Metagenomics uncovers dietary adaptations for chitin digestion in the gut microbiota of convergent myrmecophagous mammals

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Abstract

In mammals, myrmecophagy (ant and termite consumption) represents a striking example of dietary convergence. This trait evolved independently at least five times in placentals with myrmecophagous species comprising aardvarks, anteaters, some armadillos, pangolins, and aardwolves. The gut microbiome plays an important role in dietary adaptation, and previous analyses of 16S rRNA metabarcoding data have revealed convergence in the composition of the gut microbiota among some myrmecophagous species. However, the functions performed by these gut bacterial symbionts and their potential role in the digestion of prey chitinous exoskeletons remain open questions. Using long- and short-read sequencing of fecal samples, we generated 29 gut metagenomes from nine myrmecophagous and closely related insectivorous species sampled in French Guiana, South Africa, and the USA. From these, we reconstructed 314 high-quality bacterial genome bins of which 132 carried chitinase genes, highlighting their potential role in insect prey digestion. These chitinolytic bacteria belonged mainly to the family Lachnospiraceae, and some were likely convergently recruited in the different myrmecophagous species as they were detected in several host orders (i.e., *Enterococcus faecalis*, *Blautia* sp), suggesting that they could be directly involved in the adaptation to myrmecophagy. Others were found to be more host-specific, possibly reflecting phylogenetic constraints and environmental influences. Overall, our results highlight the potential role of the gut microbiome in chitin digestion in myrmecophagous mammals and provide the basis for future comparative studies performed at the mammalian scale to further unravel the mechanisms underlying the convergent adaptation to myrmecophagy.

Importance

Myrmecophagous mammals are specialized in the consumption of ants and/or termites. They do not share a direct common ancestor and evolved convergently in five distinct placental orders raising questions about the underlying adaptive mechanisms involved and the relative contribution of natural selection and phylogenetic constraints. Understanding how these species digest their prey can help answer these questions. More specifically, the role of their gut microbial symbionts in the digestion of the insect chitinous exoskeleton has not been investigated in all myrmecophagous orders. We generated 29 new gut metagenomes from nine myrmecophagous species to reconstruct more than 300 bacterial genomes in which we identified chitin-degrading enzymes. Studying the distribution of these chitinolytic bacteria among hosts revealed both shared and specific bacteria between ant-eating species. Overall, our results highlight the potential role of gut symbionts in the convergent dietary adaptation of myrmecophagous mammals and the evolutionary mechanisms shaping their gut microbiota.

Keywords

convergent evolution; myrmecophagy; mammals; gut microbiota; chitinases; metagenomics; genome assembly.

Introduction

In mammals, the gut microbiota has played a major role in dietary diversification, enabling transitions to novel carbon sources (1, 2). Several factors such as host diet, physiology, genetics, and phylogeny, but also environmental factors, shape the taxonomic composition and functional structure of the gut microbiota (3). Many studies have focused on convergent dietary adaptations and the effects of host diet, investigating how host phylogeny shapes the gut microbiota (4–10). A striking example of convergent dietary specialization is the adaptation to myrmecophagy in placental mammals. Myrmecophagous species are characterized by a diet composed of at least 90% of ants and/or termites (11). A total of 22 placental species evolved toward this diet and are found in five independent orders: Tubulidentata (aardvarks), Pilosa (anteaters), Cingulata (tolypeutine armadillos), Pholidota (pangolins), and Carnivora (aardwolves) (12–14). Their divergent evolutionary histories raise the question of how these species convergently adapted to the myrmecophagous diet and whether similar adaptive mechanisms were involved between the different species. Myrmecophagous species need to digest the chitinous exoskeleton of their prey to extract nutrients. Mammals carry chitinase genes in their genomes, which participate in chitin digestion (15–18), but their gut microbiota might also have played an important role in facilitating prey digestion (19–21).

Indeed, chitinolytic bacteria are present in a diversity of environments (i.e., soils, sediments, aquatic environments) where they ensure chitin degradation and play an important role in the carbon cycle (22–25). They also have been identified in the digestive tract of mammals where they could participate in prey digestion (26–30). Taxonomic analyses based on 16S rRNA sequences have highlighted similarities in the gut microbiota composition of myrmecophagous species (19) compared to their non-myrmecophagous sister species. For instance, genera such as *Blautia* (Lachnospiraceae), *Streptococcus* (Streptococcaceae), *Peptococcus* (Peptococcaceae), or *Eubacterium* (Lachnospiraceae) were found to be significantly more abundant in the gut microbiota of myrmecophagous species than in their sister species. Focusing on the gut archaeome of placentals, *Methanobrevibacter* has been found almost only in myrmecophagous species (10). Yet, little is known regarding the role these symbionts play in the digestion of the chitinous exoskeleton of their prey.

Microbial chitinases and N-acetylglucosaminidases are carbohydrate-active enzymes (CAZymes) (31) which ensure the degradation of chitin into chitosan and are mostly found in the Glycosyl Hydrolases (GH) families 18, 19, and 20 (23, 32). Since bacterial chitinases mainly belong to the GH18 family (32–35), they represent relevant candidates to assess the potential of the gut microbiota to digest chitin in our focal species. The GH18 enzyme family comprises

chitinases and chitin-binding proteins and within this family, bacterial chitinases are classified into three subfamilies (A, B, and C) based on sequence homology (32–34). Symbiotic chitin-degrading bacteria have been identified, as well as their chitinase genes, in the Malayan pangolin and the giant anteater (20, 21) and in other mammals having a chitin-rich diet (36, 37). This suggests that other myrmecophagous species might also carry chitinolytic gut bacteria that could participate in prey digestion. In the specific example of dietary convergence, one question resides in understanding whether these potential microbial mechanisms of chitin exoskeleton digestion have converged among the five different placental orders of myrmecophagous mammals. The different myrmecophagous mammal lineages diverged millions of years ago and evolved in diverse environments. Chitinolytic microbes might thus have been independently recruited to ensure chitin digestion, but whether similar bacteria carrying similar functions are involved still needs further investigation. Assessing whether chitinolytic bacteria present in myrmecophagous mammals are taxonomically and functionally similar will shed light on their origin.

To investigate the chitin-degrading potential of the gut microbiota of myrmecophagous mammals, we took advantage of recent advances in metagenomics to reconstruct high-quality bacterial genomes and identify Glycoside Hydrolases 18 (GH18) as this enzyme family comprises most bacterial chitinases. By combining Nanopore long-read and Illumina short-read shotgun metagenomic sequencing, we generated 29 new gut metagenomes from field-collected fecal samples of nine myrmecophagous and insectivorous species representatives of the five myrmecophagous placental orders. From these, we reconstructed 314 high-quality bacterial genomes harboring a diversity of GH18 chitinases. Identification of GH18 sequences in the same bacterial genomes revealed a potential role of gut symbionts in prey digestion through putative complex metabolic pathways. Both generalist and host-specific bacteria were identified in the different myrmecophagous species, potentially reflecting the divergent evolutionary histories of their hosts, and raising questions about the evolutionary forces (historical contingency and determinism) at play in shaping the gut microbiome of convergently evolved myrmecophagous mammals.

Results

Potential role of the gut microbiota in chitin digestion

Combining long-read and short-read assemblies, we were able to reconstruct 314 dereplicated high-quality genome bins (156 and 158 from each dataset respectively; see Table S1F) and highlighted the usefulness of using the two methods (see supplementary results part 1 available via Zenodo). The following analyses were done on this set of high-quality selected bins to assess the presence of chitinolytic bacteria in the gut microbiota of myrmecophagous mammals.

All selected bins were taxonomically assigned to bacterial genomes and their phylogeny is presented in Fig. S1. Overall, 58.3 % of the bins (n = 183) were not assigned at

148 the species level, 29.3% (n = 92) at the genus level, and 6.7% (n = 21) at the family level.
149 Taxonomically assigned bins belonged mainly to the Lachnospiraceae (n = 63),
150 Burkholderiaceae (n = 24), Acutalibacteraceae (n = 18), Ruminococcaceae (n = 12), and
151 Bacteroidaceae (n = 11) families (Table S1F). The 314 selected bins were integrated into a
152 phylogeny of prokaryote reference genomes to confirm these results (Fig. 1A). All selected
153 bins were well placed in this phylogeny, suggesting they are similar to known bacterial
154 genomes and not distantly related, even for genome bins for which taxonomic assignment
155 failed. Some of the reconstructed bacterial genome bins clustered together within the
156 Firmicutes (Fig. 1B), Bacteroidetes, and Proteobacteria (Figs. S2 A and B), in clades not
157 including reference genomes, suggesting that these genome bins could be specific to
158 myrmecophagous species (here called myrmecophagous-specific clades). Within the
159 Firmicutes, six clades containing more than two bins reconstructed from myrmecophagous
160 samples can be defined within the Lachnospiraceae family with two clades including
161 respectively 12 and 11 genome bins (Fig. 1B). In the first clade, six bins had no taxonomic
162 assignment below the family level and could represent bacterial genomes not yet described,
163 two belonged to the *Acetatifactor* genus, and four to the *CAG-510* genus. In the other clade,
164 four bins were not taxonomically assigned below the family level, three bins belonged to the
165 *CAG-590* genus, one was assigned to the *CAG-127* genus and two were assigned at the species
166 level (*Frisingiococcus caecimuris* and *Acetivibrio ethanolgignens*) (Fig. 1B). These bins were all
167 reconstructed from xenarthran gut metagenomes (Fig. 1B). Within the Acutalibacteraceae
168 family, one clade contained 18 genome bins reconstructed mostly from xenarthran gut
169 metagenomes and the three aardvark samples (Fig. 1B). Eleven had no taxonomic assignment
170 below the family level, four belonged to the *Eubacterium* genus, and three to the *UBA1227*,
171 *UBA1691*, and *UBA6857* genera (Fig. 1B). Other clades containing only genome bins from
172 myrmecophagous species were found within the Ruminococcaceae, Oscillospiraceae,
173 Anaerovoracaceae, and Erysipelotrichaceae families.

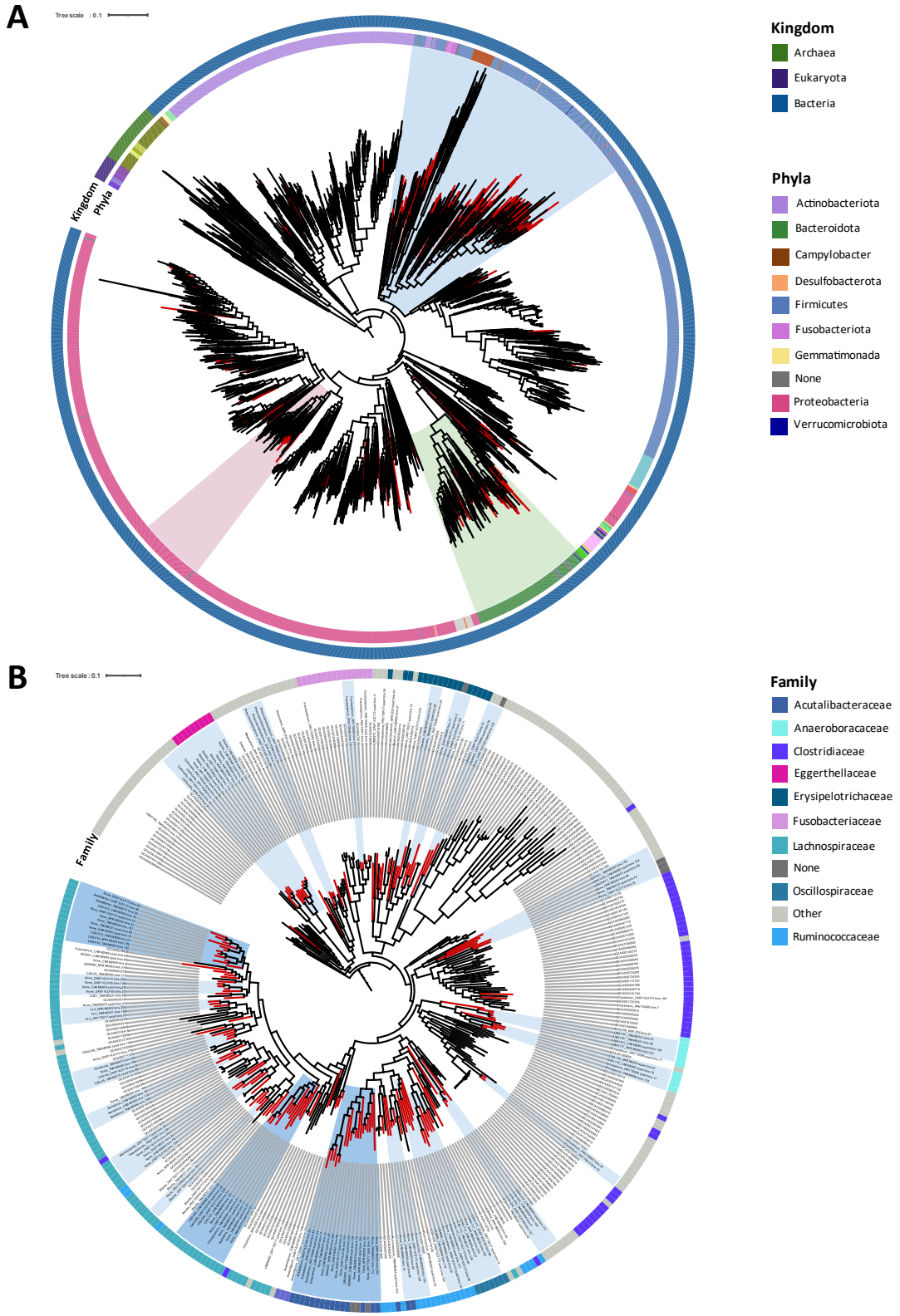


Fig 1 Phylogenetic position of the 314 high-quality selected bins reconstructed from 29 gut metagenomes of the nine focal myrmecophagous species within a reference prokaryotic phylogeny. A: Phylogeny of the 314 selected bins (red branches) with 2496 prokaryote reference genomes. Circles respectively indicate (from inner to outer circles): the bacterial

179 phyla and kingdom to which these genome bins were assigned based on the Genome
180 Taxonomy Database release 7 (38). Clades, where a subtree was defined, are highlighted in
181 blue for the Firmicutes (Fig. 1B), green for the Bacteroidetes, and pink for the Proteobacteria
182 (Figs. S2 A and B, respectively). **B**: Subtree within Fimircutes showing myrmecophagous-
183 specific clades (blue highlights; dark blue corresponds to the three clades mentioned in the
184 results, light blue to the other clades). The outer circle indicates the bacterial family to which
185 these genome bins were assigned based on the Genome Taxonomy Database. Bins' names of
186 the myrmecophagous-specific clades are indicated at leaves of the phylogenetic tree together
187 with the genus to which they were assigned to.

188

189 To understand whether these bacteria could potentially degrade chitin, these high-quality
190 genome bins were then used to search for GH18 enzymes. This resulted in 132 bins containing
191 at least one GH18 sequence (between 0 and 17 bins per sample, 4.93 on average). Having
192 complete genome bins enabled us to identify several GH18 genes in the same bin (between
193 0 and 17 per bin, 1.26 on average; Table S1F). In total, 394 GH18 sequences were identified
194 (Fig. 2) with 237 sequences presenting an active chitinolytic site (DXXDXDXE) (Fig. 2) and
195 distributed among 82 bins (here called chitinolytic bins). These chitinolytic sequences are
196 found in genome bins belonging mainly to the Lachnospiraceae (n = 183 sequences; e.g.,
197 *Blautia*, *Acetatifactor*, *Roseburia*, *Clostridium* genera), Acutalibacteraceae (n = 76; e.g.,
198 *Eubacterium* genus), and Ruminoccocaceae (n = 23; e.g., *Ruminococcus*,
199 *Acetanaerobacterium* genera) bacterial families (Fig. 2). Fifty-three sequences not presenting
200 an active site were found in a clade with sequences similar to Lysin Motif (LysM) domain-
201 containing proteins (Fig. 2), which is a 40 amino acid domain involved in peptidoglycan and
202 chitin-binding (39). Sixty-two were placed in a clade with sequences similar to src Homology-
203 3 (SH3) domain-containing proteins (Fig. 2), which is a 50 amino acid domain found in
204 intracellular and membrane proteins involved in the binding of ligands (40). These two clades
205 were used to root the tree. Finally, 278 GH18 sequences formed a clade with sequences
206 similar to known bacterial chitinases (Fig. 2). The majority had an active chitinolytic site. These
207 sequences were identified in bins reconstructed from different host species, representative
208 of the five myrmecophagous placental orders. They represent a diversity of chitinase genes,
209 as they are distributed in distinct clades (Fig. 2).
210

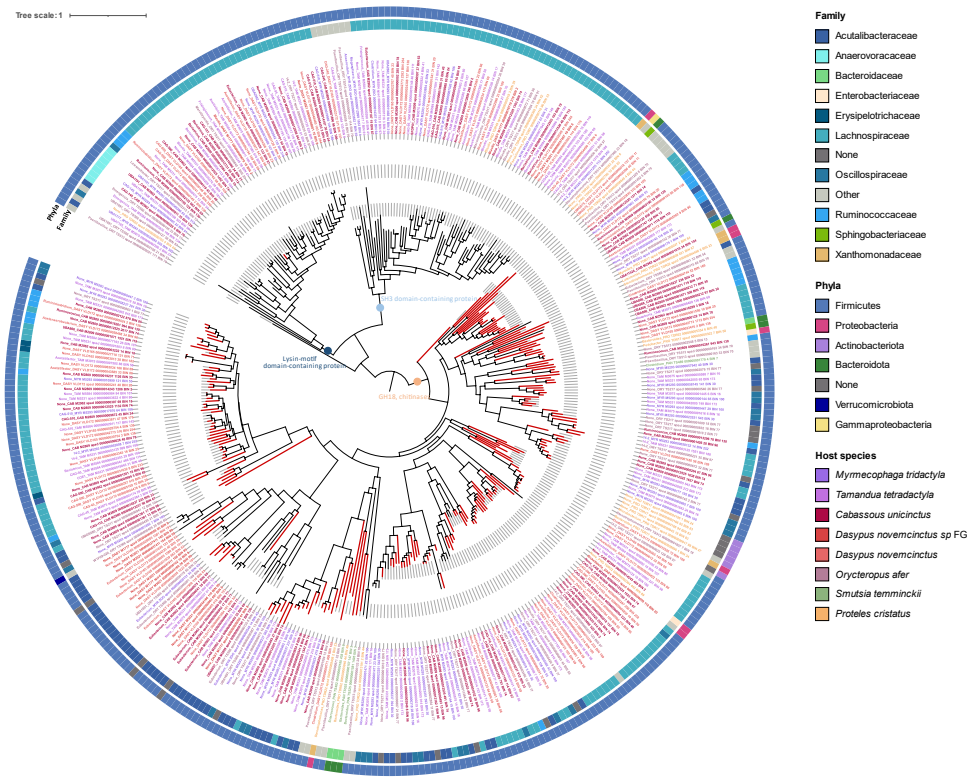


Fig 2 Phylogeny of the 394 GH18 sequences identified in 132 high-quality selected bins reconstructed from 29 gut metagenomes of the nine focal myrmecophagous species and relatives. Red branches indicate the 237 sequences having an active chitinolytic site (DXXDXDXE). Circles respectively indicate (from inner to outer circles): the bacterial family and phyla of the bin the sequence was retrieved from. Colored sequence names indicate the host species. Colored circles at certain nodes indicate enzymes to which sequences are similar when blasting them against the NCBI non-redundant protein database. Sequence names are indicated at leaves of the tree and begin with the genus to which the bin they were identified in was assigned to.

Distribution of chitinolytic bacteria among myrmecophagous mammals

Numbers of shared and specific selected bins in the nine host species were computed based on a detection threshold of 0.25 (the percentage of the reference covered by at least one read) of the selected bins across samples (Fig. 3, Table S2, and detection table available via Zenodo). According to this threshold, five selected bins were not considered to be detected in any sample. Between 6 and 124 selected bins were detected in each sample (52.62 on average) and 252 selected genomes were shared and detected in samples other than the ones they were reconstructed from (Fig. 3). Selected bins were detected in 4.86 different samples on average (between 0 and 18). Selected bins were shared between individuals of the same species but with some intra-specific variability (Fig. 3). Conversely, 57 bacterial genomes were detected in only one sample (Fig. 3, Table S2) and 194 bins were detected in more than one host species (between 0 and 7 host species; 2.73 on average), between closely related species

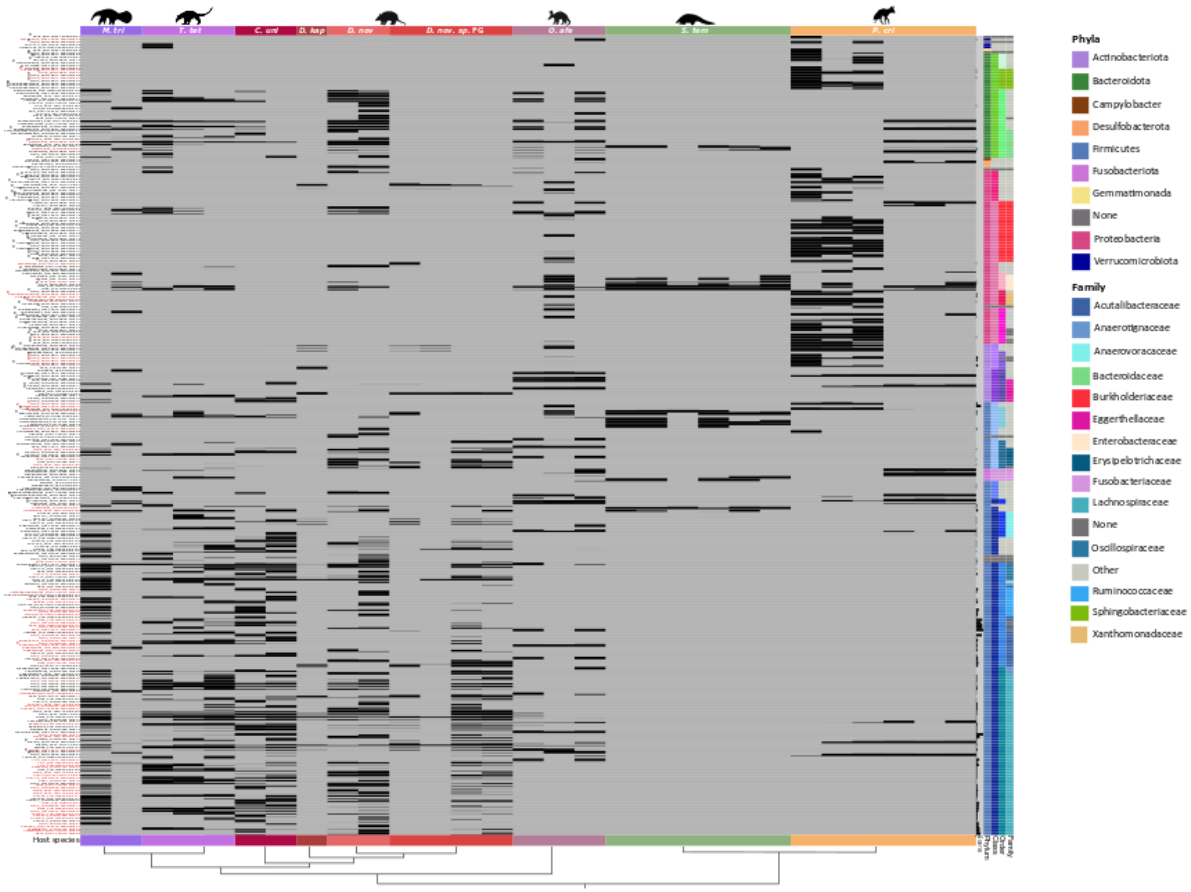
234 or distantly related species (Fig. 3, Table S2). On the other hand, 115 selected bins were
235 specific to a particular host species, meaning they were found in only one of the nine species
236 studied here (Fig. 3, Table S2). Selected bins were detected in 1.80 different host orders on
237 average (between 0 and 5) for a total of 179 bins detected in more than one host order.
238 Finally, 130 bins were detected in only one host order.

239 There were more shared selected bins between host species carrying GH18 genes than
240 shared bins with no GH18 gene (Fig. S3). Among the 194 bins shared between host species,
241 110 had at least one GH18 gene. These bins belonged to 15 different bacterial families, mainly
242 the Lachnospiraceae (n = 60; e.g., *Blautia*, *Acetatifactor*, *Roseburia* genera) and
243 Acutalibacteraceae (n = 10; e.g., *Eubacterium* genus) (Fig. S3). The 84 shared selected bins
244 without chitinases belonged to a more diverse range of bacterial families (n = 33), mainly
245 Burkholderiaceae (n = 8; e.g., *CAG-521*, *Sutterella*, *Bordetella* genera) and Bacteroidaceae (n
246 = 7; e.g., *Bacteroidetes*, *Prevotella* genera) (Fig. S3). On the contrary, among bins found in only
247 one host species, there were fewer chitinase-carrying bins. Among the 115 host-species
248 specific bins, 21 had at least one GH18 gene. They were found in 12 different bacterial families
249 (Fig. S3). The 94 specific bins with no chitinase genes belonged to 37 different bacterial
250 families, mainly Burkholderiaceae (n = 16) (Fig. S3). A similar pattern was observed at the host
251 order level (see supplementary results part 2 available via Zenodo).

252 Three high-quality selected bins carrying GH18 were shared between species
253 belonging to the five different myrmecophagous orders (Table S1F): one bin of *Bacteroides*
254 *fragilis* (mean absolute abundance across samples 2.39, see abundance table available via
255 Zenodo) and two bins of Enterobacteriaceae (mean absolute abundances across samples 0.58
256 and 3.66). Two of these three bins shared across orders were a bit more abundant, on
257 average, than the mean absolute abundance of selected bins across samples of 0.89 (the
258 minimal abundance of a bin across samples was 2.82e-6 and the maximal absolute abundance
259 was 198). Seven selected bins carrying GH18 were shared among four myrmecophagous
260 orders (Table S1F). Two bins belonged to *Enteroccocus faecalis* and were reconstructed from
261 pangolin samples, along with bins belonging to Lachnospiraceae bacteria (notably one from
262 *Blautia* sp., one from *Faecalimonas* sp. and one from an unknown genus), one from
263 *Bacteroides* sp. (Bacteroidaceae) and one from *Emergencia timonensis* (Anaerovoracaceae)
264 with mean absolute abundances across samples ranging from 0.21 (*E. timonensis*) to 4.74
265 (*Bacteroides sp*), the latter being found to be abundant in several samples (for example: one
266 *D. novemcinctus* (12.2), one *T. tetradactyla* (21.2), one *O. afer* (41.2)). One bin of *Enteroccus*
267 *faecalis* also had a mean absolute abundance (3.43) above the mean absolute abundance of
268 selected bins and was found to be abundant in several samples as well (one *D. sp. nov* FG
269 (33.8), three pangolin samples (10.8, 13.3 and 21.8), and one *T. tetradactyla* (15.3)).

270 At least one chitinolytic bin (a high-quality selected bin carrying at least one GH18
271 sequence with an active chitinolytic site) was detected in the gut microbiota of each host
272 species (Fig. 4, Table S2). Consistent with our previous results (Fig. 2) these bins mainly
273 belonged to the Firmicutes (Fig. 4). More chitinolytic bins were detected in gut metagenomes
274 of xenarthran species compared to the aardvark, the southern aardwolf, and the ground

275 pangolin, except for *Dasypus kappleri* for which we only had one sample (Fig. 4; Table S3).
276 When compared to the total number of genome bins detected per species, xenarthran species
277 had higher proportions of chitinolytic genome bins (Table S3).
278



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280 **Fig 3 Detection of the 314 high-quality bacterial genomes** (lines) **in the 29 gut metagenomes**
281 (columns) **of the nine focal species.** Each square indicates the detection of a genome bin in a
282 sample as estimated by anvi'o v7 (41). Names of bins are indicated on the left with red
283 indicating chitinolytic bins (Table S2). The names begin with the genus to which the bin was
284 assigned to. Asterisks (*) indicate bins detected in at least one soil sample (detection > 0.25)
285 (Fig. S4, Table S2, and detection table available via Zenodo). Phylogenetic relationships of host
286 species distinguished by different color strips are represented at the bottom of the graph.
287 Columns on the right indicate (from left to right): the number of GH18 sequences identified
288 in each bin (from 0 to 17), the bin's taxonomic phylum, class, order, and family. The phylogeny
289 of the 314 selected bins inferred with PhyloPhlAn v3.0.58 (42) is also represented on the right
290 of the graph (see Fig. S1). Silhouettes were downloaded from phylopic.org.

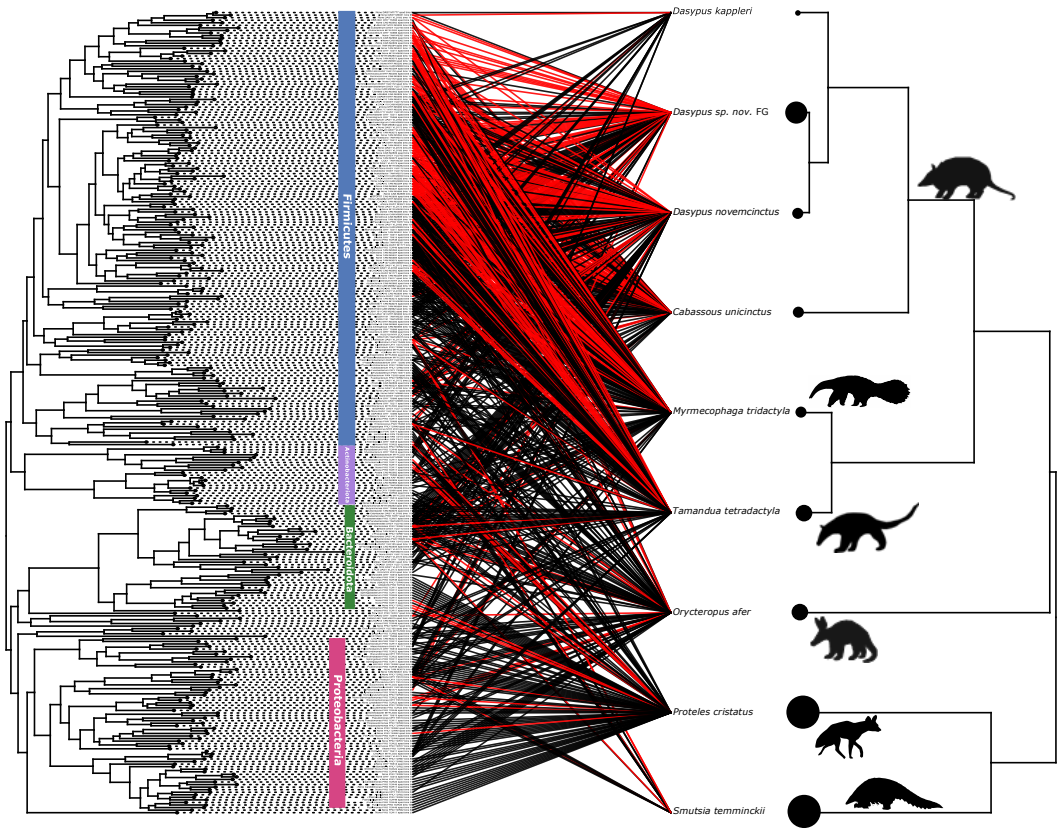

Fig 4 Distribution of chitinolytic selected bins (red links) **among the nine focal myrmecophagous species and relatives.** Phylogenies of the 314 high-quality selected bins (Fig. S1) and of the nine host species (downloaded from timetree.org) are represented respectively on the left and the right of the graph. Links illustrate, for each bin, in which host species the bin was detected (detection threshold > 0.25). Red links indicate bins in which at least one GH18 sequence with an active chitinolytic site (DXXDXDXE) was found (chitinolytic bins). The size of the circles at the tips of the host phylogeny is proportional to the number of samples (n = 1 for *D. kap*; n = 2 for *D. nov*, *C. uni* and *M. tri*; n = 3 for *T. tet* and *O. afe*; n = 4 for *D. sp. nov* FG; n = 6 for *P. cri* and *S. tem*). Bins' names are indicated at the tip of the bins' phylogeny and main bacterial phyla are indicated by colored vertical bars. This graph was done with the cophylo R package within the phytools suite (43). Silhouettes were downloaded from phylopic.org.

Discussion

Potential role of the gut microbiota in prey digestion in myrmecophagous mammals

The gut microbiota plays an important role in host digestion, which has led to several cases of gut microbiome convergence in distantly related species that share similar diets (5, 19, 44). In animals with a chitin-rich diet, symbiotic chitinolytic bacteria could be involved in prey

digestion, as they have been identified in species as diverse as nine-banded armadillos (45), insectivorous bats (28), insectivorous monkeys (30), or crustacean-eating whales (46). Here, we focused on reconstructing high-quality bacterial genome bins from gut metagenomes of species representative of the five myrmecophagous orders and identified sequences belonging to the main bacterial chitinases (i.e., GH18) (32, 33, 47). This allowed us to assess the potential of chitin degradation of gut symbionts in myrmecophagous placentals and better understand their convergent adaptation to this highly specialized diet.

Among the high-quality bacterial genome bins reconstructed, several clustered together in myrmecophagous-specific clades. These clades were found mainly within the Lachnospiraceae and Actualibacteraceae families within Firmicutes but also within the Oscillospiraceae and Ruminococcaceae. Several of these genomes had no taxonomic assignment below the family level, suggesting they could represent bacterial taxa not described yet and could be linked with the adaptation to myrmecophagy. Most of these bacterial taxa (i.e., Lachnospiraceae like *Blautia* or *Roseburia* genera or Ruminococcaceae) were previously found to be significantly more abundant in the gut microbiota of the aardwolf, giant anteater, and southern tamandua when compared to their non-myrmecophagous sister-species based on 16S rRNA metabarcoding data (19), which suggests that their presence may be associated with dietary adaptations. Besides, chitin-degrading metabolic pathways were identified in the gut metagenome of the Malayan pangolin (*Manis javanica*) as well as genes encoding chitinases and chitin-binding proteins belonging to the GH18 and GH19 enzyme families (20). This allowed the identification of chitinolytic symbionts such as *Enterococcus faecalis* (Enterococcaceae), *Clostridium paraputrificum* (Clostridiaceae), and *Bacteroides fragilis* (Bacteroidaceae) (20), whose genomes were also reconstructed here from Temminck's pangolin (*Smutsia temminckii*) gut metagenomes, confirming their probable role in prey digestion in pangolins.

Numerous GH18 genes were identified within the high-quality bacterial genomes we reconstructed here. We were able to identify 132 genome bins carrying at least one GH18 gene among which 83 had at least one GH18 sequence with an active chitinolytic site (DXXDXDXE) (48, 49), here called chitinolytic bins, and representing bacteria that might thus play a role in insect prey digestion. Among genome bins found in the three myrmecophagous-specific clades mentioned previously, almost all had at least one GH18 sequence. This further suggests that these genome bins could represent bacterial taxa playing a significant role in chitin digestion in myrmecophagous species. Studying the distribution of these bacteria in other insectivorous mammals will allow further understanding their role in the adaptation toward these specialized diets.

In addition, some of the bacterial taxa we identified here as being potentially involved in chitin digestion in myrmecophagous species belonged to or were closely related to bacterial taxa known for their chitinolytic properties. For example, *Clostridium* species are known for their chitinolytic activity (50–52). Chitinases have also been identified and studied in *Enterococcus faecalis* (53, 54). More generally, Lachnospiraceae, for which we reconstructed the most genomes, are known to degrade complex polysaccharides (55) and

351 our results suggest they could be involved in chitin hydrolysis. Moreover, several genomes of
352 Ruminococcaceae bacteria reconstructed here encode a GH18. Within this family,
353 *Ruminococcus* species have been suggested to play a role in chitin digestion in the gut
354 microbiota of insectivorous mammals (56). Several GH18 sequences were found in the same
355 bacterial genomes, suggesting that complex chitin-degrading pathways are present in the gut
356 microbiota of the myrmecophagous species, consistent with previous analyses of the
357 chitinolytic properties of certain bacteria. Indeed, several enzymes are involved in the
358 different steps of chitin hydrolysis (23). For example, two types of chitin-binding and chitinase
359 enzymes have been described in *E. faecalis* (54). This bacteria also carries endo-β-N-
360 acetylglucosaminidases (57) which are part of the chitin hydrolysis pathway and degrade
361 chitin into chitosan (23). This further highlights the complex chitinolytic machinery of these
362 bacteria.
363 Finally, some sequences do not have the ability to hydrolyze chitin because no active
364 site was identified, but could still bind chitin. For example, the Lysin-motif (LysM) domain of
365 certain proteins can bind peptidoglycan (39). It is present in certain eukaryotes chitinases (i.e.,
366 algae, nematodes) and participates in the recognition of symbiotic rhizobial bacteria by
367 leguminous plants and is thus thought to bind chitin (39, 58). The SH3 domain is also known
368 to have binding properties and is involved in protein interactions (40). Therefore, sequences
369 similar to LysM and SH3 domain-containing proteins could still be part of the chitin hydrolysis
370 process by influencing molecular interactions. In addition, enzymes carrying the
371 carbohydrate-binding module 37 (CBM37), which is known to have binding properties notably
372 to chitin, could also be involved in the chitin degrading process. The presence of other
373 enzymes potentially involved in insect digestion could be investigated, such as trehalases,
374 enzymes that break down trehalose, a sugar found in insect blood. The gut microbiota of the
375 Malayan pangolin and giant anteater were found to be significantly enriched in these enzymes
376 when compared to non-myrmecophagous species (21). The gut microbiota also plays a role
377 in the detoxification of ingested compounds (26, 44), such as insect toxins. For example,
378 metabolic pathway analyses have revealed the potential of microbial symbionts to detoxify
379 formic acid in some myrmecophagous species (21).

380 **Shared and species-specific gut bacteria among myrmecophagous mammals**

381 Morphological and genomic comparative studies have shown that the convergent adaptation
382 to myrmecophagy in mammals involved different mechanisms between the different species
383 and that phylogenetic constraints played a central role (15, 18, 59–61). As diet is one of the
384 main factors shaping the evolution of the gut microbiota (e.g., (5)), this raises the question of
385 whether the same bacterial symbionts with similar functions were convergently recruited
386 between the different ant-eating species, or whether it was different bacteria with similar
387 functions (functional convergence).
388 Analysis of the distribution of selected genome bins across samples revealed both
389 shared and host-specific bacteria among myrmecophagous species. Selected genomes were
390 mainly shared between closely related species and mainly among Xenarthra but less so

391 between distantly related orders such as pangolins (Pholidota) and anteaters (Pilosa),
392 highlighting the influence of host evolutionary history. In mammals, phylosymbiosis is
393 particularly strong (9, 62) in part because of mammalian-specific traits such as viviparity or
394 parental care (63) but also limited microbial dispersal abilities (64) facilitating the vertical
395 transmission of microbes, which may explain these results. Some bacterial genomes were
396 shared between distantly related host species and those shared across more than one order
397 mainly belong to the family Lachnospiraceae. One selected genome of *Bacteroides fragilis* and
398 two of Enterobacteriaceae were shared between species belonging to the five different
399 myrmecophagous orders and carry GH18. Similarly, seven selected genome bins were shared
400 among four host orders and belong to *Enteroccocus faecalis*, the Lachnospiraceae (i.e., *Blautia*
401 sp., *Faecalimonas* sp.), *Bacteroides* sp. (Bacteroidaceae) and *Emergencia timonensis*
402 (Anaerovoracaceae). These shared high-quality selected genome bins carrying GH18 may thus
403 participate in the adaptation to myrmecophagy and highlight the influence of the host diet,
404 consistent with previous studies showing that it is an important factor in shaping the
405 mammalian gut microbiota (e.g., (1, 4, 5, 8)). Each host species appears to carry chitinolytic
406 bacteria in its gut microbiota, confirming their potential role in adapting to an insect-based
407 diet. However, differences in the distribution of these chitinolytic selected bins could highlight
408 divergent microbiota adaptations, especially in xenarthran species, which carry more
409 chitinolytic bacteria than aardvark, ground pangolin, and southern aardwolf. Some bins were
410 found to be abundant in some samples raising questions on how the gut microbiota is
411 involved in prey digestion. Having few highly abundant chitinolytic bacterial species producing
412 large amounts of chitinases might be sufficient for a specific host to digest its prey, whereas
413 having diverse chitinolytic bacterial species might have been selected for in other host
414 species.

415 The distribution of high-quality selected genome bins across samples and species
416 revealed how shared and host-specific bacteria may reflect the divergent evolutionary
417 histories of host species and the effect of adapting to a similar diet despite phylogenetic
418 constraints. This relates to the evolution of the mammalian gut microbiota. From a
419 mammalian ancestor that probably had an insectivore-like gut microbiota (62), its
420 composition might have changed as placentals diversified and occupied new niches,
421 constraining its composition in certain species. Chitinolytic bacteria shared by all
422 myrmecophagous orders may represent ancient bacterial lineages inherited from a common
423 ancestor, while bacteria found in only certain host species may reflect more recent
424 adaptations with bacteria acquired in specific lineages.

425 The environment and biogeography also influence host-associated microbial
426 communities, which could explain the distribution pattern of selected bins across samples,
427 with large differences observed between South Africa and South America. Anteaters and
428 armadillos (Xernarthra) diverged anciently from aardvarks, pangolins, and aardwolves (~80
429 Ma) (14), and these lineages have evolved in very distinct environments and biogeographic
430 contexts for most of their evolutionary history. Soil samples from the fecal sampling sites have
431 been collected for the South African samples. Some selected bins reconstructed from gut

metagenomes of the ground pangolin, southern aardwolf, and aardvark belonged to bacterial taxa that were indeed not expected to be found in the gut but rather in the environment (e.g., Sphingobacteriaceae, Xanthomonadaceae, Burkholdericaeae). The distribution of selected genome bins reconstructed from gut metagenomes of these species was examined in soil samples and showed that 91 selected bins were also detected in at least one soil sample (detection > 0.25; Fig. S4, Table S2). For example, among the Proteobacteria selected genome bins found in myrmecophagous-specific clades (Fig. S2B), some belonged to Burkholderiaceae bacteria and were detected in at least one soil sample. This may not necessarily reflect environmental contamination, as the center of the feces was specifically sampled to minimize contamination. Rather, it may reflect the fact that these species ingest soil and thus environmental microbes while foraging (11). This could be beneficial to the host (65), as it may compensate for the lack of mastication and/or help to deal with toxins that might be present in prey (11). Environmental acquisition of microbes has also been suggested in lemurs (66) and wild echidnas (67), species that ingest soil during foraging and in which soil bacteria have been identified in their gut microbiota. These environmental bacteria could therefore represent transient bacteria in the gut, reflecting what is ingested by the host. They could also represent resident bacteria that have been recruited from the environment into the gut microbiota of myrmecophagous species. Indeed, some beneficial microorganisms could be acquired from soil microbiomes (68), which could allow the host to better adapt to its environment through horizontal gene transfer of beneficial genes (69). Moreover, it has also been suggested that environmental bacteria could be a source of chitinase genes to digest prey (22). Thus, their recruitment into the gut microbiota may have been selected to participate in the host digestion and further contribute to the adaptation to its environment. This would highlight the potential influence of the environment on the gut microbiota of myrmecophagous mammals.

Role of the holobiont in prey digestion in myrmecophagous mammals

The influence of the microbiota on host evolution and their coevolution is increasingly recognized as studies of the diverse microbiota of captive and wild animals proliferate. Many scientists now recognize the term holobiont to describe the host and all its associated symbionts, which would be the unit of natural selection as defined by the hologenome theory of evolution (70). Integrative studies (e.g., (21, 71)) and initiatives such as the “Earth Hologenome Initiative'' (http://www.earthhologenome.org/) to study hologenomic adaptations are now becoming more common. In the specific case of myrmecophagous mammals, a hologenomic approach would help to better understand the adaptive mechanisms involved. Indeed, if chitinolytic bacteria can digest chitin, prey digestion can also be ensured by host-produced chitinases. In mammals, from a placental ancestor that probably carried five functional chitinase paralogs (*CHIA*), some of these paralogs were subsequently lost during placental diversification in non-insectivorous species, leading to a positive correlation between the number of functional chitinase paralogs and the proportion of invertebrates in the diet (15); a correlation also observed in primates (72). Among

myrmecophagous species, this chitinase gene repertoire has evolved differently to ensure chitin digestion, reflecting phylogenetic constraints (15, 18). Thus, both endogenous and microbial chitinases may be involved in prey digestion, raising the question of the relative contribution of the host and its symbionts in providing the same function. For example, myrmecophagous mammals with only one functional *CHIA* paralog (*CHIA5*), such as aardwolves and pangolins, may compensate for this by overexpressing their only functional paralog or by relying more on their chitinolytic symbionts to digest their prey. In pangolins, *CHIA5* has been found to be overexpressed in all digestive organs (18). In the aardwolf, which recently diverged from the other Hyaenidae species (~10 Ma) (73), and does not present strong morphological adaptations to myrmecophagy, no host chitinases have been found to be expressed in its salivary glands (Allio *et al*, 2023) and expression of these enzymes in other digestive organs needs further investigation. Therefore, this species could rely more on its gut microbiota to ensure prey digestion. Based on our results, fewer chitinolytic bacteria were found in the gut microbiota of the southern aardwolf compared to other species but they could still ensure chitin digestion, for instance, by overexpressing their chitinases. The combination of the host genomic and transcriptomic data with metagenomic and metatranscriptomic data of its microbiota would allow answering this type of question by comparing host gene repertoires in light of the chitinolytic abilities of the gut microbiota. This will shed light on the adaptation to chitin digestion in placentals and, more generally, on the role of the holobiont in the adaptation to a specific function.

Materials and methods

1. Sampling

Thirty-three fecal samples were collected from nine species representative of the five myrmecophagous placental orders (Table 1). For armadillos and anteaters provided by the JAGUARS collection (Cayenne, French Guiana), fecal samples from roadkill and deceased zoo animals were obtained after unfreezing the specimens and dissecting the lower part of the digestive tract in the lab facilities provided by Institut Pasteur de la Guyane (Cayenne, French Guiana). Roadkill armadillos collected in the USA were also dissected to sample feces. For the aardvark, ground pangolin, and southern aardwolf, fresh fecal samples were collected directly in the field during fieldwork sessions conducted in Tswalu Kalahari and Tussen-die-Riviere reserves (South Africa). The inner part of the feces was sampled with a sterile scalpel blade to avoid soil contamination. In the South African reserves, eight soil samples were also collected near feces sampling sites to serve as a control for potential environmental contamination (Table S4). All fecal and soil samples were stored at -20°C in 96% ethanol before DNA extraction.

511 **Table 1. Detailed sample information for the 33 fecal samples collected.**

Sample name	Species name	Common name	Order	Diet	Sex	Age	Wild / Captive	Country of origin	Location	Accession number	
										Illumina	ONT
CAB M2809	*Cabassous unicinctus*	Southern naked-tailed armadillo	Cingulata	Myrmecophagous	Male	Adult	Wild	French Guiana	coll. JAGUARS	SRR23925023	SRR23925022
CAB M2982	*Cabassous unicinctus*	Southern naked-tailed armadillo	Cingulata	Myrmecophagous	Male	Adult	Wild	French Guiana	coll. JAGUARS	SRR23925011	SRR23925000
CAB M3141	*Cabassous unicinctus*	Southern naked-tailed armadillo	Cingulata	Myrmecophagous	Female	Adult	Wild	French Guiana	coll. JAGUARS	SRR23924989	SRR23924978
DASY M1748	*Dasypus novemcinctus sp FG*	Guianan long-nosed armadillo	Cingulata	Omnivorous	Male	Adult	Wild	French Guiana	coll. JAGUARS	NA	SRR23924967
DASY M1777	*Dasypus novemcinctus sp FG*	Guianan long-nosed armadillo	Cingulata	Omnivorous	Male	Juvenile	Wild	French Guiana	coll. JAGUARS	SRR23924962	SRR23924961
DASY M2256	*Dasypus novemcinctus sp FG*	Guianan long-nosed armadillo	Cingulata	Omnivorous	NA	Adult	Wild	French Guiana	coll. JAGUARS	SRR23924960	SRR23925021
DASY M2866	*Dasypus novemcinctus sp FG*	Guianan long-nosed armadillo	Cingulata	Omnivorous	Female	Adult	Wild	French Guiana	coll. JAGUARS	SRR23925020	SRR23925019
DASY M3021	*Dasypus novemcinctus sp FG*	Guianan long-nosed armadillo	Cingulata	Omnivorous	Male	Adult	Wild	French Guiana	coll. JAGUARS	SRR23925018	SRR23925017
DASY VLD165	*Dasypus novemcinctus*	Nine-banded armadillo	Cingulata	Omnivorous	Female	Adult	Wild	USA	Valdosta (GA)	SRR23925016	SRR23925015
DASY VLD168	*Dasypus novemcinctus*	Nine-banded armadillo	Cingulata	Omnivorous	Male	Adult	Wild	USA	Valdosta (GA)	NA	SRR23925014
DASY VLD172	*Dasypus novemcinctus*	Nine-banded armadillo	Cingulata	Omnivorous	Female	Adult	Wild	USA	Valdosta (GA)	SRR23925013	SRR23925012
KAP M2867	*Dasypus kappleri*	Greater long-nosed armadillo	Cingulata	Insectivorous	Male	Adult	Wild	French Guiana	coll. JAGUARS	SRR23925010	SRR23925009
MYR M5293	*Myrmecophaga tridactyla*	Giant anteater	Pilosa	Myrmecophagous	Female	Adult	Wild	French Guiana	coll. JAGUARS	SRR23925008	SRR23925007
MYR M5295	*Myrmecophaga tridactyla*	Giant anteater	Pilosa	Myrmecophagous	NA	Juvenile	Wild	French Guiana	coll. JAGUARS	SRR23925006	SRR23925005
MYR ZOO	*Myrmecophaga tridactyla*	Giant anteater	Pilosa	Myrmecophagous	Female	Adulte	Captive	France	Montpellier zoo	SRR23925004	SRR23925003
ORY T8217	*Orycteropus afer*	Aardvark	Tubulidentata	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23925002	SRR23925001
ORY T8513	*Orycteropus afer*	Aardvark	Tubulidentata	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23924999	SRR23924998
ORY T8588	*Orycteropus afer*	Aardvark	Tubulidentata	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23924997	SRR23924996
PAN T8471	*Smutsia temminckii*	Ground pangolin	Pholidota	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23924995	SRR23924994
PAN T8476	*Smutsia temminckii*	Ground pangolin	Pholidota	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23924993	SRR23924992
PAN T8482	*Smutsia temminckii*	Ground pangolin	Pholidota	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23924991	SRR23924990
PAN T8488	*Smutsia temminckii*	Ground pangolin	Pholidota	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23924988	SRR23924987
PAN T8489	*Smutsia temminckii*	Ground pangolin	Pholidota	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23924986	SRR23924985
PAN T8626	*Smutsia temminckii*	Ground pangolin	Pholidota	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23924984	SRR23924983
PRO TDR7	*Proteles cristatus*	Southern aardwolf	Carnivora	Myrmecophagous	NA	NA	Wild	South Africa	Tussen-die-Riviere reserve	SRR23924982	SRR23924981
PRO TDR17	*Proteles cristatus*	Southern aardwolf	Carnivora	Myrmecophagous	NA	NA	Wild	South Africa	Tussen-die-Riviere reserve	SRR23924980	SRR23924979
PRO TDR22	*Proteles cristatus*	Southern aardwolf	Carnivora	Myrmecophagous	NA	NA	Wild	South Africa	Tussen-die-Riviere reserve	SRR23924977	SRR23924976
PRO TDR49	*Proteles cristatus*	Southern aardwolf	Carnivora	Myrmecophagous	NA	NA	Wild	South Africa	Tussen-die-Riviere reserve	SRR23924975	SRR23924974
PRO TDR82	*Proteles cristatus*	Southern aardwolf	Carnivora	Myrmecophagous	NA	NA	Wild	South Africa	Tussen-die-Riviere reserve	SRR23924973	SRR23924972
PRO TDR87	*Proteles cristatus*	Southern aardwolf	Carnivora	Myrmecophagous	NA	NA	Wild	South Africa	Tussen-die-Riviere reserve	SRR23924971	SRR23924970
TAM M3075	*Tamandua tetradactyla*	Southern tamandua	Pilosa	Myrmecophagous	Male	Adult	Wild	French Guiana	coll. JAGUARS	SRR23924969	SRR23924968
TAM M5331	*Tamandua tetradactyla*	Southern tamandua	Pilosa	Myrmecophagous	Male	Adult	Wild	French Guiana	coll. JAGUARS	SRR23924966	SRR23924965
TAM M5584	*Tamandua tetradactyla*	Southern tamandua	Pilosa	Myrmecophagous	Female	Adult	Wild	French Guiana	coll. JAGUARS	SRR23924964	SRR23924963

512 *N.B.*: Diet was determined based on field observations (i.e., dissections) and the literature (11, 74-78).

513 ## 2. DNA extraction

514 Whole DNA was extracted from fecal samples following the optimized protocol of (79) using
515 the Genomic DNA from soil kit (NucleoSpin, Macherey-Nagel). Two successive extractions
516 were done and a purification step was added to retrieve high molecular weight DNA suitable
517 for long-read sequencing (79). The same kit was used to extract DNA from soil samples. Before
518 the extraction, samples were incubated with 700 µL of the lysis buffer (SL1) (79) and 30 µl of
519 proteinase K at 56°C for 30 minutes.

520 ## 3. Library preparation and DNA sequencing

521 ### 3.1. Long-read sequencing

522 Long-read libraries were constructed using the SKQ-LSK109 and SKQ-LSK110 library
523 preparation kits (ONT, Oxford Nanopore Technologies, https://nanoporetech.com/). Shotgun
524 metagenomic sequencing was done using the MinION, MK1C, and GridION devices using one
525 R9 flowcell per sample. Between 150 and 988 ng of DNA were loaded per flowcell, which were
526 run for 48 to 72 hours (Table S1A). For samples sequenced on the GridION, super-accurate
527 basecalling was performed with Guppy v5+ (Qscore = 10) whereas on the MinION and MK1C
528 fast basecalling was used (Qscore = 7). Sequencing output statistics were checked using
529 PycoQC v2.5.2 (80) with a minimum quality score set to 7 or 10 depending on the sequencing
530 device used (Table S1A). Rebasecalling of samples sequenced on the MinION and MK1C was
531 done on a GPU machine with Guppy v5.0.16 (super accurate mode, Qscore = 10, config_file =
532 dna_r9.4.1_450bps_sup.cfg) (ONT).

533 ### 3.2. Short-read sequencing

534 Short-read shotgun metagenomic Illumina sequencing was done for all samples to generate
535 data for long-read assemblies polishing. Either the first or second extraction was used for
536 sequencing as they have previously been shown to be both suitable for short-read sequencing

537 (79). Library preparation and Illumina sequencing on a NovaSeq instrument were outsourced
538 to Novogene Europe (Cambridge, UK) to generate metagenomes using 50 million of 150 bp
539 paired-end reads each (15 Gb of raw data per sample) (Table S1A). Two samples of two
540 *Dasypus* species (DASY M1746 and DASY VLD168) could not be sequenced using short reads
541 due to low quantity of starting material and were not included in the following analyses;
542 resulting in 31 samples being analyzed. Short-read metagenomic sequencing of soil samples
543 was performed following the same protocol.

544 **4. Data filtering**

545 **4.1. Long-read data**

546 Sequencing adapters were removed with Porechop v0.2.4 (81) used with default parameters.
547 Reads shorter than 200 bp were removed using Filtlong v0.2.1 (82). No quality filtering was
548 performed at this stage as it was previously done during basecalling (Qscore > 10).

549 Host mitogenomes were downloaded for the nine myrmecophagous species of our
550 dataset from the National Center for Biotechnology Information (NCBI) Genbank database
551 (Table S1A). Long-read metagenomes were mapped to the host mitogenome with Minimap2
552 v2.17 (83, 84) with the ONT preset (*-ax map-ont*) for Oxford Nanopore reads. This allowed us
553 to confirm the host species. Host mitogenomes were then assembled from the mapping reads
554 with Flye v2.8.3 (85) with default parameters for ONT raw reads (*--nano-raw*). Mapping of
555 host mitogenomic reads on host mitogenomes was visually checked with Geneious Prime
556 2022.0.2 (86), and mitochondrial reads were removed from the metagenomes for
557 downstream analyses.

558 The same was done to remove host nuclear reads by mapping long-read
559 metagenomes against the host genome using Minimap2 v2.17 (83, 84) using the Nanopore
560 read option (*-ax map-ont*) (Table S1A). The *Dasypus novemcinctus* RefSeq genome assembly
561 (Dasnov 3.0; GCF_000208655.1) was downloaded from Genbank. The *Orycteropus afer*
562 genome was downloaded from the DNA Zoo database (87) (HiC assembly based on the draft
563 assembly of Di Palma *et al*, unpublished). *Myrmecophaga tridactyla*, *Tamandua tetradactyla*,
564 *Smutsia gigantea,* and *Proteles cristatus* genomes have been previously reconstructed using
565 long (ONT) and short (Illumina) reads assembled with MaSuRCA v3.2.9 (88) (see (89) for a
566 detailed description of the hybrid assembly process). For *Cabassous unicinctus*, we used the
567 Discovar draft genome assembly generated by (90). For species lacking an available reference
568 genome, the genome of the closest relative in our dataset was used: *Smutsia gigantea* for *S.*
569 *temminckii* and *Dasypus novemcinctus* for *D. kappleri* and *Dasypus sp. nov.* FG. Host reads
570 were removed from the metagenomes for downstream analyses. One southern naked-tailed
571 armadillo sample (CAB M3141) and one giant anteater sample (MYR M5295) presented a high
572 proportion of host reads (> 95%) and were excluded from the following analyses. The final
573 dataset thus included 29 samples.

574 Finally, contaminant human reads were removed following the same approach and
575 using the telomere-to-telomere human genome assembly (GCA009914755.3) as reference
576 (Table S1A).

577 ### 4.2. Short-read data

578 Illumina sequencing adapters were removed using FASTP v0.20.0 with default quality filtering
579 parameters (91). Host and human reads were removed using the same approach as for long-
580 read metagenomes but the mapping was done with bowtie2 v2.3.5 (92) with default
581 parameters (Table S1A).

582 Samtools v1.7 (93) was used to manipulate long- and short-read mapping files.

583 ## 5. Metagenome assembly

584 Long-read metagenomes were assembled for each sample using metaFlye v2.9 (94) with
585 default parameters for ONT reads basecalled with Guppy v5+ (*--nano-hq*, recommended
586 mode when < 5% of sequencing errors is expected) and the strain mode (*--keep-haplotypes*),
587 which prevents closely related strains represented by different paths in the assembly graph
588 to be collapsed. Long-read metagenome assemblies were then polished with short-reads
589 using Pilon v1.24 (95) with default parameters. Short-read metagenomes were assembled
590 using metaSPAdes v3.11.0 (96) and MEGAHIT v1.1.2 (97) with default parameters.

591 Assembly statistics were computed with anvi'o v7 (41) (Tables S1B and C). Anvi'o first
592 generates a contig database in which k-mer frequencies for each contig are computed (k = 4).
593 Prodigal v2.6.3 (98) is then used to predict archaeal and bacterial open reading frames and
594 estimate the number of genes. Finally, microbial single-copy core genes are searched using
595 HMMER (99) with the hmmscan v3.2.1 program (default in anvi'o).

596 ## 6. Reconstruction of bacterial genomes

597 ### 6.1. Genome binning and bins selection

598 Prior to binning, reads were mapped to metagenome assemblies to obtain the coverage
599 information needed for binning. This was done using Bowtie2 v2.2.9 (92) for short reads and
600 Minimap2 v2.17 (83, 84) for long reads. Genome binning was conducted on single long-read
601 polished assemblies and short-read assemblies separately (trimmed to keep contigs longer
602 than 1000 bp) using metaBAT2 v2.15 (100) with default parameters (minimum size of contigs
603 set to 2500 bp and bin minimal length set to 200 kb).

604 Completeness and redundancy of bins were estimated using anvi'o v7 (Tables S1D and
605 E), which relies on the detection of single-copy core genes (SCG). Bins with more than 90%
606 completeness and less than 5% redundancy were selected for downstream analyses. Based
607 on these criteria, 239 genome bins were selected from the long-read assemblies and 254 from
608 the short-read assemblies. Genome bins statistics were computed using anvi'o v7 as done for
609 the metagenome assemblies (Tables S1D and E).

610 ### 6.2. Genome bins dereplication

611 To remove potentially redundant bins, a dereplication step was performed on the set of
612 selected bins using dRep v3.3.0 (101). dRep first filters genomes based on their length (> 50
613 000 by default) and their completeness and redundancy as computed with CheckM v1.1.11
614 (102) (> 75% completeness, < 25% redundancy by default). dRep then clusters genomes based

615 on an Average Nucleotide Identity (ANI) threshold of 90% using the Mash algorithm v2.3
616 (103). Genomes having at least 90% ANI are then clustered using fastANI v1.33 (104), here
617 with a secondary ANI threshold of 98% (recommended to avoid mis-mapping of metagenomic
618 reads against bins, for example, to study their distribution across metagenomes). By default,
619 at least 10% of the genome is compared (minimum alignment fraction). Two bins did not pass
620 dRep quality filtering in each dataset and were not included in the analysis. In the long-read
621 dataset, 38 genomes were removed by dRep with an ANI of 98%, resulting in 201 unique
622 genome bins. In the short-read dataset, 48 genomes were removed, resulting in 206 unique
623 genome bins. Selected genome bin statistics were compared between bins reconstructed
624 from long-read and short-read assemblies using anvi'o v7 (see supplementary results part 1
625 available via Zenodo).
626 To determine whether similar genome bins were reconstructed using long-read
627 polished assemblies (n = 201) or short-read assemblies (n = 206), dRep was also run on the
628 set of all selected bins (n = 407) with 98% ANI to remove redundant genome bins (93 genomes
629 removed, resulting in 314 non-redundant high-quality bins). To capture the majority of the
630 bacterial taxa present in our samples, we decided to combine high-quality selected bins
631 reconstructed from both long- and short-read assemblies dereplicated with an ANI of 98% in
632 our final dataset for downstream analyses (n = 314; Table S1F) (see supplementary results
633 part 1 and dRep output results available via Zenodo).
634
635 **6.3. Taxonomic assignment of selected bins**
636 The taxonomy of selected genome bins reconstructed from long- and short-read assemblies
637 was assessed using anvi'o v7 which uses 22 single-copy core genes (SCGs) and the taxonomy
638 of the genomes defined by the Genome Taxonomy Database release 7 (GTDB) (38) from which
639 these genes have been extracted (Table S1F).
640 The 314 selected bins were placed in a phylogeny of 2566 reference prokaryotic
641 genomes downloaded from Genbank using PhyloPhlAn v3.0.58 (42). PhyloPhlAn first searches
642 universal prokaryotic marker genes (option *-d* phylophlan, a database comprising 400
643 markers) (105), here using Diamond v2.0.6 (106). Marker genes were then aligned using
644 MAFFT v7.475 (107), and cleaned using trimAl v1.4 (108). Next, marker gene alignments were
645 concatenated. Finally, the phylogeny was inferred using IQ-TREE v2.0.3 (109) under the LG
646 model. PhyloPhlAn was run with the *–diversity* high and *–fast* options, which together set a
647 range of parameters for the reconstruction of high-ranked taxonomic level phylogenies (42).
648 By default, at least 100 markers of the PhyloPhlAn database must be present in a genome for
649 it to be included in the analysis (*--min_num_markers* 100) and each marker should be found
650 in at least four genomes to be included in the analysis (*--min_num_entries* 4). Seventy of the
651 reference genomes had less than 100 markers and were excluded from the analysis resulting
652 in a total of 2810 genomes in the final reconstructed tree. The phylogeny was rooted with
653 Archaea so the Bacteria were monophyletic. Using the same PhyloPhlAn parameters, a
654 phylogeny of the set of the 314 selected bacterial genome bins was also inferred (Fig. S1). This
655 phylogeny was rooted according to (110), placing the root of the bacterial tree separating

656 Terrabacteria (e.g., Firmicutes, Actinobacteriota) and Gracilicutes (e.g., Proteobacteria,
657 Desulfobacterota, Campylobacterota, Fibrobacteres-Chlorobi-Bacteroidetes group).

658 ### 6.4. Distribution of selected bins in gut metagenomes

659 Short-read metagenomes were mapped against the 314 selected bins using bowtie2 v2.3.5
660 with local sensitive alignment parameters. The distribution of bins across samples was
661 explored using anvi'o v7. Anvi'o enables us to compute the percentage of mapped reads on
662 each bin for each metagenome and computes statistics such as the coverage, percentage of
663 recruited reads, abundance, and detection (horizontal coverage) of bins across metagenomes
664 (samples). The distribution across samples was studied using the anvi'o interface by
665 visualizing the detection of selected genome bins across samples. A detection threshold of
666 0.25 % of the reference covered by at least one read was chosen to consider a bin detected
667 in a sample. The number of bins shared among the different samples and host species and
668 bins specific to a sample or host species were calculated. Applying the same approach, the
669 distribution of selected bins reconstructed from the aardvark, ground pangolin, and southern
670 aardwolf samples (n = 140) was explored in the eight soil samples collected in South Africa
671 near feces sampling sites (Fig. S4).

672 ## 7. Identification of bacterial chitinase genes

673 Chitinase genes were searched in selected genome bins. More specifically, sequences of
674 enzymes belonging to the Glycoside Hydrolase 18 (GH18) family (comprising chitinases and
675 chitin-binding proteins) as determined by the classification of Carbohydrate Active enzymes
676 (CAZymes) (31) were scanned using dbCAN2 (111) with default parameters. dbCAN2 is
677 designed to annotate CAZymes sequences in genomes using an HMMER search (99) against
678 the dbCAN HMM database containing HMM models for each CAZyme family. Proteins were
679 predicted using Prodigal (98).

680 The amino acid sequences of 420 identified GH18 genes were imported in Geneious
681 Prime 2022.0.2. Sequences were aligned using MAFFT v7.450 with default parameters. The
682 alignment was cleaned by removing sites not present in at least 50% of the sequences,
683 resulting in an alignment of 390 amino acids. The chitinase gene tree was inferred with RAxML
684 v8.2.11 (112) under the LG+G model and the rapid hill-climbing algorithm for topology
685 rearrangements. After removing 26 sequences that did not align correctly and had very long
686 branches, the final alignment included 394 sequences. The phylogeny was rooted with the
687 clades containing sequences without chitinolytic sites as they were divergent from the other
688 sequences. The conserved chitinolytic site (DXXDXDXE) (48, 49), typical of chitin-degrading
689 enzymes, was searched in the aligned sequences. BLAST searches against the NCBI non-
690 redundant protein database were conducted to assess whether identified GH18 genes were
691 similar to known microbial chitinases.

8. Data availability

Raw RNAseq short Illumina and long ONT reads have been submitted to the Short Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) and are available under BioProject number PRJNA942254. Metagenome assemblies, genome bins (all and selected), phylogenetic datasets (alignments and corresponding trees), and other supplementary materials and results are available from zenodo.org (https://doi.org/: 10.5281/zenodo.7995394).

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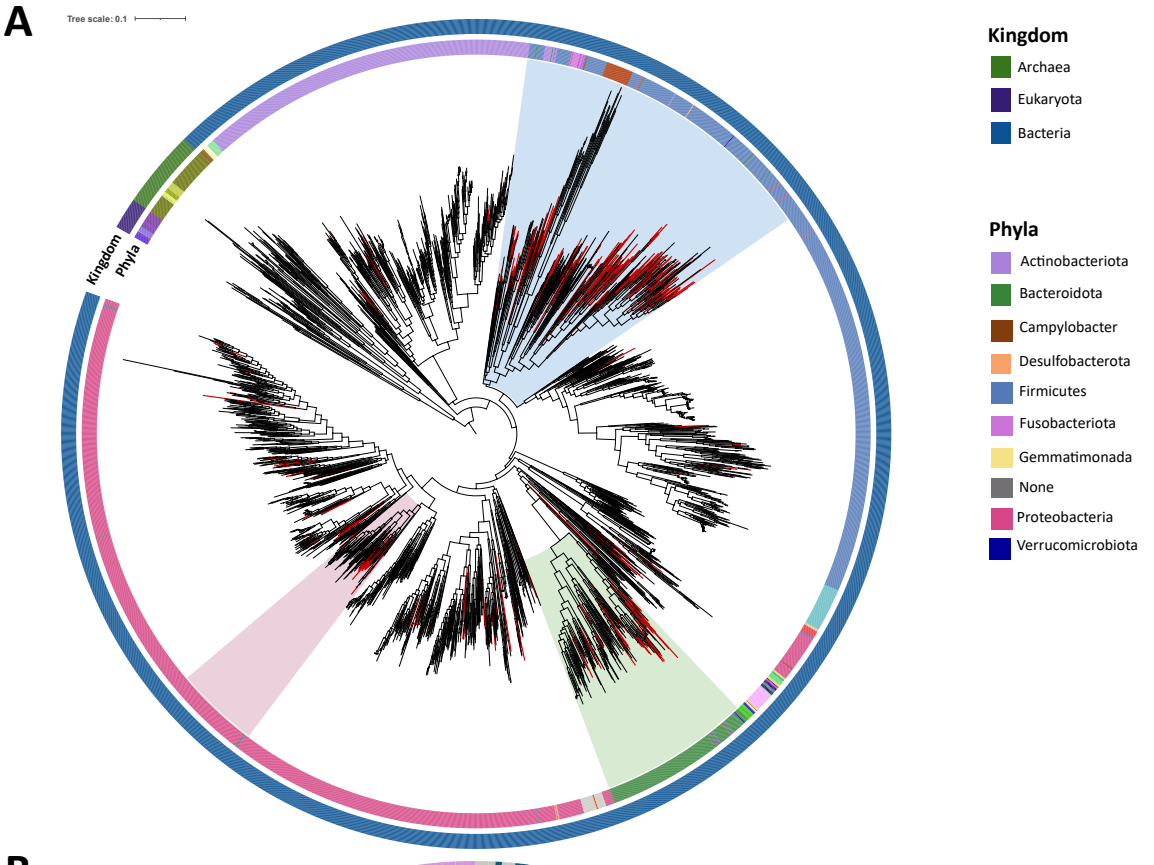
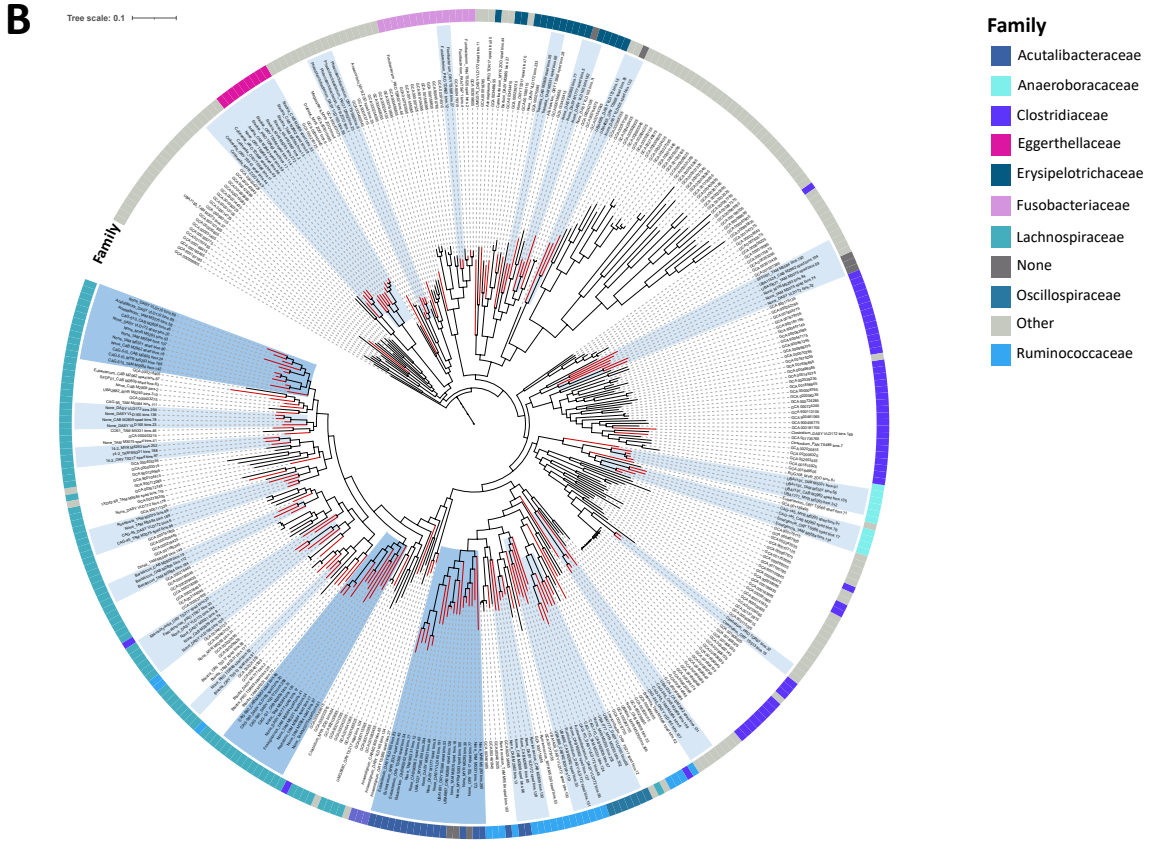
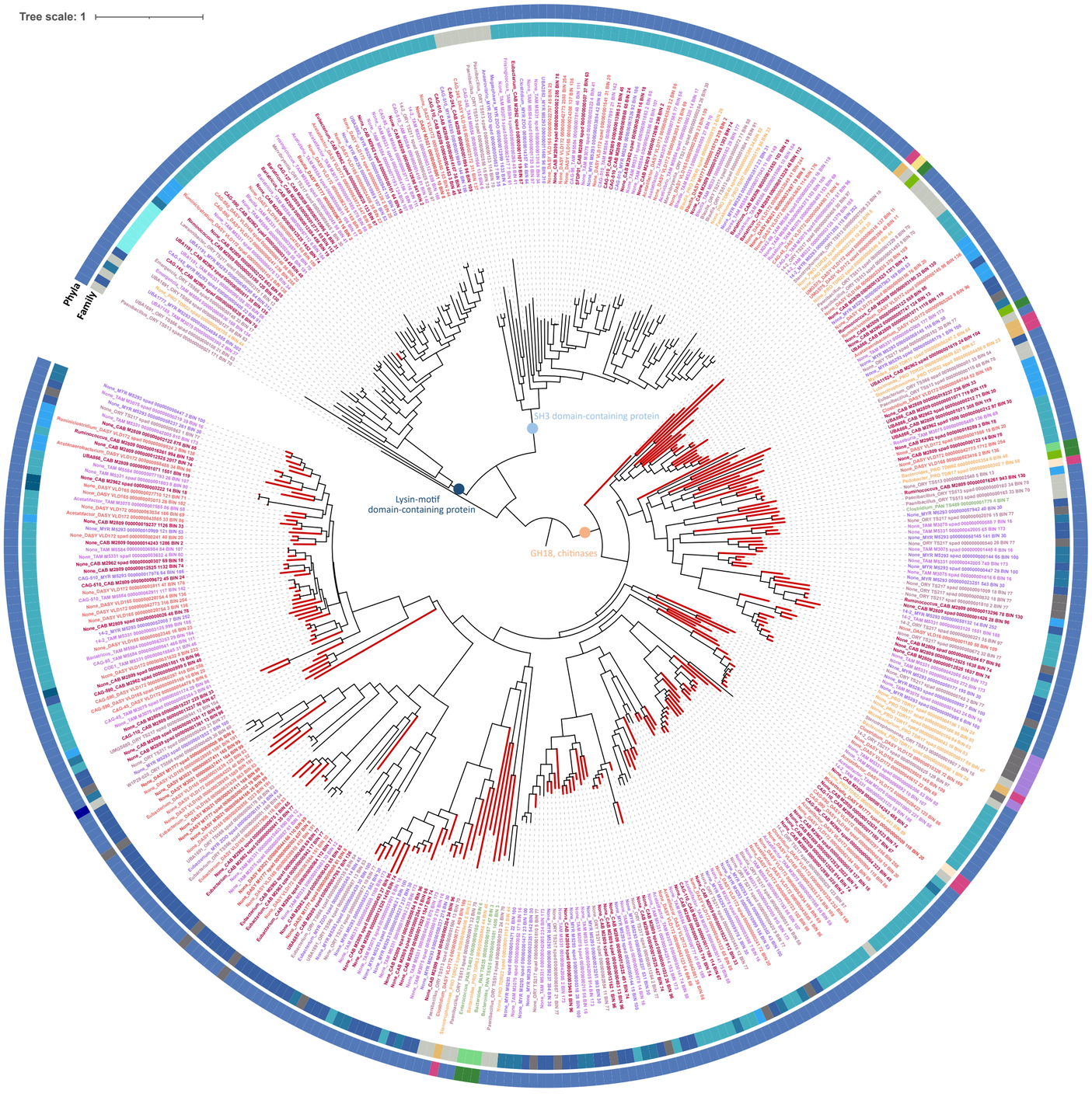

A
Tree scale: 0.1
Kingdom
Phyla
Kingdom
Archaea
Eukaryota
Bacteria
Phyla
Actinobacteriota
Bacteroidota
Campylobacter
Desulfobacterota
Firmicutes
Fusobacteriota
Gemmatimonada
None
Proteobacteria
Verrucomicrobiota


B
Tree scale: 0.1
Family
Family
Acutalibacteraceae
Anaeroboracaceae
Clostridiaceae
Eggerthellaceae
Erysipelotrichaceae
Fusobacteriaceae
Lachnospiraceae
None
Oscillospiraceae
Other
Ruminococcaceae



Family

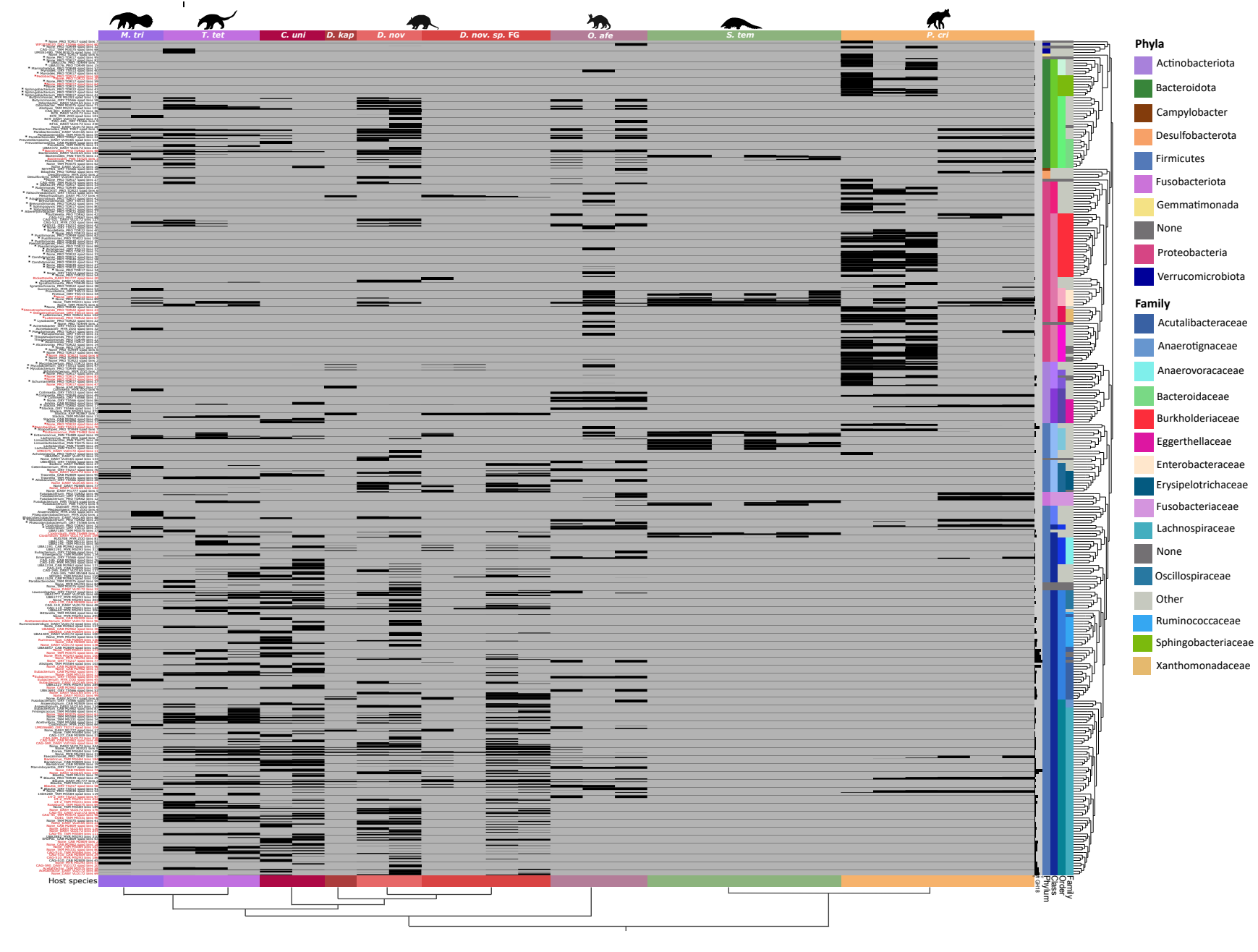
- Acutalibacteraceae
- Anaerovoracaceae
- Bacteroidaceae
- Enterobacteriaceae
- Erysipelotrichaceae
- Lachnospiraceae
- None
- Oscillospiraceae
- Other
- Ruminococcaceae
- Sphingobacteriaceae
- Xanthomonadaceae

Phyla

- Firmicutes
- Proteobacteria
- Actinobacteriota
- Bacteroidota
- None
- Verrucomicrobiota
- Gammaproteobacteria

Host species

- *Myrmecophaga tridactyla*
- *Tamandua tetradactyla*
- *Cabassous unicinctus*
- *Dasypus novemcinctus sp* FG
- *Dasypus novemcinctus*
- *Orycteropus afer*
- *Smutsia temminckii*
- *Proteles cristatus*


M. tri
T. tet
C. uni
D. kap
D. nov
D. nov. sp. FG
O. afe
S. tem
P. cri
Host species
Phylum
Class
Order
Family
Phyla
Actinobacteriota
Bacteroidota
Campylobacter
Desulfobacterota
Firmicutes
Fusobacteriota
Gemmatimonada
None
Proteobacteria
Verrucomicrobiota
Family
Acutalibacteraceae
Anaerotignaceae
Anaerovoracaceae
Bacteroidaceae
Burkholderiaceae
Eggerthellaceae
Enterobacteraceae
Erysipelotrichaceae
Fusobacteriaceae
Lachnospiraceae
None
Oscillospiraceae
Other
Ruminococcaceae
Sphingobacteriaceae
Xanthomonadaceae

Dasypus kappleri
Dasypus sp. nov. FG
Dasypus novemcinctus
Cabassous unicinctus
Myrmecophaga tridactyla
Tamandua tetradactyla
Orycteropus afer
Proteles cristatus
Smutsia temminckii
Firmicutes
Actinobacteriota
Bacteroidota
Proteobacteria

Sample name	Species name	Common name	Order	Diet	Sex	Age	Wild / Captive	Country of origin	Location	Accession number	
										Illumina	ONT
CAB M2809	*Cabassous unicinctus*	Southern naked-tailed armadillo	Cingulata	Myrmecophagous	Male	Adult	Wild	French Guiana	coll. JAGUARS	SRR23925023	SRR23925022
CAB M2962	*Cabassous unicinctus*	Southern naked-tailed armadillo	Cingulata	Myrmecophagous	Male	Adult	Wild	French Guiana	coll. JAGUARS	SRR23925011	SRR23925000
CAB M3141	*Cabassous unicinctus*	Southern naked-tailed armadillo	Cingulata	Myrmecophagous	Female	Adult	Wild	French Guiana	coll. JAGUARS	SRR23924989	SRR23924978
DASY M1746	*Dasypus novemcinctus sp FG*	Guianan long-nosed armadillo	Cingulata	Omnivorous	Male	Adult	Wild	French Guiana	coll. JAGUARS	NA	SRR23924967
DASY M1777	*Dasypus novemcinctus sp FG*	Guianan long-nosed armadillo	Cingulata	Omnivorous	Male	Juvenile	Wild	French Guiana	coll. JAGUARS	SRR23924962	SRR23924961
DASY M2255	*Dasypus novemcinctus sp FG*	Guianan long-nosed armadillo	Cingulata	Omnivorous	NA	Adult	Wild	French Guiana	coll. JAGUARS	SRR23924960	SRR23925021
DASY M2865	*Dasypus novemcinctus sp FG*	Guianan long-nosed armadillo	Cingulata	Omnivorous	Female	Adult	Wild	French Guiana	coll. JAGUARS	SRR23925020	SRR23925019
DASY M3021	*Dasypus novemcinctus sp FG*	Guianan long-nosed armadillo	Cingulata	Omnivorous	Male	Adult	Wild	French Guiana	coll. JAGUARS	SRR23925018	SRR23925017
DASY VLD165	*Dasypus novemcinctus*	Nine-banded armadillo	Cingulata	Omnivorous	Female	Adult	Wild	USA	Valdosta (GA)	SRR23925016	SRR23925015
DASY VLD168	*Dasypus novemcinctus*	Nine-banded armadillo	Cingulata	Omnivorous	Male	Adult	Wild	USA	Valdosta (GA)	NA	SRR23925014
DASY VLD172	*Dasypus novemcinctus*	Nine-banded armadillo	Cingulata	Omnivorous	Female	Adult	Wild	USA	Valdosta (GA)	SRR23925013	SRR23925012
KAP M2867	*Dasypus kappleri*	Greater long-nosed armadillo	Cingulata	Insectivorous	Male	Adult	Wild	French Guiana	coll. JAGUARS	SRR23925010	SRR23925009
MYR M5293	*Myrmecophaga tridactyla*	Giant anteater	Pilosa	Myrmecophagous	Female	Adult	Wild	French Guiana	coll. JAGUARS	SRR23925008	SRR23925007
MYR M5295	*Myrmecophaga tridactyla*	Giant anteater	Pilosa	Myrmecophagous	NA	Juvenile	Wild	French Guiana	coll. JAGUARS	SRR23925006	SRR23925005
MYR ZOO	*Myrmecophaga tridactyla*	Giant anteater	Pilosa	Myrmecophagous	Female	Adulte	Captive	France	Montpellier zoo	SRR23925004	SRR23925003
ORY TS217	*Orycteropus afer*	Aardvark	Tubulidentata	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23925002	SRR23925001
ORY TS513	*Orycteropus afer*	Aardvark	Tubulidentata	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23924999	SRR23924998
ORY TS566	*Orycteropus afer*	Aardvark	Tubulidentata	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23924997	SRR23924996
PAN TS471	*Smutsia temminckii*	Ground pangolin	Pholidota	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23924995	SRR23924994
PAN TS475	*Smutsia temminckii*	Ground pangolin	Pholidota	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23924993	SRR23924992
PAN TS482	*Smutsia temminckii*	Ground pangolin	Pholidota	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23924991	SRR23924990
PAN TS488	*Smutsia temminckii*	Ground pangolin	Pholidota	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23924988	SRR23924987
PAN TS489	*Smutsia temminckii*	Ground pangolin	Pholidota	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23924986	SRR23924985
PAN TS525	*Smutsia temminckii*	Ground pangolin	Pholidota	Myrmecophagous	NA	NA	Wild	South Africa	Tswalu Kalahari reserve	SRR23924984	SRR23924983
PRO TDR7	*Proteles cristatus*	Southern aardwolf	Carnivora	Myrmecophagous	NA	NA	Wild	South Africa	Tussen-die-Riviere reserve	SRR23924982	SRR23924981
PRO TDR17	*Proteles cristatus*	Southern aardwolf	Carnivora	Myrmecophagous	NA	NA	Wild	South Africa	Tussen-die-Riviere reserve	SRR23924980	SRR23924979
PRO TDR22	*Proteles cristatus*	Southern aardwolf	Carnivora	Myrmecophagous	NA	NA	Wild	South Africa	Tussen-die-Riviere reserve	SRR23924977	SRR23924976
PRO TDR49	*Proteles cristatus*	Southern aardwolf	Carnivora	Myrmecophagous	NA	NA	Wild	South Africa	Tussen-die-Riviere reserve	SRR23924975	SRR23924974
PRO TDR62	*Proteles cristatus*	Southern aardwolf	Carnivora	Myrmecophagous	NA	NA	Wild	South Africa	Tussen-die-Riviere reserve	SRR23924973	SRR23924972
PRO TDR67	*Proteles cristatus*	Southern aardwolf	Carnivora	Myrmecophagous	NA	NA	Wild	South Africa	Tussen-die-Riviere reserve	SRR23924971	SRR23924970
TAM M3075	*Tamandua tetradactyla*	Southern tamandua	Pilosa	Myrmecophagous	Male	Adult	Wild	French Guiana	coll. JAGUARS	SRR23924969	SRR23924968
TAM M5331	*Tamandua tetradactyla*	Southern tamandua	Pilosa	Myrmecophagous	Male	Adult	Wild	French Guiana	coll. JAGUARS	SRR23924966	SRR23924965
TAM M5584	*Tamandua tetradactyla*	Southern tamandua	Pilosa	Myrmecophagous	Female	Adult	Wild	French Guiana	coll. JAGUARS	SRR23924964	SRR23924963

N.B.: Diet was determined based on field observations (i.e., dissections) and the literature (11, 74-78).