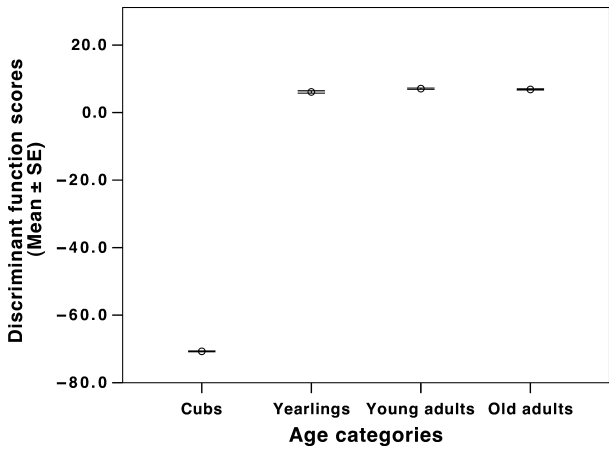

Fig. 3. Discriminant function scores (mean ± SE) from DFA of 79 subcaudal gland secretion samples differentiating T-RFLP profiles of cubs (age 0; $n = 6$), yearlings (age = 1; $n = 12$), young adults (age 2–5; $n = 33$), and old adults (age > 5; $n = 17$). The function explained 100% of the variance in the grouping variable.

Firmicutes, was more common and abundant in cubs (66.6% presence) than in adults (6.8% presence). TRF 160.6, which mostly comprised Gammaproteobacteria, was only found in cubs (relative abundance = 0.8%; Fig. 2).

Discussion

This study provides the first detailed molecular characterization of the microbiota inhabiting the subcaudal pouch of *M. meles*. We discovered 56 OTUs in the subcaudal gland secretion of *M. meles*. The high diversity in the bacterial community was unusual when compared to the scent organs of 13 other mammalian species reviewed in Archie & Theis (2011). However, only very few studies (e.g. Roze *et al.*, 2010) had used culture-independent methods, limiting the diversity of identified species.

The bacterial community in the subcaudal pouch was dominated by Actinobacteria, which co-existed with

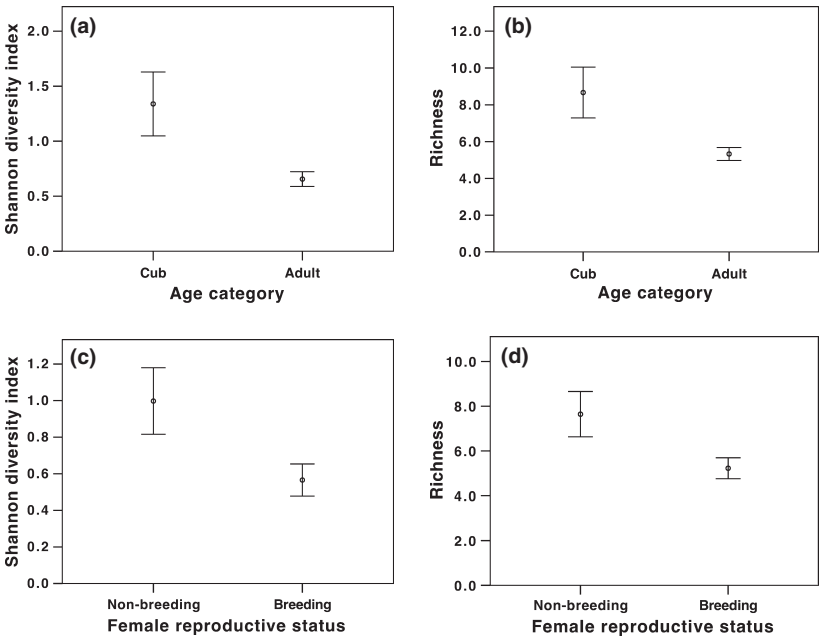

Fig. 4. Mean (± SE) difference of (a) Shannon index of diversity of cubs (n = 6) and adults (n = 73); (b) richness of cubs and adults; (c) Shannon index of diversity of nonbreeding (n = 14) and breeding females (n = 30); and (d) richness of nonbreeding and breeding females. Richness signified the total number of TRF, and the Shannon index of diversity was calculated from the relative abundance of TRFs of badger subcaudal gland secretion bacterial communities.

Table 3. The percentage contribution of the top 10 TRFs to the overall difference between cubs and adults

TRF	Cum.%*	Clone
140.4	16.43	SGS-17, 18
212.0	27.93	SGS-44
160.6	36.73	SGS-28 to 33
138.9	43.06	SGS-19 to 26
133.7	49.37	SGS-7 to 16
194.2	54.84	NA†
212.8	60.10	SGS-45 to 48
189.3	64.42	SGS-36 to 38
214.1	68.08	SGS-49 to 56
208.2	71.13	SGS-41 to 43

*Cumulative percentage.
†No related sequence.

numerous minor OTUs classified under Firmicutes, Bacteroidetes, Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria. Some Actinobacteria have been shown to play a major role in the transformation of odorless steroids into odorous derivatives. For example, in the human axilla, different bacteria such as *Corynebacterium* metabolize the odorless sebaceous and apocrine gland secretions (e.g. steroids and sebum) into odorous compounds (Gower *et al.*, 1986; Kohl *et al.*, 2001), generating the typical armpit smell. Moreover, studies showed humans to have individually distinctive axillary bacterial communities and thus resulting odor profiles, which may serve as a cue for individual (Natsch *et al.*, 2006; Xu *et al.*, 2007; Kuhn & Natsch, 2009) and kin (Weisfeld *et al.*, 2003) recognition. Badger subcaudal gland secretion has been shown to be rich in short- and medium-chain fatty acids (Buesching *et al.*, 2002b, c) that are likely to be pheromonally active end products of Actinobacteria-metabolizing long-chain fatty acids (Albone & Perry, 1975; Gorman, 1976), as has been suggested for the perineal glands of *Erethizon dorsatum*, whose bacterial community is also dominated by Actinobacteria (Roze *et al.*, 2010).

Other less abundant species are also likely to contribute to odor generation. For example, *Lactobacillus*, *Streptococcus*, and *Enterococcus* produce lactic acid as their major fermentation product and were also identified in the scent organ of other mammals [e.g. the perineal glands of *E. dorsatum* (Roze *et al.*, 2010) and tarsal tufts of white-tailed deer (*Odocoileus virginianus*; Alexy *et al.*, 2003)]. *Clostridium*, which is also found in the anal sac of the red fox (*Vulpes vulpes*; Ware & Gosden, 1980), generates an unpleasant odor during anaerobic decomposition of proteins owing to the production of fatty acids, ammonia, hydrogen sulfide, and amines. *Bacteroides* and *Escherichia*, identified in the anal glands of beavers (*Castor canadensis*) as well as in the scent organs of other species (for details see Archie & Theis, 2011), are able to produce a mixture of organic acids as fermentation end products. They are widespread in mammalian digestive systems and can easily be transferred from the anus to the subcaudal pouch. Thus, their ability to produce odorants may have resulted in the evolution of a long-term mutualistic association between a variety of bacteria and *M. meles*. However, our study focused on a single population, and it is theoretically possible for the bacterial taxa to be population

specific. Thus, additional studies on other populations are needed to understand whether the bacterial communities found here are typical for badgers throughout their geographic range.

Interestingly, cubs and adults differed highly significantly in the bacterial communities populating their subcaudal pouches, indicating that the acquisition of a 'mature bacterial community' is an ontogenetic process related to physiological changes during maturation. As cubs start to produce subcaudal gland secretion at approximately 4 months of age (Buesching *et al.*, 2002a; Fell *et al.*, 2006), different species of bacteria may start to colonize the subcaudal pouch at this stage. It is highly possible that some opportunistic bacteria would take advantage of this habitat and achieve high abundances before a 'mature bacterial community' is established. Succession of symbiotic bacterial communities is common (Deplancke *et al.*, 2000; Favier *et al.*, 2002; Lu *et al.*, 2003), for example, the colonization of bacteria on human skin has been related to maturity and increase in skin substrate availability (Nordstrom & Noble, 1984).

In badgers, mutual allo-marking is commonly assumed to facilitate the exchange, and thus gradual assimilation, of pouch bacteria between group members (Kruuk *et al.*, 1984; Buesching *et al.*, 2003). While very young cubs have been shown to anoint themselves with the scent of adult group members by brushing past their subcaudal pouch region (a behavior termed scent-theft: Fell *et al.*, 2006), older cubs producing their own subcaudal gland secretion have to obtain the typical bacterial community from other badgers that harbor the specific 'mature bacterial community' to enable them to produce typical adult scent profiles. Hence, the subcaudal pouch microbial communities of cubs is likely to undergo a succession from the more diverse and variable pioneering communities dominated by Firmicutes to less diverse and more specific communities dominated by Actinobacteria found in older individuals (yearlings and adults).

In addition, the composition of the microbial community is affected by the primary gland products. Some Actinobacteria are capable of metabolizing sex steroids (Gower *et al.*, 1986; Kohl *et al.*, 2001), which will only be present in the primary gland secretion in measurable amounts after the host reaches sexual maturity. The chemical composition of the subcaudal gland secretion changes significantly with maturity (Buesching *et al.*, 2002b). Hence, the shift from Firmicutes in cubs to Actinobacteria as the most prevalent bacteria in adults is likely to be influenced by the onset of puberty (such as in humans; Nordstrom & Noble, 1984), making the resources and environment progressively more suitable for Actinobacteria. On the other hand, there was no significant difference between age categories in badgers older than 1 year (i.e. yearlings, young, and old adults), indicating that bacterial communities stay relatively stable once established during the first year of life and are not liable to major changes through senescence (Buesching *et al.*, 2009; Dugdale *et al.*, 2011b).

Previous studies on humans have shown that the levels of blood androgens contribute to the differences in axillary bacterial community in men and women (Kohl *et al.*, 2001). Similarly, the difference in bacterial diversity between recently breeding and nonbreeding female badgers is likely due to diverging hormone levels and thus the composition of their primary gland products, resulting in the differences in the chemical composition (e.g. dodecanoic acid, tetradecanoic acid, and heptadecanoic acid) reported previously (Buesching *et al.*, 2002b). The accuracy of species richness estimation from T-RFLP has been criticized because of its limited ability to detect rare taxa (Bent *et al.*, 2007), particularly in highly diverse communities (Orcutt *et al.*, 2009). However, it is generally considered to be robust for the purpose of relative comparisons, identifying dominant species and examining richness of low-diversity communities (Engebretson & Moyer, 2003; Orcutt *et al.*, 2009).

Bacterial communities differed with badger body condition in spring but not in summer. As some of the chemicals in the subcaudal gland secretion are correlated with badger body condition in some seasons, a possible explanation for this observation is that the composition and/or amounts of primary gland products serving as food sources for different bacteria also vary with body condition (Buesching *et al.*, 2002a, b), although this variation is not ubiquitous throughout the year (Buesching *et al.*, 2002b).

The chemical composition of *M. meles* subcaudal gland secretion encodes a diverse array of information about the scent owner (Buesching *et al.*, 2002b, c). Surprisingly, our results showed that highly significant differences in the bacterial communities existed only between cubs and adults, whereas other factors (except spring body condition) did not affect the microbial communities significantly. However, it is possible that factors such as kinship (Weisfeld *et al.*, 2003), social group membership (Buesching *et al.*, 2003), and host genotypes (Kuhn & Natsch, 2009), particularly the major histocompatibility complex (Lanyon *et al.*, 2007; Zomer *et al.*, 2009; Sin *et al.*, 2012 a, b), have a greater influence on shaping the community of the symbiotic bacteria. Future studies examining bacterial communities in badger subcaudal gland secretion should thus include the previously mentioned key factors together with analyses of the semio-chemical profiles to determine the role of symbiotic bacteria in the olfactory communication of badgers.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. 16S rRNA gene sequencing results from *M. meles* subcaudal gland secretion.

Table S2. Relative abundance of forward and reverse TRFs in T-RFLP data of all individuals ($n = 79$).

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