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Evidence for a bacterial mechanism for group-specific social odors among hyenas

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Symbiotic microbes can benefit their animal hosts by enhancing the diversity of communication signals available to them. The fermentation hypothesis for chemical recognition posits that 1) fermentative bacteria in specialized mammalian scent glands generate odorants that mammals co-opt to communicate with one another, and 2) that variation in scent gland odors is due to underlying variation in the structure of bacterial communities within scent glands. For example, group-specific social odors are suggested to be due to members of the same social group harboring more similar bacterial communities in their scent glands than do members of different social groups. We used 16S rRNA gene surveys to show that 1) the scent secretions of spotted hyenas are densely populated by fermentative bacteria whose closest relatives are well-documented odor producers, and that 2) these bacterial communities are more similar among hyenas from the same social group than among those from different groups.

The evolutionary history of animals spans 600 million years, and over its course no animal has evolved independently of symbiotic microbes[1,2]. Instead, each animal has maintained intimate associations with microbial symbionts that have likely had profound effects on the animal's biology[1–3]. Associations between animals and their symbiotic microbes can certainly be antagonistic, but many appear to be mutualistic. For instance, animal-associated bacteria effectively prime host immune systems, competitively exclude host pathogens, afford hosts access to otherwise inaccessible vitamins and nutrients, and facilitate the development and functioning of host tissues[2,4,5]. It is also becomingly increasingly clear that symbiotic bacteria can beneficially affect animal behavior, and that their contributions may be particularly prominent in the realm of chemical communication[6]. Communication via chemical means appears to be the oldest and most widespread mode of signaling among animals[7,8]. The components of animal chemical signals can be synthesized by signalers themselves, obtained directly from signalers' environments, or acquired from the metabolic products of symbiotic microbes[9]. Many mammals communicate chemically by scent marking with secretions from specialized integumental scent glands[7,10]. These scent glands are warm, moist, organic-rich and largely anaerobic, and thus appear highly conducive to the proliferation of fermentative symbiotic bacteria[10].

The fermentation hypothesis for chemical recognition, introduced over 30 years ago, posits that, as bacteria ferment the protein and lipid-rich substrates in scent glands, they produce odorous metabolites that are co-opted by their mammalian hosts as components of chemical signals[6,10]. The hypothesis further posits that variation in many mammalian chemical signals, both among and within species, is due, at least in part, to underlying variation in the composition or structure of odor-producing bacterial communities within scent glands. For example, individual-specific scents are postulated to be a product of individuals harboring unique bacterial communities in their scent glands, whereas group-specific scents—which are not mutually exclusive of individual-specific scents—are suggested to be due to members of the same social group harboring more similar odor-producing bacterial communities in their scent glands than do members of different social groups[6,10]. Group-specific bacterial communities could arise through cross-infection among group members, as a consequence of members occupying the same space, coming into frequent bodily contact with one another, and/or consistently scent marking the same sites (e.g. communal scent posts). This mechanism was recently proposed as an explanation for the existence of observed group-specific social odors in the big brown bat, *Eptesicus fuscus*, Bechstein's bat, *Myotis bechsteinii*, European badger, *Meles meles*, and the spotted hyena, *Crocuta crocuta*[11–15]. From a functional standpoint, group-specific odors could facilitate rapid discrimination of group members from others, thereby enabling appropriate conciliatory or agonistic responses to encountered conspecifics[6,16].

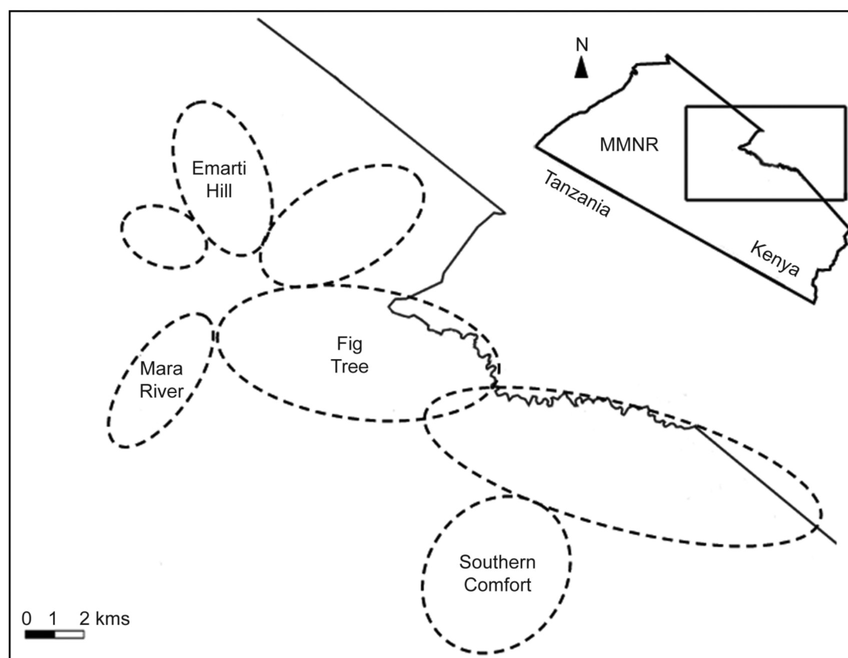

Figure 1 | Relative locations of the four sampled hyena clans within the Masai Mara National Reserve (MMNR), Kenya. The dashed lines represent estimated territorial boundaries of clans—sampled or otherwise—in the north-central area of the Reserve during the study period[39]. We deeply surveyed the bacterial communities in the scent pouches of four lactating females from each of the four sampled clans.

If the fermentation hypothesis for group-specific social odors among scent marking mammals is correct, then minimally 1) mammalian scent glands should contain substantive populations of odor-producing bacteria, and 2) the composition and/or structure of bacterial communities inhabiting the scent glands of members of the same social group should be more similar than those associated with members of different social groups. Historically, technical limitations associated with cultivation-based surveys of symbiotic bacteria have impeded the ability to effectively test predictions of the fermentation hypothesis for chemical recognition and, as a consequence, evaluations of the hypothesis have typically yielded either ambiguous results or concluded that the bacterial diversity in integumental scent glands is insufficient to underlie the observed diversity in chemical signals[6]. However, contemporary culture-independent, molecular survey tools can more effectively elucidate the diversity and metabolic potential of symbiotic bacterial communities directly from nucleotide sequences[6]. The objective of the current study was to use sequence-based community survey tools to evaluate first predictions of the hypothesis that symbiotic bacterial communities underlie the existence of group-specific social odors among wild spotted hyenas.

Spotted hyenas are large carnivores found throughout sub-Saharan Africa. They live in complex social groups, called clans, which typically consist of 40-80 individuals[17,18]. Clans contain multiple breeding males and multiple overlapping generations of females. The adult members of each clan cooperatively maintain and defend their group's territory against neighboring hyena clans, and they also direct hostility toward same-sex foreign hyenas intruding into their group's territory[17,19–21]. Despite being cohesive units, hyena clans are fission-fusion societies, in which members seldom all occupy the same place at the same time. Instead, subgroups of clan members form and dissolve such that these subgroups change in size and composition several times per day[22,23]. To mediate the complex social relationships both within and among clans, spotted hyenas employ a rich suite of tactile, visual, vocal and chemical signaling behaviors[17,20]. A particularly common and conspicuous chemical signaling behavior among hyenas is 'pasting,' a form of scent marking wherein a hyena typically straddles a grass stalk, extrudes its anal scent pouch, and drags the exposed pouch across the top of the stalk, leaving behind a thin layer of secretion, called 'paste'[17,20,24]. Paste is composed of lipid-rich sebum and presumably desquamated epithelial cells, and it is produced by a pair of lobulated sebaceous glands that secrete their products directly into the anal scent pouch[10,20,24].

The major volatile constituents of paste are fatty acids, esters, hydrocarbons, alcohols and aldehydes[25–27]. Collectively, they give paste a pungent, sour mulch odor that persists, detectable by the human nose, for more than a month after paste is deposited on grass stalks[20]. Previous investigations have shown that the odor of spotted hyena paste varies based on the individual identity, sex and group membership of the scent donor[14,26–28]. Hyenas' group-specific odors, in particular, are due to underlying variation in the structure of short-chain fatty acid and ester profiles of paste[14,26]. These odorants are well-documented products of bacterial fermentation[29]. Therefore, it has been suggested that group-specific social odors among spotted hyenas are a product of hyenas harboring group-specific bacterial communities in their scent pouches[14,15]. Here we initiate evaluation of this hypothesis.

This study provides the first in-depth, next-generation sequencing survey of the bacterial communities inhabiting the specialized scent glands of any mammal. It illustrates that individual glands can support far greater bacterial diversity than was previously reported[6], and that, among hyenas, this diversity comprises bacteria whose closest relatives are well-documented odor producers. Furthermore, by evaluating the diversity of bacterial communities in the scent glands of hyenas belonging to multiple clans in an African Reserve (Figure 1), this study provides the first cultivation-independent test of the prediction that the structure of bacterial communities in the scent glands of group-mates should be more similar than those among members of different social groups. The data support these predictions and, therefore, also the fermentation hypothesis for group-specific chemical recognition among spotted hyenas.

Results

Characterization of scent pouch bacterial communities. Scanning electron micrographs (SEMs) revealed that spotted hyena paste

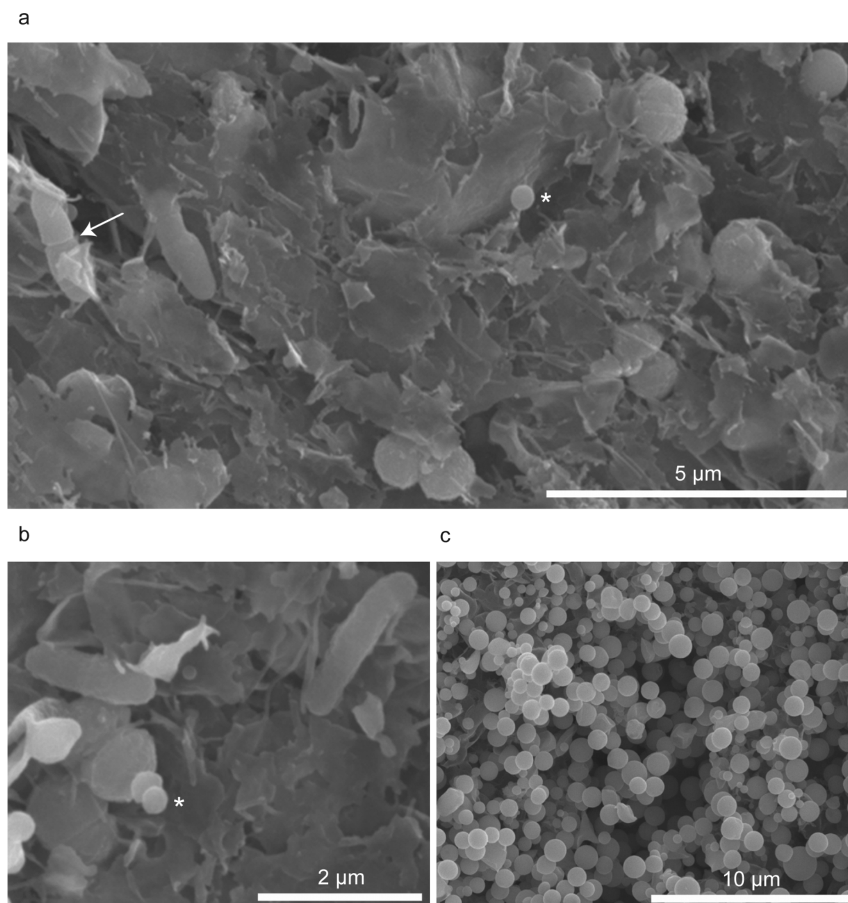

Figure 2 | Scanning electron micrographs of spotted hyena scent pouch secretions (called 'paste'). Panels A and B reveal rod and coccus-shaped bacteria embedded in the paste substrate. The arrow indicates an apparent division ring on a rod-shaped bacterium, and asterisks indicate presumed lipid droplets. Panel C illustrates the abundant clumping of presumed lipid droplets at the edge of the sample post-processing. A, B and C were x5000, x12000 and x2500 magnifications, respectively.

supports substantive populations of coccus and rod-shaped bacteria, and that some of these bacteria appeared to be in the process of dividing when sampling occurred—as indicated, for instance, by the presence of fission rings among rods (Figure 2). Subsequent 16S rRNA gene surveys of the bacterial communities in paste further revealed that these bacteria are primarily members of the phyla Firmicutes, Actinobacteria, Bacteroidetes and Proteobacteria (Table 1). The sampled pouch bacteria represented 78 previously characterized genera but, importantly, this is a minimum estimate of genus-level bacterial richness because more than half of the sampled sequences could not be confidently ($\geq$ 80% confidence threshold) assigned to a pre-established bacterial genus, suggesting the presence of many bacterial types new to science. We found 16 prominent operational taxonomic units (OTUs)—each constituting, on average, at least 0.5% of sampled sequences—in the scent pouches of female spotted hyenas. Collectively, these 16 OTUs accounted for more than 90% of the sampled sequences, and seven of these OTUs were found in the pouches of all 16 hyenas. When representative 16S rRNA gene sequences of these prominent OTUs were integrated into a phylogenetic tree alongside those of neighboring bacterial type strains, it became evident that the scent pouches of female spotted hyenas are primarily populated by bacteria most closely related to the genera *Anaerococcus*, *Anaerovorax*, *Corynebacterium*, *Eubacterium*, *Fastidiosipila*, *Helcococcus*, *Porphyromonas* and *Propionibacterium* (Figure 3).

Variation in the structure of scent pouch bacterial communities among spotted hyena clans. There was not a pronounced effect of clan membership on the composition of hyena scent pouch bacterial communities (Table 2). In other words, the scent pouches of female hyenas generally contained the same bacterial OTUs, regardless of which clan the hyena belonged to. There was, however, an evident effect of clan membership on the structure of these symbiotic communities, with the relative abundances of OTUs being more similar among clan-mates than among hyenas from different clans (Table 2; Figure 4). The degree of variation in the structure of scent pouch bacterial communities within clans did not significantly vary among clans, suggesting that the clans' symbiotic communities varied in their multivariate locations, not their dispersions (permutation analysis of multivariate dispersions, PERMDISP2; Bray-Curtis; distance to spatial medians; 9999 permutations of the least-absolute-deviation (LAD) residuals; $P = 0.106$)[30]. Specifically, the bacterial communities inhabiting the pouches of Southern Comfort, Mara River and Fig Tree hyenas were distinguishable. The structure of the scent pouch communities of Emarti Hill hyenas were different from those of Southern Comfort hyenas, but were not consistently different from those of Mara River or Fig Tree hyenas. Similarity Percentage (SIMPER) analyses indicated that OTUs 1, 4, 6, 7, 9, 55 and 63 were largely responsible for the observed differences in scent pouch bacterial communities among the hyena clans (Table 3). Each of these OTUs is a prominent member of hyena scent pouch communities (Figure 3). Aside from OTU 1, which is a *Propionibacterium*, the others are generally unclassified Firmicutes.

Discussion

The fermentation hypothesis for chemical recognition predicts that specialized mammalian scent glands 1) harbor odor-producing

Table 1 | Phylum(and genus)-level assignments of operational taxonomic units (OTUs) in the scent pouches of female hyenas

Phylum	Number of OTUs	Percentage of Sequences (± SD)	Genera
Firmicutes	236 (55)	73.3 ± 13.9 (22.9 ± 14.6)	*Anaerococcus, Anaerovorax, Bacillus, Blautia, Clostridium, Coprobacillus, Dialister, Eubacterium, Facklamia, Finegoldia, Fusibacter, Helcococcus, Holdemania, Ignavigranum, Macrococcus, Peptococcus, Peptoniphilus, Peptostreptococcus, Sedimentibacter, Sporacetigenium, Staphylococcus, Streptococcus, Tissierella*
Actinobacteria	53 (34)	17.1 ± 14.0 (17.0 ± 14.0)	*Actinomyces, Arthrobacter, Blastococcus, Brachybacterium, Cellulomonas, Corynebacterium, Curtobacterium, Dietzia, Friedmanniella, Janibacter, Kineosporia, Marmoricola, Micromonospora, Olsenella, Phycicoccus, Propionibacterium, Rubrobacter, Virgisporangium*
Bacteroidetes	27 (14)	3.1 ± 3.2 (3.1 ± 3.1)	*Bacteroides, Flavisolibacter, Paludibacter, Parabacteroides, Paraprevotella, Pedobacter, Petrimonas, Porphyromonas, Prevotella*
Proteobacteria	27 (23)	1.1 ± 3.1 (1.0 ± 3.1)	*Acinetobacter, Aeromonas, Anaeromyxobacter, Arcobacter, Bradyrhizobium, Comamonas, Enhydrobacter, Herbaspirillum, Massilia, Neisseria, Novosphingobium, Pantoea, Phenylobacterium, Pseudomonas, Sphingomonas, Stenotrophomonas, Sutterella*
Chloroflexi	13 (3)	0.1 ± 0.1 (< 0.1 ± 0.0)	*Bellilinea, Longilinea, Sphaerobacter*
Fusobacteria	4 (3)	0.1 ± 0.3 (0.1 ± 0.3)	*Cetobacterium, Fusobacterium*
Acidobacteria	6 (6)	< 0.1 ± 0.0 (< 0.1 ± 0.0)	*Gp4, Gp6, Gp7, Gp17*
Tenericutes	1 (1)	< 0.1 ± 0.0 (< 0.1 ± 0.0)	*Anaeroplasma*
Gemmatimonadetes	1 (1)	< 0.1 ± 0.0 (< 0.1 ± 0.0)	*Gemmatimonas*
Classified Totals	**368 (140)**	**94.8 ± 4.1 (44.1 ± 24.5)**	**78 Characterized Genera**

Taxonomic assignments of OTUs were determined using the Ribosomal Database Project's Classifier tool with an 80% confidence threshold[51]. Most of the sequences that remained unclassified at the phylum level (4.9/5.2%) belonged to OTU 21 (see Figure 3), which fell just short of being placed in the phylum Firmicutes with a confidence of 79%.

bacteria, and that 2) the structure of bacterial communities in these glands varies with the host trait of interest—here, clan membership. The data from scanning electron microscopy (SEM) were consistent with the prior suggestion that, similar to the scent pouch secretions of many other mammals[10], hyena paste is a mixture of lipid-rich sebum and desquamated epithelial cells[20,24]. The micrographs also revealed that paste supports substantive communities of coccus and rod-shaped bacteria and, furthermore, provided evidence, such as fission rings, that some of these bacteria were in the process of dividing, and were therefore necessarily metabolically active when paste sample collection occurred. The 16S rRNA gene surveys of paste revealed that more than half of the analyzed sequences could not be confidently assigned to a previously characterized genus. The hyena scent pouch microbiota therefore contains many novel bacterial types. Still, the 16S sequences that could be assigned to a genus represented 78 genera from nine different bacterial phyla. Collectively, the 15 prior surveys of specialized mammalian scent glands—covering 14 species from five mammalian orders—recovered bacteria from just 63 genera within five bacterial phyla[6,31]. Therefore, as expected, the next-generation sequencing approach used in this study afforded a far more comprehensive view of the bacterial communities inhabiting the specialized scent glands of a mammal than was previously available and, consequently, overturned the general conclusion from earlier studies that the bacterial diversity in integumental scent glands appears insufficient to underlie the observed diversity of chemical signals within scent marking mammalian species[6].

In this study, the prominent OTUs—some assigned to a bacterial genus, some not—were incorporated into a phylogenetic tree to gain additional taxonomic information about the hyena scent pouch microbiota. The phylogenetic analysis showed that the scent pouch microbiota are largely obligate or facultative anaerobes whose closest characterized relatives are well-documented odor producers. Specifically, the pouch bacteria belong, or are closely related, to genera that produce a diverse array of short-chain fatty acids (see Supplementary Table S1 online). Consistent with the fermentation hypothesis for chemical recognition, there is also documented variation in SCFA production at the species/strain level within these genera. Therefore, variation in the structure of symbiotic communities composed of these bacteria could very feasibly result in variation in host odor profiles.

SCFAs are prominent odorants in spotted hyena paste[14,26]. Furthermore, they largely constitute group-specific paste odors among hyena clans[14,26]. Group-specific paste odors, similar to the group-specific social odors of other mammals[11,12,32], and even social insects[16], are due to variation in the structure of odor profiles (i.e. quantitative variation in odorants) among social groups. In this study, there was a significant effect of clan membership on the structure of bacterial communities (i.e. quantitative variation in OTUs) in the scent pouches of female hyenas simultaneously residing in a Reserve in Kenya. Furthermore, the structure of three of the four clans' scent pouch communities varied discernibly. The scent pouch bacterial communities of a fourth clan, Emarti Hill, were different from those of one clan, but not significantly so from the other two. There are several possible explanations for why Emarti Hill hyenas did not possess a strong group-specific bacterial community: the Emarti Hill clan might have only recently formed, it may have experienced a recent influx of immigrants, or it may be less cohesive, in general, than the others. Unfortunately, these potential explanations cannot be evaluated *post hoc* with this particular data set. Notably, however, similar patterns exist in the group-specific social odor data sets as well. Specifically, in each of the studies illustrating group-specific social odors among scent marking mammals[11,12,14,32], including the spotted hyena, there was not complete clustering of individual odorant profiles by social group, with some groups exhibiting more variable profiles than others. This indicates that there are underlying factors of the phenomenon still to be identified.

Group-specific microbial communities—and consequently odor profiles—among social animals could be a product of shared environments (e.g. cross-infection)[13–15,33], host genetic similarity (e.g. family groups)[34–36], or, a product of repeated interactions between these two mechanisms[16,37,38]. Spotted hyenas frequently scent mark the same grass stalks as their clan-mates (i.e. overmarking), and they often do so in rapid succession to one another[14,15,17,20]. For hyenas, therefore, overmarking appears to be a viable pathway for the transmission of bacterial communities among members of hyena clans[14,15]. Although average genetic relatedness within hyena clans

OTU 65 (0.9%, 15, *Corynebacterium*)
Corynebacterium auris (X81873)
Corynebacterium striatum (X84442)
Corynebacterium pilosum (X84246)
OTU 16 (1.2%, 11, *Corynebacterium*)
Propionibacterium jensenii (AJ704571)
Propionibacterium thoenii (AJ704572)
Propionibacterium microaerophi (AF234623)
OTU 1 (14.1%, 16, *Propionibacterium*)
Actinobacteria
OTU 7 (15.9%, 16, *Anaerococcus*)
OTU 63 (4.1%, 16, *Anaerococcus*)
OTU 4 (6.9%, 15, n/a)
OTU 21 (4.9%, 16, n/a)
Anaerococcus lactolyticus (AF542233)
Anaerococcus prevotii (AF542232)
Anaerococcus octavius (Y07841)
OTU 11 (2.9%, 15, n/a)
OTU 36 (0.8%, 16, n/a)
OTU 9 (24.4%, 16, n/a)
Helcococcus sueciensis (AJ579914)
Helcococcus ovis (Y16279)
Helcococcus kunzii (X69837)
OTU 15 (1.3%, 12, *Anaerovorax*)
Eubacterium nodatum (U13041)
Eubacterium sulci (AJ006963)
Anaerovorax odorimutans (AJ251215)
Fastidiosipila sanguinis (AJ575187)
OTU 55 (2.3%, 10, n/a)
OTU 6 (8.2%, 15, n/a)
OTU 387 (0.6%, 7, n/a)
Firmicutes
OTU 5 (0.7%, 16, *Porphyromonas*)
OTU 24 (2.1%, 10, *Porphyromonas*)
Porphyromonas bennonis (EU414673)
Porphyromonas somerae (AY968205)
Porphyromonas cangingivalis (X76259)
Bacteroidetes
0.10

Figure 3 | Phylogenetic characterization of the prominent operational taxonomic units (OTUs) in the scent pouches of female hyenas. The 16S rRNA-based phylogenetic tree illustrates the evolutionary relationships among OTUs constituting, on average, $\geq$ 0.5% of the surveyed sequences and nearest-neighbor type strains. For each of the OTUs, the respective information in parentheses indicates the average percent abundance of the OTU among the 16 scent pouches, the total number of pouches in which the OTU was found, and, if applicable, the genus to which the OTU was assigned by the RDP Classifier tool using an 80% confidence threshold. An accession number is provided for each type strain. The tree—rooted with *Deinococcus radiodurans* (Y11332)—was generated using the Randomized Accelerated Maximum Likelihood (RAxML) rapid bootstrap algorithm across 1000 iterations. Single and double asterisks denote bootstrap values $\geq$ 90 and 95, respectively. The scale bar, or branch length, reflects the mean number of nucleotide substitutions per site.

is low, it is higher within than among clans[39,40]. Given that the bacterial communities in the urine marks of laboratory mice vary with the major histocompatibility complex haplotype and broad background genotype of the host[35,36], similar effects of these factors on the scent pouch bacterial communities of hyenas ought to be considered as well. Importantly, however, the underlying mechanisms for the formation of bacterial communities in the scent glands of mammals, although deserving of further investigation, are ancillary to the evaluation of the fermentation hypothesis for group-specific chemical recognition presented here.

This study employed scanning electron microscopy and next-generation sequencing technology to test necessary first predictions of the fermentation hypothesis for group-specific chemical recognition in the spotted hyena. It demonstrated that the scent pouches of spotted hyenas harbor diverse communities of anaerobic, fermentative bacteria whose close relatives are known odor-producers. Furthermore, the odors they produce are the same odors shown to constitute group-specific social odors among hyena clans. This study also revealed the existence of group-specific bacterial communities in the scent pouches of hyenas, illustrating that their diversity is sufficient to underlie group-specific social odors in this mammal. It does not, however, definitively demonstrate that they do so. Therefore, we are currently testing further predictions of the fermentation hypothesis for chemical recognition in the spotted hyena. Specifically, we

Table 2 | Results of analyses of similarity (ANOSIM) evaluating variation in the composition and structure of scent pouch bacterial communities among hyena clans. The abbreviations refer to the sampled clans: SC = Southern Comfort, MR = Mara River, FT = Fig Tree, EH = Emarti Hill

	Composition	Structure
	Sorensen Index	Bray-Curtis Index
Global effect	**R = 0.17, *P* = 0.033**	**R = 0.36, *P* = 0.002**
SC vs. MR	R = 0.31, *P* = 0.084	R = 0.98, *P* = 0.028
SC vs. FT	R = 0.19, *P* = 0.087	R = 0.41, *P* = 0.028
SC vs. EH	R = 0.41, *P* = 0.063	R = 0.37, *P* = 0.028
MR vs. FT	R = 0.05, *P* = 0.288	R = 0.41, *P* = 0.028
MR vs. EH	R = −0.01, *P* = 0.544	R = 0.03, *P* = 0.434
FT vs. EH	R = 0.00, *P* = 0.488	R = 0.05, *P* = 0.229

are testing the predictions that the odor and bacterial profiles of individual paste samples co-vary, and that specific members of the scent pouch microbiota, isolated and grown in pure culture, generate SCFAs and other odorants found in paste.

Methods

Collection of scent pouch secretions (called 'paste'). From 1999 – 2000, paste samples were collected directly from the anal scent pouches of 16 lactating female hyenas in the Masai Mara National Reserve, Kenya. Each had been anesthetized using Telazol (6.5 mg/kg) delivered from a CO_2-powered darting rifle. The paste samples were placed in sterile cryogenic vials, stored in liquid nitrogen, and transported to Michigan State University, where they remained frozen at −80°C until being used in this study. The sampled females represented four distinct hyena clans within the north-central region of the Reserve: Southern Comfort, Fig Tree, Mara River and Emarti Hill (Figure 1).

Scanning electron microscopy. Paste samples were fixed for 1 hr in 4% glutaraldehyde buffered with 0.1 M sodium phosphate at pH 7.4. They were then rinsed and postfixed in 1% osmium tetroxide for 1 hr, before being rinsed again and dehydrated in an ethanol series (25%, 50%, 75%, 95% vol/vol) for 15 mins at each gradation and for three 15 min changes in pure ethanol. The samples were dried in a Balzers Model 010 critical point dryer using liquid CO_2 as the transitional fluid, and mounted on aluminum stubs using Quick Cure-5 epoxy. They were then coated with ~ 10 nm osmium and examined in a JEOL JSM-6400V scanning electron microscope. Digital images were acquired using Olympus analysis pro software (v 3.2).

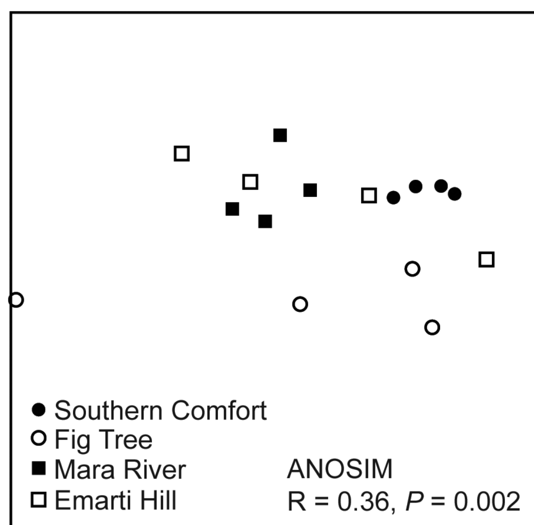

Figure 4 | Nonmetric multidimensional scaling (NMS) plot illustrating variation in scent pouch bacterial community structure among hyena clans. In NMS plots, the distance of sampled communities to one another reflects their underlying distance in multivariate space—here based on the Bray-Curtis similarity index. The X and Y axes were symmetrical, and the plot's stress (0.12)—a measure of its goodness-of-fit—indicated that the plot was an informative representation of community-level data[56].

16S rRNA-encoding gene amplification and sequencing. DNA was extracted from each paste sample (~ 0.1 g) using a MO BIO UltraClean fecal DNA kit, and each extraction was subsequently diluted tenfold with nuclease-free water. Bacterial 16S rRNA-encoding genes in diluted extractions were amplified and purified following the provisional protocol of the National Institute of Health's Human Microbiome Project (http://www.hmpdacc.org/tools_protocols/tools_protocols.php). Specifically, 16S rRNA genes were PCR-amplified using two broadly conserved primers targeting the V5-V3 variable regions of the bacterial 16S rRNA gene (926R: 5′ – CCG TCA ATT CMT TTR AGT – 3′; 357F: 5′ – CCT ACG GGA GGC AGC AG – 3′). The PCR program consisted of an initial dissociation step of 95°C for 2 min, followed by 30 cycles of 95°C for 20 s (denaturing), 50°C for 30 s (annealing), and 72°C for 5 minutes (extending). Each sample was PCR-amplified in duplicate and each amplification reaction included the incorporation of a unique molecular barcode to enable multiplex sequencing of samples[41]. Following amplification, PCR products were purified using solid-phase reversible immobilization ('SPRI bead clean-up'; Agencourt AMPure XP), quantified using an Invitrogen high sensitivity Quant-iT dsDNA assay kit, and pooled at equimolar concentrations. Nucleotide sequencing was performed on a 454 GS FLX Titanium instrument (Roche Diagnostics) at the Baylor College of Medicine's Human Genome Sequencing Center.

Sequence processing and operational taxonomic unit (OTU) formation. During initial processing, sequences were culled if they 1) contained any ambiguous base calls, 2) did not have perfect barcode and forward primer matches, 3) did not span the entire 900 – 500 base position region of the *Escherichia coli* (J01695) 16S rRNA gene, or 4) if they did not have a minimum average exponential quality score of 25 or greater over this region. The remaining sequences were truncated at the *E. coli* positions 900 and 500, which correspond to taxonomically-conserved regions of the 16S rRNA gene[42]. Sequences were then aligned using the Infernal Aligner tool provided by the Ribosomal Database Project (RDP) and were binned into operational taxonomic units (OTUs)—based on their percent nucleotide sequence similarity—using RDP's complete-linkage clustering tool[43] (v 10.23). In this study, we binned sequences using 90, 97 and 98% sequence similarity cutoffs. Results consequent of a 97% cutoff are presented here, and those from 90 and 98% cutoffs—which tell a very similar story—are included as supplementary materials online (Table S2, Figure S3). For perspective, we note that bacterial 16S rRNA sequences sharing at least 97% of their homologous bases are often referred to as coming from conspecifics, but we caution that the strength and functional relevance of this correlation remains uncertain[44].

To minimize the potential influences of PCR bias and sequencing errors on community analyses[45–47], OTUs were culled if they occurred in only a single sequencing reaction, or if their representative sequences were flagged as chimeric by the Chimera Slayer tool in Mothur[47,48] (v 1.17.0, Silva Gold database). A single exception was made for OTU 21 (see Figure 3), which was recovered in high abundance in all sequencing reactions and appeared legitimate when it was manually compared to unflagged OTUs in this study and to 16S rRNA reference sequences using Arb software[49,50] (v 5.1; Silva *rRNA* database, v 104). Experimentally removing OTU 21 from our data set did not affect analyses. Lastly, OTUs were culled if the RDP Naïve Bayesian rRNA Classifier tool[51] (v 2.2, 80% confidence threshold) deemed them chloroplast DNA sequences. In total, 3.5% (3958/111896) of sequences were culled. When the OTU-culling process was complete, we verified that each sample was most similar to its technical replicate, and then combined the data from each female's replicate samples, except for hyena EH489, for whom only a single sequencing reaction was successful. Ultimately, 107,938 sequences were binned into 403 OTUs, with each female contributing 6746 ± 1547 sequences representing 119 ± 43 OTUs (means ± SD throughout, unless otherwise noted). The Good's coverage estimations for the samples were 99.3% ± 0.2, and the rarefaction curves for all samples had plateaued (see Supplementary Fig. S4 online). Together, these data indicated that sample coverage was consistently very high for communities sampled in this study.

Characterizing the taxonomic identities of scent pouch bacteria. Representative sequences of the 403 OTUs were obtained using the Dereplicate tool in the RDP (GenBank JX051873 – JX052266; see Supplementary Information S5, S6 online). The RDP Naïve Bayesian *rRNA* Classifier tool was then used to assign phylum and genus-level taxonomic identities to the representative sequences[51] (v 2.2, 80% confidence threshold). Even though the RDP Classifier performs particularly well when classifying sequences spanning the V3–V5 region of the 16S rRNA gene[51], many of the OTUs in this study could not be confidently assigned to previously characterized genera (see Results). Therefore, to obtain approximate genus-level taxonomic information for the prominent OTUs in female scent pouches, representative sequences were incorporated into a phylogenetic tree alongside sequences from bacterial type strains. Specifically, representative sequences from OTUs constituting, on average, at least 0.5% of sequences among sampled hyenas were aligned using Silva's SINA Webaligner[49] (http://www.arb-silva.de/), uploaded into Arb[49,50] (v 5.1; Silva *rRNA* database, v 104), and incorporated into a phylogenetic tree alongside neighboring type strains using the Randomized Accelerated Maximum Likelihood (RAxML) rapid bootstrap algorithm[52] (v 7.0.3, filters = positions 10351 – 27583, pos_var_Bacteria_102). The 16 OTUs represented in this tree constitute more than 90% of the sequences analyzed in this study.

Table 3 | Results of SIMPER analyses indicating the contribution of specific operational taxonomic units (OTUs) to observed differences in scent pouch community structure among spotted hyena clans. The abbreviations refer to the sampled clans: SC = Southern Comfort, MR = Mara River, FT = Fig Tree, EH = Emarti Hill

Clan A vs. B	Overall Average Dissimilarity	Five Most Influential OTUs	Percent Contribution to Difference	Average Percent Abundance in Clan A ± SEM	Average Percent Abundance in Clan B ± SEM
SC vs. MR	**49.7**	7	16.6	6.07 ± 2.41	22.58 ± 2.25
		1	13.8	4.63 ± 0.66	18.33 ± 1.91
		4	12.0	16.53 ± 1.29	4.62 ± 2.55
		6	10.0	11.08 ± 0.42	1.17 ± 0.41
		9	8.9	29.28 ± 2.88	25.96 ± 4.92
SC vs. FT	**54.6**	4	14.2	16.53 ± 1.29	1.05 ± 0.55
		6	14.1	11.08 ± 0.42	15.50 ± 9.07
		1	13.3	4.63 ± 0.66	18.67 ± 12.59
		9	11.2	29.28 ± 2.88	18.26 ± 6.30
		55	7.0	0.02 ± 0.02	7.62 ± 4.69
SC vs. EH	**47.7**	7	24.6	6.07 ± 2.41	26.14 ± 13.68
		4	11.6	16.53 ± 1.29	5.47 ± 2.60
		1	10.8	4.63 ± 0.66	14.89 ± 3.50
		9	8.7	29.28 ± 2.88	24.10 ± 4.08
		63	6.7	1.39 ± 0.72	6.46 ± 5.70
MR vs. FT	**53.9**	1	17.3	18.33 ± 1.91	18.67 ± 12.59
		6	14.3	1.17 ± 0.41	15.50 ± 9.07
		7	12.8	22.58 ± 2.25	8.74 ± 2.58
		9	11.0	25.96 ± 4.92	18.26 ± 6.30
		55	6.9	1.40 ± 0.89	7.62 ± 4.69

Evaluating variation in scent pouch bacterial communities among hyena clans. OTU count data were standardized through conversion into proportions, and the contributions of highly prominent OTUs to quantitative similarity index calculations were tempered by $\log_{10}(x + 1)$ transformations of the data[53]. However, nearly identical results to those presented here were obtained using untransformed proportion data, as well as OTU count data normalized for sample size. Given that the majority of the OTUs in this study could not be confidently assigned to particular known genera (see Results), and that among those which could 16S rRNA gene copy number was largely unknown or variable, it was not possible to adjust OTU data to reflect potential variation in 16S rRNA gene copy number among community members[54,55]. Therefore, although our OTU data accurately reflect patterns in bacterial community structure among sampled hyenas, they do not necessarily provide definitive information about the absolute or rank abundances of specific bacteria in scent pouch communities.

Variation in the composition (qualitative) and structure (quantitative) of scent pouch bacterial communities was characterized using Sorensen (Dice coefficient) and Bray-Curtis similarity indices, respectively[56]. Community similarities were then visualized through nonmetric multidimensional scaling (NMS) plots, and statistically evaluated via analyses of similarity (ANOSIM)[53,56,57]. In NMS plots, samples' vicinity to other samples in portrayable space—typically two dimensions—reflects their underlying similarity in multivariate space. It is the preferred method for illustrating relationships among ecological communities[56,57]. ANOSIM is a multivariate, non-parametric permutation test for evaluating differences in community composition or structure between two or more pre-defined treatment groups (e.g. hyena clans). Given the permutational nature of ANOSIM, the application of Bonferroni corrections to planned pairwise comparisons is overly conservative and substantially increases the likelihood of Type II statistical errors, especially when modest sample sizes exist[58,59]. When differences in bacterial community structure between hyena clans were observed, similarity percentage (SIMPER) analyses were conducted to elucidate the contributions of specific OTUs to those differences[57]. To maximize interpretability, SIMPER analyses were performed using untransformed percent abundance data. All statistical and graphical analyses were completed using the PAST data analysis package[60] (v 2.12).

Animal Use and Care. Our research, described in Animal Research Protocol IACUC 05/11-110-00, was approved most recently on June 15, 2011 by the Institutional Animal Care and Use Committee at Michigan State University, and complies with Kenyan law.

1. Rosenberg, E., Sharon, G., Atad, I. & Zilber-Rosenberg, I. The evolution of animals and plants via symbiosis with microorganisms. *Environ. Microbiol. Rep.* **2**, 500–506 (2010).
2. Bosch, T. C. G. & McFall-Ngai, M. J. Metaorganisms as the new frontier. *Zoology* **114**, 185–190 (2011).
3. Savinov, A. Autocenosis and democenosis as individual- and population-level ecological categories in terms of symbiogenesis and systems approach. *Russian J. Ecol.* **42**, 179–185 (2011).
4. Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A. & Gordon, J. I. Host-bacterial mutualism in the human intestine. *Science* **307**, 1915–1920 (2005).
5. Fraune, S. & Bosch, T. C. G. Why bacteria matter in animal development and evolution. *Bioessays* **32**, 571–580 (2010).
6. Archie, E. A. & Theis, K. R. Animal behaviour meets microbial ecology. *Anim. Behav.* **82**, 425–436 (2011).
7. Wyatt, T. D. *Pheromones and Animal Behaviour: Communication by Smell and Taste.* (Cambridge University Press, 2003).
8. Steiger, S., Schmitt, T. & Schaefer, H. M. The origin and dynamic evolution of chemical information transfer. *P. Roy. Soc. B-Biol. Sci.* **278**, 970–979 (2011).
9. Wyatt, T. Pheromones and signature mixtures: defining species-wide signals and variable cues for identity in both invertebrates and vertebrates. *J. Comp. Physiol. A* **196**, 685–700 (2010).
10. Albone, E. S. *Mammalian Semiochemistry* (John Wiley, 1984).
11. Bloss, J., Acree, T. E., Bloss, J. M., Hood, W. R. & Kunz, T. H. Potential use of chemical cues for colony-mate recognition in the big brown bat, *Eptesicus fuscus*. *J. Chem. Ecol.* **28**, 819–834 (2002).
12. Safi, K. & Kerth, G. Secretions of the interaural gland contain information about individuality and colony membership in the Bechstein's bat. *Anim. Behav.* **65**, 363–369 (2003).
13. Buesching, C. D., Stopka, P. & MacDonald, D. W. The social function of allo-marking in the European badger (*Meles meles*). *Behaviour* **140**, 965–980 (2003).
14. Burgener, N., East, M. L., Hofer, H. & Dehnhard, M. Do spotted hyena scent marks code for clan membership? in *Chemical Signals in Vertebrates 11* (eds J. L. Hurst, R. J. Beynon, S. C. Roberts & T. D. Wyatt) 169-178 (Springer, 2008).
15. Theis, K. R., Heckla, A. L., Verge, J. R. & Holekamp, K. E. The ontogeny of pasting behavior in free-living spotted hyenas, *Crocuta crocuta*. in *Chemical Signals in Vertebrates 11* (eds J. L. Hurst, R. J. Beynon, S. C. Roberts & T. D. Wyatt) 179–188 (Springer, 2008).
16. van Zweden, J. S. & d'Ettorre, P. Nestmate recognition in social insects and the role of hydrocarbons. in *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology* (eds G. J. Blomquist & A-G. Bagnères) 222–243 (Cambridge University Press, 2010).
17. Kruuk, H. *The Spotted Hyena: A Study of Predation and Social Behavior* (University of Chicago Press, 1972).
18. Trinkel, M., Fleischmann, P. H. & Kastberger, G. Comparison of land-use strategies of spotted hyenas (*Crocuta crocuta*, Erxleben) in different ecosystems. *Afr. J. Ecol.* **44**, 537–539 (2006).
19. Boydston, E. E., Morelli, T. L. & Holekamp, K. E. Sex differences in territorial behavior exhibited by the spotted hyena (Hyaenidae, *Crocuta crocuta*). *Ethology* **107**, 369–385 (2001).
20. Mills, M. G. L. *Kalahari Hyenas: Comparative Behavioral Ecology of Two Species* (Chapman & Hall, 1990).
21. Henschel, J. R. & Skinner, J. D. Territorial behaviour by a clan of spotted hyaenas, *Crocuta crocuta*. *Ethology* **88**, 223–235 (1991).
22. Smith, J. E., Kolowski, J. M., Graham, K. E., Dawes, S. E. & Holekamp, K. E. Social and ecological determinants of fission-fusion dynamics in the spotted hyaena. *Anim. Behav.* **76**, 619–636 (2008).

23. Holekamp, K. E. *et al.* Patterns of association among female spotted hyenas (*Crocuta crocuta*). *J. Mammal.* **78**, 55–64 (1997).
24. Matthews, L. H. Reproduction in the spotted hyaena, *Crocuta crocuta* (Erxleben). *Philos. T. Roy. Soc. B.* **230**, 1–78 (1939).
25. Buglass, A. J., Darling, F. M. C. & Waterhouse, J. S. Analysis of the anal sac secretion of the Hyaenidae. in *Chemical Signals in Vertebrates 5* (eds D.W. Macdonald, D. Muller-Schwarze & S. E. Natynczuk) 65-69 (Kluwer Academic / Plenum Publishers, 1990).
26. Hofer, H., East, M. L., Sammang, I. & Dehnhard, M. Analysis of volatile compounds in scent-marks of spotted hyenas (*Crocuta crocuta*) and their possible function in olfactory communication. in *Chemical Signals in Vertebrates 9* (eds A. Marchlewska-Koj, J. L. Lepri & D. Muller-Schwarze) 141–148 (Kluwer Academic / Plenum Publishers, 2001).
27. Burgener, N., Dehnhard, M., Hofer, H. & East, M. L. Does anal gland scent signal identity in the spotted hyaena? *Anim. Behav.* **77**, 707–715 (2009).
28. Drea, C. M., Vignieri, S. N., Kim, H. S., Weldele, M. L. & Glickman, S. E. Responses to olfactory stimuli in spotted hyenas (*Crocuta crocuta*): II. Discrimination of conspecific scent. *J. Comp. Psychol.* **116**, 342–349 (2002).
29. Agler, M. T., Wrenn, B. A., Zinder, S. H. & Angenent, L. T. Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform. *Trends Biotechnol.* **29**, 70–78 (2011).
30. Anderson, M. J. Distance-based tests for homogeneity of multivariate dispersions. *Biometrics* **62**, 245–253 (2006).
31. Sin, Y. W., Buesching, C. D., Burke, T. & Macdonald, D. W. Molecular characterization of the microbial communities in the subcaudal gland secretion of the European badger (*Meles meles*). *FEMS Microbiol. Ecol.* **81**, 648–659 (2012).
32. Buesching, C. D., Waterhouse, J. S. & Macdonald, D. W. Gas-chromatographic analyses of the subcaudal gland secretion of the European badger (*Meles meles*) Part I: Chemical differences related to individual parameters. *J. Chem. Ecol.* **28**, 41–56 (2002).
33. Albone, E. S., Eglinton, G., Walker, J. M. & Ware, G. C. The anal sac secretion of the red fox (*Vulpes vulpes*); its chemistry and microbiology. A comparison with the anal sac secretion of the lion (*Panthera leo*). *Life Sci.* **14**, 387–400 (1974).
34. Svendsen, G. E. & Jollick, J. D. Bacterial contents of the anal and castor glands of beaver. *J. Chem. Ecol.* **4**, 563–569 (1978).
35. Lanyon, C. V. *et al.* Murine scent mark microbial communities are genetically determined. *FEMS Microbiol. Ecol.* **59**, 576–583 (2007).
36. Zomer, S. *et al.* Consensus multivariate methods in gas chromatography mass spectrometry and denaturing gradient gel electrophoresis: MHC-congenic and other strains of mice can be classified according to the profiles of volatiles and microflora in their scent-marks. *Analyst* **134**, 114–123 (2009).
37. Benson, A. K. *et al.* Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *P. Natl. Acad. Sci. USA* **107**, 18933–18938 (2010).
38. Spor, A., Koren, O. & Ley, R. Unravelling the effects of the environment and host genotype on the gut microbiome. *Nat. Rev. Micro.* **9**, 279–290 (2011).
39. Van Horn, R. C., Engh, A. L., Scribner, K. T., Funk, S. M. & Holekamp, K. E. Behavioural structuring of relatedness in the spotted hyena (*Crocuta crocuta*) suggests direct fitness benefits of clan-level cooperation. *Mol. Ecol.* **13**, 449–458 (2004).
40. Holekamp, K. E., Smith, J. E., Strelioff, C. C., Van Horn, R. C. & Watts, H. E. Society, demography and genetic structure in the spotted hyena. *Mol. Ecol.* **21**, 613–632 (2012).
41. Binladen, J. *et al.* The use of coded PCR primers enables high-throughput sequencing of multiple homolog amplification products by 454 parallel sequencing. *Plos One* **2**, e197 (2007).
42. Cannone, J. *et al.* The Comparative RNA Web (CRW) Site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. *BMC Bioinformatics* **3**, 2 (2002).
43. Cole, J. R. *et al.* The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res.* **37**, D141–D145 (2009).
44. Lozupone, C. A. & Knight, R. Species divergence and the measurement of microbial diversity. *FEMS Microbiol. Rev.* **32**, 557–578 (2008).
45. Kunin, V., Engelbrektson, A., Ochman, H. & Hugenholtz, P. Wrinkles in the rare biosphere: pyrosequencing errors can lead to artificial inflation of diversity estimates. *Environ. Microbiol.* **12**, 118–123 (2010).
46. Kanagawa, T. Bias and artifacts in multitemplate polymerase chain reactions (PCR). *J. Biosci. Bioeng.* **96**, 317–323 (2003).
47. Haas, B. J. *et al.* Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res.* **21**, 494–504 (2011).
48. Schloss, P. D. *et al.* Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microb.* **75**, 7537–7541 (2009).
49. Pruesse, E. *et al.* SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* **35**, 7188–7196 (2007).
50. Ludwig, W. *et al.* ARB: a software environment for sequence data. *Nucleic Acids Res.* **32**, 1363–1371 (2004).
51. Wang, Q., Garrity, G. M., Tiedje, J. M. & Cole, J. R. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microb.* **73**, 5261–5267 (2007).
52. Stamatakis, A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688–2690 (2006).
53. Ramette, A. Multivariate analyses in microbial ecology. *FEMS Microbiol. Ecol.* **62**, 142–160 (2007).
54. Rastogi, R., Wu, M., DasGupta, I. & Fox, G. Visualization of ribosomal RNA operon copy number distribution. *BMC Microbiol.* **9**, 208 (2009).
55. Lee, Z., Bussema, C. & Schmidt, T. rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. *Nucleic Acids Res.* **37**, D489–D493 (2009).
56. McCune, B. & Grace, J. B. *Analysis of Ecological Communities* (MjM Software Design, 2002).
57. Clarke, K. R. Non-parametric multivariate analysis of changes in community structure. *Aust. J. Ecol.* **18**, 117–143 (1993).
58. Hammer, O. *PAST: PAleontological STatistics manual.* 2.07 edn, (Natural History Museum, 2011).
59. Clarke, K. R. & Warwick, R. M. *Change in Marine Communities: An Approach to Statistical Analysis and Interpretation.* 2nd edn, (PRIMER-E Ltd., 2001).
60. Hammer, O., Harper, D. A. T. & Ryan, P. D. PAST: PAleontological STatistics software package for education and data analysis. *Palaeontol. Electron.* **4**, 1–9 (2001).

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Author contributions

K.R.T., T.M.S. and K.E.H. conceived and designed the study. K.R.T. completed the analyses. K.R.T., T.M.S. and K.E.H. wrote the manuscript.

Additional information

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