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Association of maternal genetics with the gut microbiome and eucalypt diet selection in captive koalas

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Background. Koalas (*Phascolarctos cinereus*), an Australian arboreal marsupial, depend on eucalyptus tree leaves (*Eucalyptus* spp.) for their diet. They selectively consume only a few of the hundreds of available eucalypt species. Since the koala gut microbiome is essential for the digestion and detoxification of eucalypts, their individual differences in the gut microbiome may lead to variations in their eucalypt selection and eucalypt metabolic capacity. Despite a strong relationship between the gut microbiome and eucalypt foraging in koalas, research focusing on the relationship between the gut microbiome and differences in food preferences is very limited. Therefore, we aimed to determine whether individual and regional differences exist in the gut microbiome of koalas as well as the mechanism by which these differences influence eucalypt selection. **Methods.** Foraging data and fecal samples of koalas were collected from two zoos in Japan. The mitochondrial phylogenic analysis was conducted to estimate the maternal origin of each koala. In addition, the koala's 16S-based gut microbiome was analyzed to determine the composition and diversity of each koala's gut microbiome. We used these data to investigate the relationship among maternal origin, gut microbiome and eucalypt diet selection. **Results and Discussion.** This research revealed that diversity and composition of the gut microbiome and that eucalypt diet selection of koalas differs among regions. We also revealed that the gut microbiome alpha diversity was correlated with foraging diversity in koalas. These individual and regional differences would result from vertical (maternal) transmission of the gut microbiome and represent an intraspecific variation in koala foraging strategies. Further, we demonstrated that certain gut bacteria were strongly correlated with both maternal origin and eucalypt foraging patterns. Bacteria found to be associated with maternal origin included bacteria involved in fiber digestion and degradation of secondary metabolites, such as the families Rikenellaceae

and Synergistaceae. These bacteria may cause differences in metabolic capacity between individual and regional koalas and influence their eucalypt selection. **Conclusion.** We showed that the characteristics of the gut microbiome and eucalyptus diet selection of koalas differ by individuals and regional origins. In addition, some gut bacteria that would influence eucalypt foraging of koalas showed the relationships with both maternal origin and eucalypt foraging pattern. Given the importance of the gut microbiome to koalas foraging on eucalyptus and their strong symbiotic relationship, future studies should focus on the symbiotic relationship and coevolution between koalas and the gut microbiome to understand individual and regional differences in eucalypt diet selection by koalas.

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Abstract

Background. Koalas (*Phascolarctos cinereus*), an Australian arboreal marsupial, depend on eucalyptus tree leaves (*Eucalyptus* spp.) for their diet. They selectively consume only a few of the hundreds of available eucalypt species. Since the koala gut microbiome is essential for the digestion and detoxification of eucalypts, their individual differences in the gut microbiome may lead to variations in their eucalypt selection and eucalypt metabolic capacity. Despite a strong relationship between the gut microbiome and eucalypt foraging in koalas, research focusing on the relationship between the gut microbiome and differences in food preferences is very limited. Therefore, we aimed to determine whether individual and regional differences exist in the gut microbiome of koalas as well as the mechanism by which these differences influence eucalypt selection.

Methods. Foraging data and fecal samples of koalas were collected from two zoos in Japan. The mitochondrial phylogenic analysis was conducted to estimate the maternal origin of each koala. In addition, the koala's 16S-based gut microbiome was analyzed to determine the

composition and diversity of each koala's gut microbiome. We used these data to investigate the relationship among maternal origin, gut microbiome and eucalypt diet selection.

Results and Discussion. This research revealed that diversity and composition of the gut microbiome and that eucalypt diet selection of koalas differs among regions. We also revealed that the gut microbiome alpha diversity was correlated with foraging diversity in koalas. These individual and regional differences would result from vertical (maternal) transmission of the gut microbiome and represent an intraspecific variation in koala foraging strategies. Further, we demonstrated that certain gut bacteria were strongly correlated with both maternal origin and eucalypt foraging patterns. Bacteria found to be associated with maternal origin included bacteria involved in fiber digestion and degradation of secondary metabolites, such as the families Rikenellaceae and Synergistaceae. These bacteria may cause differences in metabolic capacity between individual and regional koalas and influence their eucalypt selection.

Conclusion. We showed that the characteristics of the gut microbiome and eucalyptus diet selection of koalas differ by individuals and regional origins. In addition, some gut bacteria that would influence eucalypt foraging of koalas showed the relationships with both maternal origin and eucalypt foraging pattern. Given the importance of the gut microbiome to koalas foraging on eucalyptus and their strong symbiotic relationship, future studies should focus on the symbiotic relationship and coevolution between koalas and the gut microbiome to understand individual and regional differences in eucalypt diet selection by koalas.

Introduction

The marsupial koalas (*Phascolarctos cinereus*) live in eucalypt forests in eastern and southeastern Australia. They consume eucalypt tree leaves (*Eucalyptus* spp.) almost exclusively, which are potentially toxic and not suitable for other animals. They select and depend on a few eucalypt species and even conspecific eucalypt individuals. Although 120 eucalypt species have been recorded as koala food sources, each koala may only consume 1–10 eucalypt species [1, 2]. The Cape Otway population in Victoria reportedly experienced starvation and collapse of the individual number due to the high density of koalas. The overbrowsing of their preferred trees (*Eucalyptus viminalis*) resulted in the defoliation of these trees [3]. Notably, another eucalyptus tree species, *E. obliqua*, is preferred by some living koalas. Other koalas that preferred *E. viminalis* did not feed on *E. obliqua* and therefore suffered starvation, leading to their death. Thus, with regard to their extreme diet preferences, we aimed to determine the factors that lead to differences in food preferences among individual koalas.

Koalas have evident regional differences in morphology, such as size and color [4, 5], population density [6], and genetic diversity [7]. Due to these regional differences, the koalas are divided into northern (Queensland and northern New South Wales) and southern (Victorian and southern New South Wales) koalas for husbandry management and maintained under specific conditions (rearing temperature, feeding eucalypt species) suitable for each group. Previously, koalas were thought to have three subspecies based on morphological differences (*P. c. adustus*, *P. c. cinereus*, and *P. c. victor*) [4, 8]. Currently, it is known that koalas can be divided into four

distinct groups (two northern lineages, a central lineage, and a southern lineage) based on mtDNA analysis [9]. The genome-wide analysis also revealed that koalas are divided into five distinct groups [7]. Eucalypt vegetation potentially preferred by koalas varies from region to region [1]. The nitrogen and fiber content and composition of potentially toxic plant secondary metabolites in eucalyptus are known to vary by region and species [10–12]. Therefore, koalas have had to adapt to local vegetation in terms of chemistry and change their dietary preferences [13].

The role and impact of symbiotic microorganisms on host animals have been highly recognized, including koalas [14, 15]. The gut microbiome contributes to many biological functions such as host metabolism [16], detoxification of secondary metabolites [17–19], immune system [20, 21] and behavior [22, 23]. The gut microbiome is particularly important in koalas, which use the enlarged hindgut to ferment eucalyptus: highly fibrous, low in nutrition, and rich in secondary metabolites [24, 25]. They have specifically developed their characteristics: morphology, such as large cecum and colon fermentation tanks [26]; physiology, such as adjustment of the speed of the passage of substances through the gastrointestinal tract according to their size [26]; genetics, such as the expanded repertoire of bitter taste and olfactory receptor gene family and cytochrome P450 monooxygenase (CYP) gene family [7] to adapt to appropriate eucalypt selection, ability to digest and detoxify the leaves of eucalyptus, leading to dependence on the gut microbiome [27, 28]. Koala juveniles consume their mother's feces, called pap, which contains a high concentration of microorganisms and digested eucalypt residues [29]; it enables juveniles to gain gut microorganisms necessary for their growth, development, and eucalypt digestion [30]. Despite such a strong relationship between the gut microbiome and eucalypt foraging in koalas, research focusing on the relationship between the gut microbiome and differences in food preferences is limited to only two studies in Cape Otway [12, 31].

Herein, we hypothesized that the adaptation of the gut microbiomes of koalas to region-specific eucalypt vegetation is associated with regional differences in eucalyptus selection. A previous study reported the food preferences of individual koalas from Japanese zoos [32]. We investigated the maternal genetics (mitochondrial lineage and gut microbiome) of captive koalas in Japan and its relationship with eucalypt diet selection. Subsequently, we investigated the relationship among mitochondrial phylogeny, eucalypt diet selection, and 16S-based gut microbiomes in captive koalas from two Japanese zoos.

Materials & Methods

Ethics statement

This study adhered to the Animals in Research: Reporting On Wildlife (ARROW) guidelines. Sample collection and behavioral recordings were approved by Hirakawa Zoological Park and Awaji Farm Park England Hill as collaborative projects with Hayakawa Lab of Hokkaido University and Ogura Lab of Kitasato University and were fully performed through noninvasive

approaches, except for blood collection. To minimize suffering, blood samples were not collected for the purpose of this study; instead, used residues from routine health examinations were employed. Behavioral recordings were completely non-invasively conducted in the zoo-visitors' area and did not artificially control koalas' behavior for the purpose of this study. The koalas were healthily kept for the purpose of public exhibitions in the zoos in the enough size of enclosure (>5 m wide, >5 m depth, and >5 m height). Their environments were enriched. They were always provided branches of a variety of eucalypt species scattered in the enclosure. Since koalas are arboreal animals, logs were arranged in a three-dimensional manner for the koalas to enjoy moving in any direction. The animal experimentation protocol was approved by the President of Kitasato University through the judgment of the Institutional Animal Care and Use Committee of Kitasato University (Approval No. 21-069).

Animals

To perform sufficient statistical analysis, a total of 15 captive koalas were selected and examined in this study (Tables 1, S1). Of these, nine koalas obtained care at Hirakawa Zoological Park, whereas the other six obtained care at Awaji Farm Park England Hill. Four of the Awaji koalas were southern koalas (Yuki, Daichi, Nozomi, and Midori), whereas the others were northern koalas. To be blind test, behavioral recording was performed by MA, CA and RW, and statistical analysis was performed by KK.

Mitochondrial phylogenetic analysis

Mitochondrial phylogenetic analysis, based on the study by Neaves et al. [9], was conducted to estimate the maternal origin of each koala. Blood or fecal samples of koalas were collected to extract and purify genomic DNA. The collected blood samples were immediately mixed with anticoagulants (EDTA or heparin). All samples were frozen at −20°C prior to DNA extraction. Total DNA was extracted from blood samples using Qiagen DNeasy Blood and Tissue Kit (Qiagen GmbH) and from fecal samples using QIAamp Fast DNA Stool Mini Kit (Qiagen GmbH). Next, using TaKaRa Ex Taq Hot Start Version (Takara Bio Inc.), the mitochondrial DNA control region (D-loop) was amplified via polymerase chain reaction (PCR) using the following primers: MaL15999M (ACC ATC AAC ACC CAA AGC TGA) and MaH16498M (CCT GAA GTA GCA ACC AGT AG) (Fumagalli et al. 1997). The PCR conditions were as follows: initial denaturation (94°C for 10 min); followed by 35 cycles of denaturation (94°C for 10 s), annealing (60°C for 30 s), and extension (72°C for 60 s); and final extension (72°C for 10 min). The PCR products were purified via precipitation with isopropanol. Next, the purified PCR products were directly sequenced using PCR primers for complete coverage in both strand orientations via BigDye Terminator v3.1 Cycle Sequencing Kit and 3130 Genetic Analyzer (Applied Biosystems). Chromatograms were imported into FinchTV (Geospiza Inc.) and analyzed.

The phylogeny of the sequenced D-loop region of mitochondrial DNA was analyzed with the sequences of 48 koalas reported by Neaves et al. [9]. A multiple alignment was

constructed by MUSCLE [33]. A phylogenetic tree was reconstructed using the Maximum-Likelihood (ML) method with 1,000 bootstrap resamplings using MEGA11 [34].

Foraging data and gut microbiome

Foraging data was collected from six koalas in Hirakawa Zoological Park for 8 days between 21–30 November 2021. Subjects were housed independently (Boonda and Ito) or in cohabitation with two individuals (Himawari with Kibou and Sora with Itsuki). During foraging and feeding, all koala pairs that use the same enclosure rarely interfere with each other. At Hirakawa, four of the five eucalypt species (*E. camaldulensis*, CR; *E. microcorys*, M; *E. punctata*, P; *E. robusta*, R; *E. tereticornis*, T) were fed twice a day (9:00 and 16:00). The combination of eucalyptus fed at the same time and the frequency of feeding was counterbalanced. The method of behavioral recoding was determined *a priori*. Eucalypt species consumed by each koala were observed using the instantaneous sampling method in 30-s intervals [35]. The observation time was 1 hour each at 9:00 am and 4:00 pm immediately after feeding by caretakers and 1 hour each at 11:00 am and 2:00 pm outside of the immediate feeding period. Finally, the observation was carried out for 8 days (a total of 32 hours).

Fecal samples were collected during foraging observation periods. Fecal sampling was performed by collecting fresh feces immediately after defecation. In case of cohabiting housed individuals, individual identification was performed by direct observation of defecation. The fecal samples of nonsubject koalas were also collected in Hirakawa and Awaji for comparison. Fresh fecal samples were collected for sampling. Finally, 2–5 fecal samples per koala and a total of 62 fecal samples were collected and stored at −20°C until DNA extraction.

16S rRNA gene sequencing

According to Hayakawa et al. [36, 37], we performed 16S-based gut microbiome analysis using collected fecal samples. After quantifying the concentration of purified fecal DNA using Qubit dsDNA HS Assay Kit equipped with Qubit fluorometer (Thermo Fisher Scientific), we amplified the V3–V4 region of the 16S rRNA gene using KAPA HiFi Hot Start Ready Mix (Kapa Biosystem, Inc., Wilmington, MA, USA). We used the following primer pair: 1S-D-Bact-0341-b-S-17 (forward), CCT ACG GGN GGC WGC AG; S-D-Bact-0785-a-A-21 (reverse), GAC TAC HVG GGT ATC TAA TCC [38], with the specific overhang adaptors TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG - [3-6-mer Ns] - [forward primer] and GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G - [3-6mer Ns] - [reverse primer], where 3-6mer Ns can improve the sequencing quality [39]. After confirming PCR amplification via gel electrophoresis, we purified the PCR amplicons with Agencourt AMPure XP beads (Beckman Coulter, Inc.). Then, we performed index PCR using Illumina Nextera XT Index Kit (Illumina, Inc.). Additionally, we confirmed the presence and appropriate length of index PCR products via electrophoresis and then purified index PCR products with Agencourt AMPure XP beads. All index PCR products were mixed at the same molarities for constructing a library. After PhiX

spike-in (30%), we sequenced the library using the Illumina Miseq platform (Illumina, Inc.). (2 × 301 bp).

Data analysis

The MiSeq base calls were converted to FASTQ files using configureBclToFastq.pl implemented by the bcl2fastq conversion software v1.8.4 (Illumina, Inc.) (options: no-eamss, mismatches 0, and use-bases-mask Y300n,Y8,Y8,Y300n). The read pairs were demultiplexed, the primer sequences were trimmed, and those with low-quality index sequences were discarded, where the index sequences included nucleotide(s) with a quality score of <30, using clsplitseq in Claident [40] (option: minqualtag = 30).

Quality control and data analysis were performed using QIIME2 v2021.4.0 [41]. To generate amplicon sequence variants (ASVs), DADA2 v2021.4.0 [42] was used to quality filter the sequences with a read cut length of 260 (forward) and 260 (reverse) based on quality control results and denoise chimeric sequences with a read count of 1,000,000 for training the error model. The taxonomy analysis was conducted with the SILVA 138 database [43, 44]. Subsequently, sample depths were rarefied where the value was ≥0.99 for all samples using Good's coverage (4,105 sequences per sample).

We determined alpha diversity (Shannon index, Chao1, Simpson index, Simpson index of evenness, Pielou's evenness index, Faith's phylogenetic diversity, and observed features) and beta diversity (unweighted UniFrac, weighted UniFrac, Jaccard index, and Bray–Curtis dissimilarity) to analyze differences in the gut microbiome between mitochondrial lineages or between the management groups. The pairwise Kruskal–Wallis test with Benjamini–Hochberg correction was used for comparing alpha diversity, whereas permutational multivariate analysis of variance (PERMANOVA) was used to assess the effect of mitochondrial lineage on gut microbiome similarity. Individual foraging data (proportion of foraging of each eucalypt species) were used to visualize similarities in foraging patterns using nonmetric multidimensional scaling (NMDS) via *vegan* v2.6.4 in R v4.2.3. PERMANOVA was performed using the vegan package to assess the effect of mitochondrial lineage on foraging patterns.

To investigate the relationship between the diversity of individual eucalypt foraging and alpha diversity of the gut microbiome, Spearman's rank correlation coefficients were calculated. In this analysis, the Shannon index was used to indicate the diversity of individual eucalypt foraging. Analysis of composition of microbiomes (ANCOM) [45] was used to determine whether gut bacteria with characteristic relative abundances exist in each mitochondrial lineage or management group. All codes are available in Supplemental Information.

Results

Mitochondrial phylogeny

We performed phylogenetic analysis of the mitochondrial D-loop to estimate the maternal origins of the analyzed koalas (Fig. 1). A phylogenetic tree was constructed using the sequences

of five koala haplotypes from Japanese zoos as well as the sequences of 48 individuals used for phylogenetic analysis by Neaves et al. [9]. Thus, we revealed that koalas from Japanese zoos examined in this study have three different origins (northern-2, $N = 2$; central, $N = 4$; and southern, $N = 9$).

Gut microbiome diversity among mitochondrial lineages

We investigated the mechanism by which the maternal origins of koalas, which were determined using mitochondrial phylogenetic analysis, and management groups influence the gut bacterial microbiome (Dataset S1, S2). Seven indices of alpha diversity were calculated (Table S2, S3, Fig. 2). The index that considers evenness revealed that alpha diversity was the highest in the southern lineage and lowest in the central lineage (Simpson Index; South = 0.87 ± 0.07, Center = 0.75 ± 0.08, North2 = 0.84 ± 0.04; Simpson Index of Evenness; South = 0.10 ± 0.04, Center = 0.04 ± 0.01, North2 = 0.06 ± 0.01). In contrast, the index considering richness revealed that the southern lineage had the lowest alpha diversity (Chao1; South = 103.55 ± 28.36, Center = 129.84 ± 19.18, North2 = 125.31 ± 9.13; Faith's phylogenetic diversity; South = 6.78 ± 0.98, Center = 7.81 ± 0.92, North2 = 7.50 ± 0.54; and observed features; South = 91.95 ± 21.58, Center = 115.57 ± 18.18, North2 = 113.25 ± 5.36). This trend was more clearly demonstrated between the management groups (Fig. 2a, 2b) (the pairwise Kruskal–Wallis test with Benjamini–Hochberg correction; Shannon Index; South = 4.32 ± 0.22, North = 3.96 ± 0.50, $P = 0.002022$; Simpson Index; South = 0.90 ± 0.02, North = 0.81 ± 0.09, $P = 2.60E-07$; Simpson Index of Evenness; South = 0.14 ± 0.03, North = 0.06 ± 0.02, $P = 4.12E-10$; Chao1; South = 89.18 ± 11.66, North = 123.30 ± 25.96, $P = 0.000003$; Faith's phylogenetic diversity; South = 6.28 ± 0.50, North = 7.50 ± 0.98, $P = 0.000001$; and observed features; South = 79.80 ± 6.94, North = 109.67 ± 20.61, $P = 2.28E-07$).

Mitochondrial lineage have a significant impact on gut microbiome similarity (beta diversity), both qualitatively (Unweighted UniFrac) and quantitatively (weighted UniFrac) (Fig. 3) (pairwise PERMANOVA tests; Unweighted UniFrac, Center vs North2: pseudo-$F = 8.03$, $q = 0.001$, Center vs South: pseudo-$F = 8.13$, $q = 0.001$, North2 vs South: pseudo-$F = 7.29$, $q = 0.001$; number of permutations = 999; weighted UniFrac, Center vs North2: pseudo-$F = 5.78$, $q = 0.0015$, Center vs South: pseudo-$F = 11.22$, $q = 0.0015$, North2 vs South: pseudo-$F = 5.09$, $q = 0.002$; number of permutations = 999). In the same way, management groups also have a significant impact on gut microbiome similarity, both qualitatively and quantitatively (Fig. 3) (pairwise PERMANOVA tests; Unweighted UniFrac, pseudo-$F = 16.698158$, $q = 0.001$; number of permutations = 999; weighted UniFrac, pseudo-$F = 27.86$, $q = 0.0015$; number of permutations = 999).

Based on differences in gut microbiome similarity between mitochondrial lineages, we investigated whether there were bacteria that differed in relative abundance with mitochondrial lineages or management groups using ANCOM. The ANCOM results revealed significant differences in the relative abundance of 12 bacterial genera between the two management groups (Table S4, Fig. 4a). Of them, eight genera showed higher relative abundance in southern koalas,

whereas the four other genera showed higher relative abundance in northern koalas. Further, two bacterial genera showed significant differences among mitochondrial lineages (Table S5, Fig. 4b). These two genera clearly showed differences among mitochondrial lineages; they are unknown genera belonging to the families *Tannerellaceae* (significant in southern, $W = 119$) and *Rikenellaceae* (significant in northern 2, $W = 111$).

Relationship between eucalypt foraging and the gut microbiome

We recorded foraging data on zoo koalas to investigate the relationship between eucalyptus foraging and the gut microbiome (Fig. 5, Dataset S2). The proportion of each eucalyptus species in foraging varied greatly from individual to individual. These foraging data were used to visualize the similarity of NMDS foraging patterns (Fig. 6). PERMANOVA showed that mitochondrial lineage significantly influences foraging patterns ($F = 5.88$, $R^2 = 0.68$, $P = 0.014$, number of permutations = 719).

We investigated the relationship between the diversity of eucalypt foraging and the gut microbiome diversity via Spearman's rank correlation coefficients (Table S6, Fig. 7). The result shows a positive correlation between the diversity of the foraging and diversity of the gut microbiome (Spearman's rank correlation; $\rho = 0.89$, $P = 0.009$, Shannon entropy). There was a correlation with the indicator that considers evenness but not with the indicator that considers richness (Tables S6), *i.e.*, significant in Shannon, Simpson, Simpson's index of evenness, and Pielou's evenness index, but not in Chao 1, Faith's phylogenetic diversity and observed features (Spearman's rank correlation; Shannon entropy: ρ = 0.886, $P = 0.009$, Pielou evenness: $\rho = 0.770$, $P = 0.036$, Simpson: $\rho = 0.830$, $P = 0.021$, Simpson e: $\rho = 0.830$, $P = 0.021$, Chao1: $\rho = 0.030$, $P = 0.479$, Faith pd: $\rho = 0.540$, $P = 0.133$, Observed features: $\rho = 0.030$, $P = 0.479$).

We investigated whether gut bacteria at the genus level vary in relative abundance depending on the foraging proportion of the *Eucalyptus* species. We revealed that the relative abundance of some gut bacteria increased or decreased depending on the foraging proportion of each eucalypt species (Table S7, Fig. 8). The number of gut bacterial genera that showed a significant positive correlation was one with *E. microcorys* and two with *E. punctata* (Spearman's rank correlation with Benjamini–Hochberg correction; $q < 0.05$). Conversely, the number of gut bacterial genera that showed a significant negative correlation was four with *E. camaldulensis*, two with *E. microcorys,* and one with *E. robsta* (Spearman's rank correlation with Benjamini–Hochberg correction; $q < 0.05$).

Discussion

As the koala gut microbiome is known to be highly involved in eucalypt digestion and detoxification [28, 29], there is an increasing need to consider the gut microbiome in koala management, both *in situ* (within habitats) and *ex situ* (outside habitats) conservation [46]. In general, the abundance and composition of the gut microbiome varies in response to dietary changes [47]. However, the koala gut microbiome has been reported to be stable within

individuals [48] and does not change significantly in response to dietary changes [31, 49]. Furthermore, there is a report that geographic distance of habitat has an influence on the similarity of the gut microbiome of wild koalas [46]. Thus, it is considerable that this stable regional difference in the koala gut microbiome could be driving a significant difference in eucalyptus selection between regions. However, research focusing on the relationship between the gut microbiome and differences in dietary preferences and its regionality is limited. Therefore, in this study we sought to elucidate how regional differences influence koalas' gut microbiomes and their eucalypt selection, and the mechanism by which these regional differences influence eucalypt selection. As a result, we showed presence of the relationship between the gut microbiome and eucalypt diet selection as well as the effect of geographical distribution on both factors by analyzing foraging data, mitochondrial lineages as indicators of regional origin, and the gut microbiome in captive koalas in Japan.

We demonstrated the influence of mitochondrial lineages on foraging patterns in eucalypt diet selection (Fig. 6). Additionally, differences in the alpha and beta diversity values of the gut microbiome were observed between mitochondrial lineages and management groups (Table S2, Figs. 2, 3). These results indicate regional variations in the koala gut microbiome and eucalypt diet selection, suggesting that different gut microbiomes in different regions may lead to variation in eucalypt diet selection. It is well known that joey koalas are fed with cecum feces, known as pap, of their mothers as an important weaning diet, after which they acquire the gut microbiome necessary for eucalypt foraging [29, 50]. Additionally, mitochondrial DNA is inherited from the mother to child [51]. Genetic factors such as detoxification and bitter taste receptor genes can also affect eucalypt diet selection [7]. Thus, the genetic inheritance in koalas may explain the differences in eucalypt foraging and the gut microbiome.

Investigating the relationship between the diversity of gut microbiomes and the diversity of koala eucalyptus foraging, we found that the more diverse gut microbiome the koalas had, the more diverse eucalyptus that they ate (Fig. 7). Only the diversity of the gut microbiome, considering evenness rather than richness, was found with the be correlated to diversity of the foraging of eucalyptus (Table S5). This finding suggests that the uniformity of the gut microbiome, rather than the presence of many bacteria, is more important for the foraging of diverse eucalypts. Since there were differences in evenness of alpha diversity among mitochondrial lineages and management groups (both highest in the southern lineage), these differences may explain the different food habits of koalas by region, *i.e.*, this may influence the number of eucalypt species preferred in different regions.

It is known that small animals need to selectively consume a high-quality diet while large animals need to consume large amounts because their energy requirements, tolerance to toxic substances, and amount of fermentable fiber vary with their body size and organ size [52–54]. There are geographical variations in koala body size, with northern individuals known to be smaller and southern individuals larger [4, 5]. Therefore, small northern individuals likely need to selectively consume eucalyptus leaves, whereas larger southern individuals need to consume large amounts of eucalyptus leaves. Thus, this need for optimal selection of foraging by body

size may have led to the selection of a gut microbiome that allows northern individuals to consume specific types of eucalypts (a composition suitable for consuming specific eucalypts) and a gut microbiome that allows southern individuals to consume many eucalypts (highly diverse, especially even, gut microbiome) (Figs. 7).

Previous studies have reported that gut bacteria influence the host diet [19]. For example, the gut bacteria reportedly contribute to oxalate degradation in the creosote bush diet of woodrats (*Neotoma spp*.) [17] as well as mimosine degradation in the *Leucaena leucocephala* diet of Australian cattle [55, 56], thus influencing diet. In koalas, differences in the composition of the gut microbiome may explain different feeding habits, revealing that fecal inoculation alters feeding habits [12, 31]. The current study revealed that the relative abundance of several gut bacteria in koalas was associated with mitochondrial lineages, management groups, and foraging proportion of each eucalypt species (Fig. 8). The family *Rikenellaceae* was associated with the northern lineage 2. This family is known to be involved in carbohydrate degradation [57]. In addition, the genus RC9 group of *Rikenellaceae* is known to play an important role in crude fiber digestion [58]. The genus *Parabacteroides* was abundantly detected in southern koalas in the management group and has been reported to possess many oligosaccharide-degrading genes and genes associated with tannin degradation [12]. The family *Synergistaceae* was abundantly found in southern koalas in the management group. This family is known to be involved in the degradation of secondary plant metabolites [59]. These bacteria may lead to differences in the metabolic capacities of individuals and affect the foraging patterns of different individuals and regions.

Although 16S rRNA gene sequencing performed in this study provides information on the composition and taxonomy of the gut microbiome [60], the relevant functional information is limited [19]. As duplicate functions (redundancy) of bacteria have been reported in other species [61, 62], studies have also suggested a redundancy in the gut bacterial function of koalas [46, 48]. Therefore, we believe that a metagenomic sequencing approach is warranted in the future to analyze gut microbiomes at genetic and functional levels and compare them at functional and physiological levels. Other factors such as differences in past food experience, genetics, and physiology are also likely to influence eucalyptus foraging in koalas [31, 32]. Recent studies have reported that when the host and its associated microorganisms are considered as one ecosystem (holobiont), the hologenome, which is the collective term for the host and microbial genomes, can be subject to natural selection [63]. Therefore, future studies should focus on the symbiotic relationship and coevolution between koalas and the gut microbiome to better understand individual and regional differences in eucalypt diet selection by koalas.

There are several limitations in the interpretation of the present results. First, this study used the mitochondrial lineage as the region of origin of koalas. However, the mitochondrial lineage can only divide koalas into four groups, making examining the relationship with regional differences in actual vegetation difficult. A method using the nuclear genome, which allows for finer groupings [7] and groupings that consider actual vegetation, would help clarify the role of the gut microbiome in adaptation to regional vegetation. Notably, the study subjects were captive

koalas; the sample size was limited and included individuals related to each other. Although there have been reported that there are few differences in the gut microbiome between captive and wild individuals [27], it is known that the gut microbiome and preferred eucalypts of mothers and their offspring are similar [50, 64]. Therefore, additional validation is required in more unrelated individuals in the future.

Previous research has reported that geographic distance of habitat influences the similarity of the gut microbiome of wild koalas [46]. Moreover, this study revealed that captive koalas in Japan have similar gut microbiomes by region of origin. Koalas have been continuously bred and raised in Japan since the early 1980s. In Japan, breeding and cohabitation between mitochondrial lineages have also been conducted. Thus, the fact that regional characteristics in the gut microbiome have been observed even though many generations of koalas have been bred in Japan, far from their habitat, shows how robust the koala gut microbiome of koalas is and indicates that the koala gut microbiome of koalas has regional variation based on matrilineal inheritance. Given the importance of the gut microbiome for koala foraging and the strong symbiotic relationship between them, we believe that future research should focus on the three-way relationship between koalas, eucalypts, and the gut microbiome to understand koala foraging ecology and to conduct conservation management.

Conclusions

This study revealed that the diversity and composition of the gut microbiome of koalas and their eucalyptus diet selection differ by regional origin. We also found that some gut bacteria that may influence koalas' eucalypt foraging are associated with both maternal origin and eucalypt foraging patterns, and that the alpha diversity (particularly evenness) of the gut microbiome correlates with foraging diversity in koalas. These differences could result from vertical transmission of the gut microbiome based on maternal transmission and the robustness of the gut microbiome as a hindgut fermenter. These regional differences may also represent an intraspecific variation in koala foraging strategies. Investigating regional differences in eucalypt composition and genetics as well as physiology of koalas is necessary to better understand koala foraging ecology.

Acknowledgements

We thank the staff of Hirakawa Zoological Park and Awaji Farm Park England Hill for their cooperation in collecting samples and sharing individual information, especially Akira Fukumori and Ayaka Ito (Hirakawa) as well as Atsushi Goto, Masato Akai, Izumi Hirayama, and Kakeru Murai (Awaji). We also thank all members of the Ogura Lab (Kitasato University) for their helpful support, especially Chie Kawase, Honami Kikuchi, and Shuntaro Kitamori for their cooperation in fecal sampling, and all members of the Hayakawa Lab (Hokkaido University) for

support and discussion. The authors also thank Hitoshi Suzuki and Shin-ichiro Aiba for their valuable comments on the manuscript.

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Figure 1

The ML tree of the D-loop in koalas.

This is inferred using the Tamura three-parameter model with a discrete Gamma distribution (five categories), which has the lowest Bayesian Information Criterion scores (unrooted). The tree with the highest logarithmic likelihood (−990.84) is shown. The percentage (≥50%) of trees based on 1,000 bootstrap replications is shown below the branches. This tree shows four clades (Northern 1, Northen 2, Central, and Southern), which corresponding to the clades found by Neaves et al. [9].

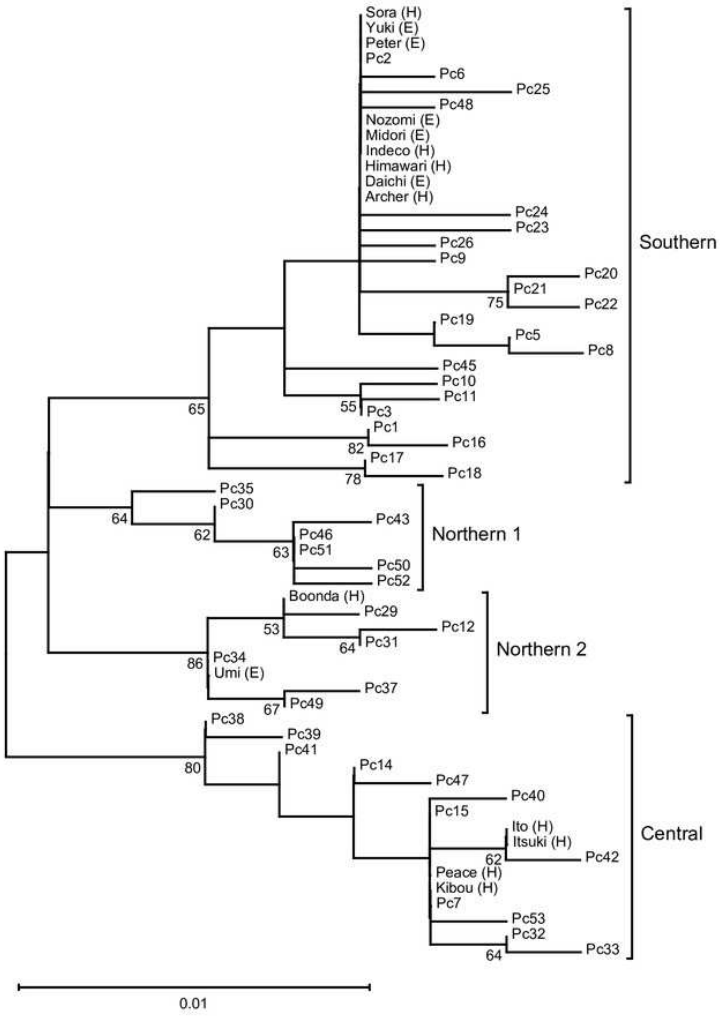
Sora (H)
Yuki (E)
Peter (E)
Pc2
Pc6
Pc25
Pc48
Nozomi (E)
Midori (E)
Indeco (H)
Himawari (H)
Daichi (E)
Archer (H)
Pc24
Pc23
Pc26
Pc9
Pc20
Pc21
75
Pc22
Pc19
Pc5
Pc8
Pc45
Pc10
Pc11
55
Pc3
65
Pc1
82
Pc16
Pc17
78
Pc18
Southern
Pc35
Pc30
64
Pc43
62
Pc46
63
Pc51
Pc50
Pc52
Northern 1
Boonda (H)
Pc29
53
Pc12
64
Pc31
86
Pc34
Umi (E)
Pc37
67
Pc49
Northern 2
Pc38
Pc39
Pc41
80
Pc14
Pc47
Pc40
Pc15
Ito (H)
Itsuki (H)
62
Pc42
Peace (H)
Kibou (H)
Pc7
Pc53
Pc32
64
Pc33
Central
0.01

Fig. 1

Figure 2

The difference in alpha diversity between management groups in (a) Shannon index and (b) Chao1 and mitochondrial lineages in (c) Shannon index and (d) Chao1.

Statistical tests are conducted by the pairwise Kruskal–Wallis test with Benjamini–Hochberg correction.

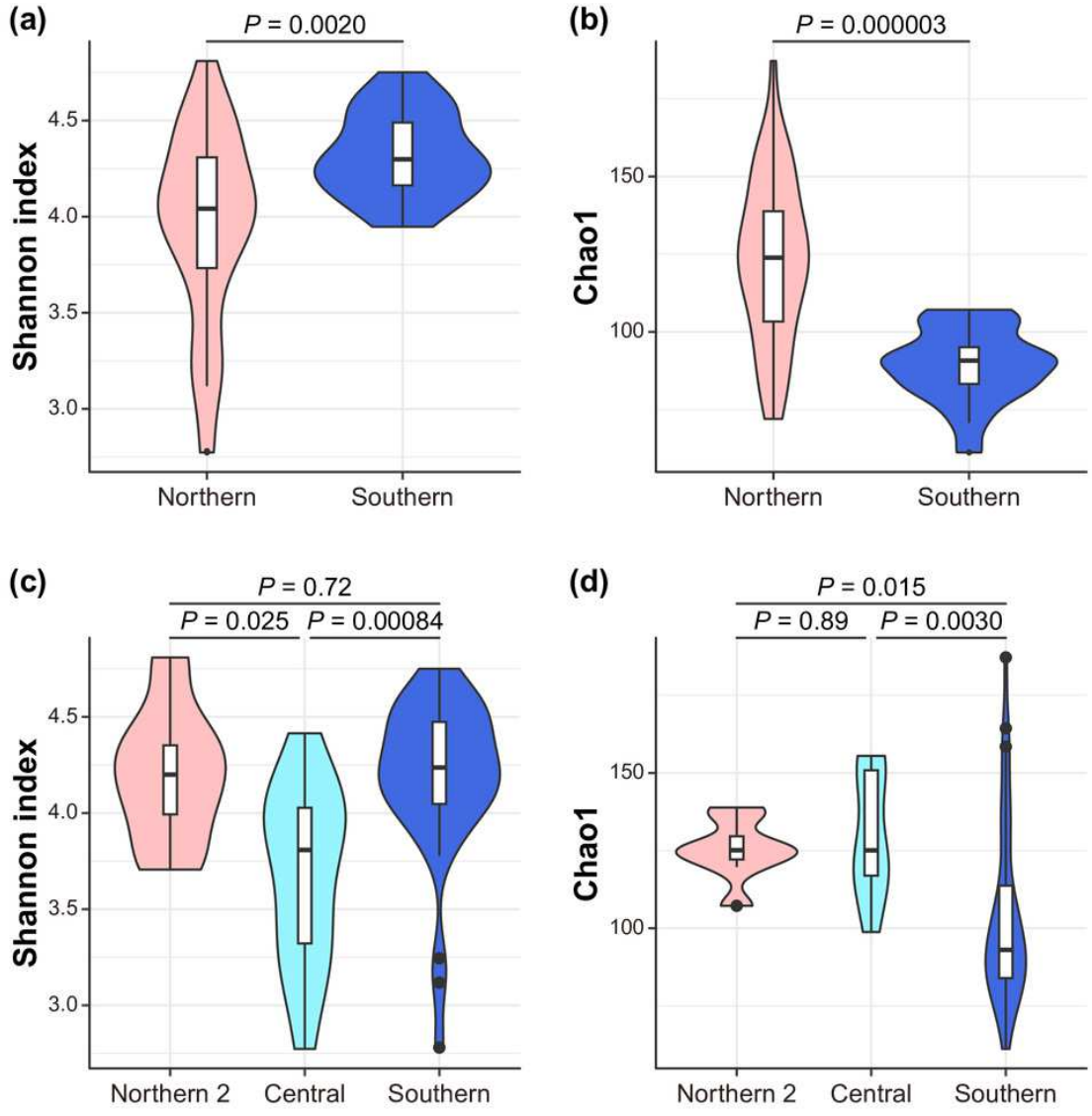
(a)
P = 0.0020
Shannon index
4.5
4.0
3.5
3.0
Northern
Southern
(b)
P = 0.000003
Chao1
150
100
Northern
Southern
(c)
P = 0.72
P = 0.025
P = 0.00084
Shannon index
4.5
4.0
3.5
3.0
Northern 2
Central
Southern
(d)
P = 0.015
P = 0.89
P = 0.0030
Chao1
150
100
Northern 2
Central
Southern

Fig. 2

Figure 3

Management groups and mitochondrial lineages significantly influenced the gut bacterial community.

Clustered by the management groups in **(a)** unweighted UniFrac (pairwise PERMANOVA tests: pseudo-*F* = 16.698158; *P* = 0.001; number of permutations = 999) and **(b)** weighted UniFrac (pairwise PERMANOVA tests: pseudo-*F* = 27.864924; *P* = 0.001; number of permutations = 999). Clustered by mitochondrial lineages in **(c)** unweighted UniFrac (pairwise PERMANOVA tests, Center vs North2: pseudo-*F* = 8.025514; *q* = 0.001; Center vs South: pseudo-*F* = 8.133812; *q* = 0.001; North2 vs South: pseudo-*F* = 7.289387; *q* = 0.001; number of permutations = 999) and **(d)** weighted UniFrac (pairwise PERMANOVA tests, Center vs North2: pseudo-*F* = 5.784462; *q* = 0.0015; Center vs South: pseudo-*F* = 11.216916; *q* = 0.0015; North2 vs South: pseudo-*F* = 5.085741; *q* = 0.002; number of permutations = 999).

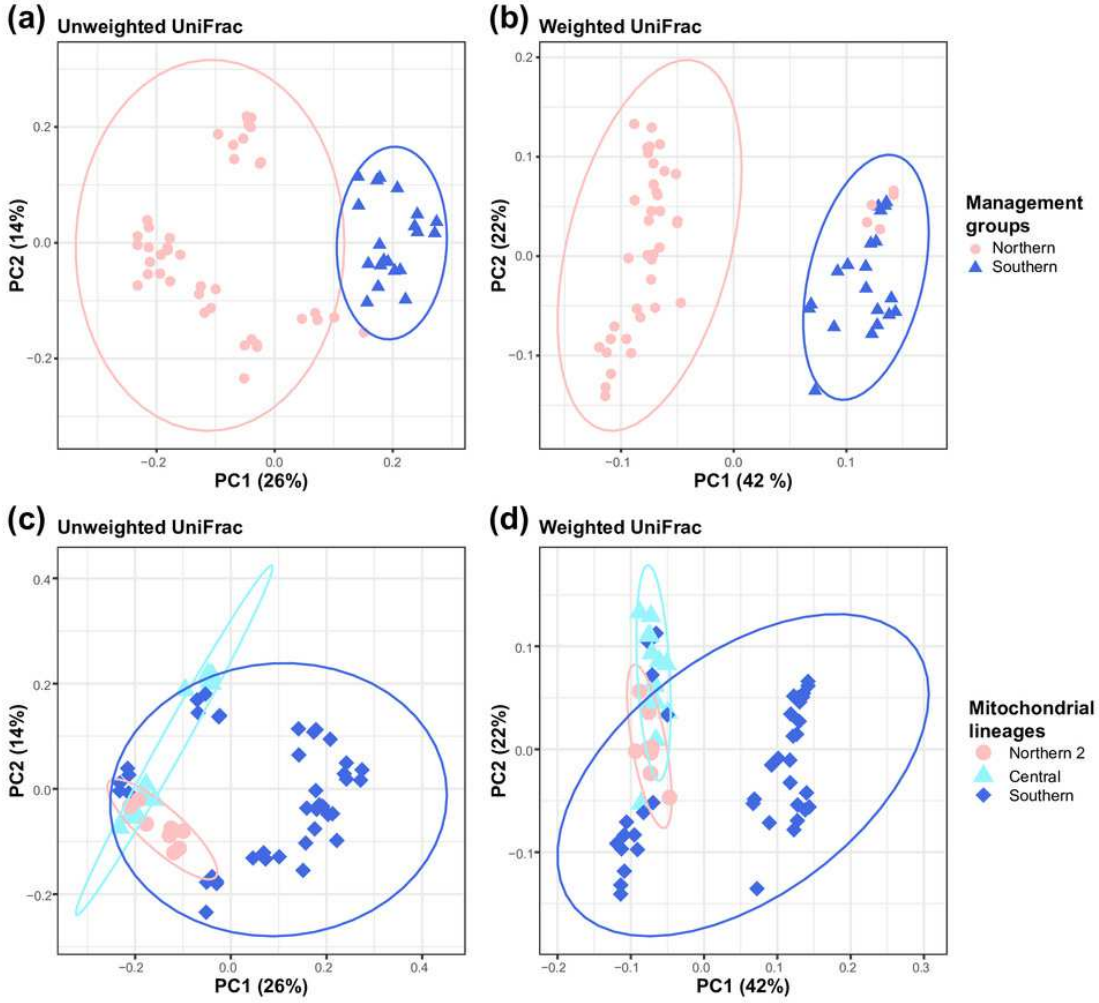
(a) Unweighted UniFrac
PC2 (14%)
PC1 (26%)
(b) Weighted UniFrac
PC2 (22%)
PC1 (42 %)
Management groups
Northern
Southern
(c) Unweighted UniFrac
PC2 (14%)
PC1 (26%)
(d) Weighted UniFrac
PC2 (22%)
PC1 (42%)
Mitochondrial lineages
Northern 2
Central
Southern

Fig. 3

Figure 4

Volcano plot of the results of the analysis of the composition of microbiomes (ANCOM) between (a) management groups or (b) mitochondrial lineages at the genus level.

Each circle represents a taxon. Those with statistically significant differences based on the *W* statistics between mitochondrial lineages are colored red.

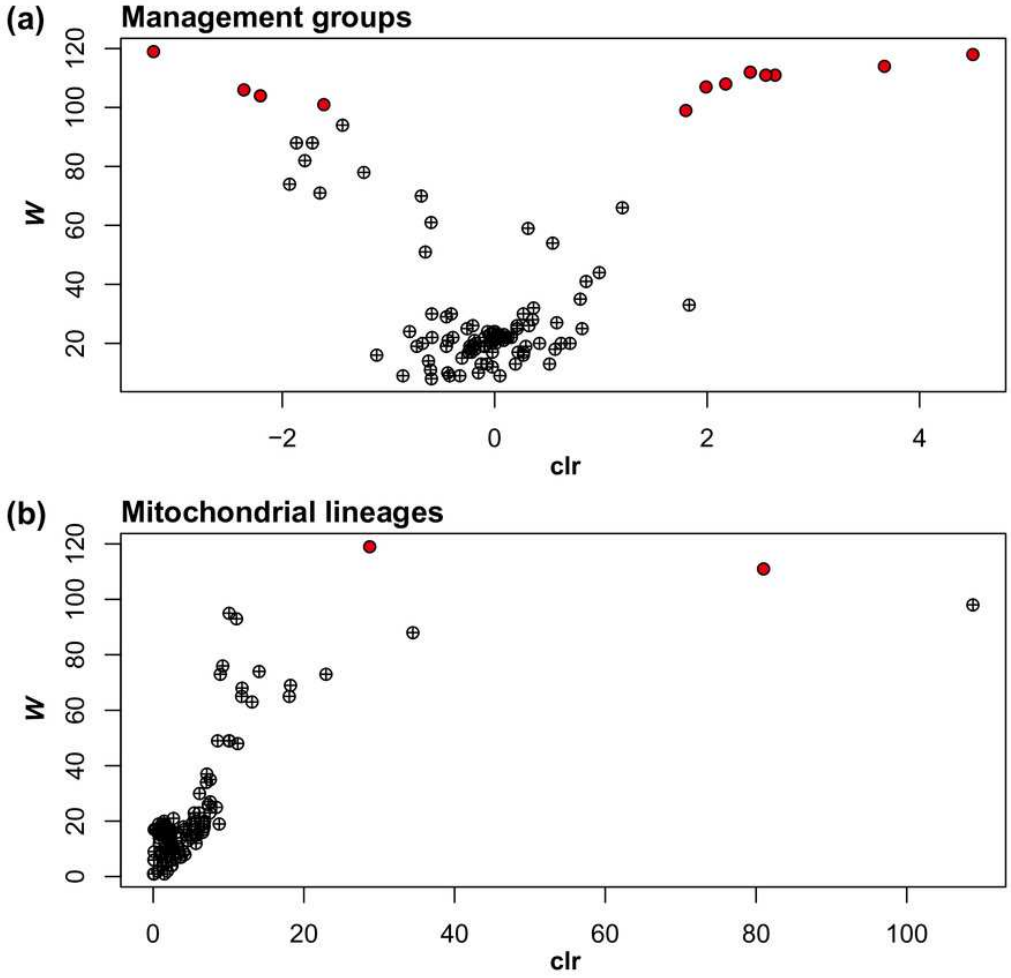

Fig. 4

Figure 5

Diet proportion of each individual. *Eucalyptus camaldulensis*, CR; *E. microcorys*, M; *E. punctata*, P; *E. robsta*, R; *E. tereticornis*, T.

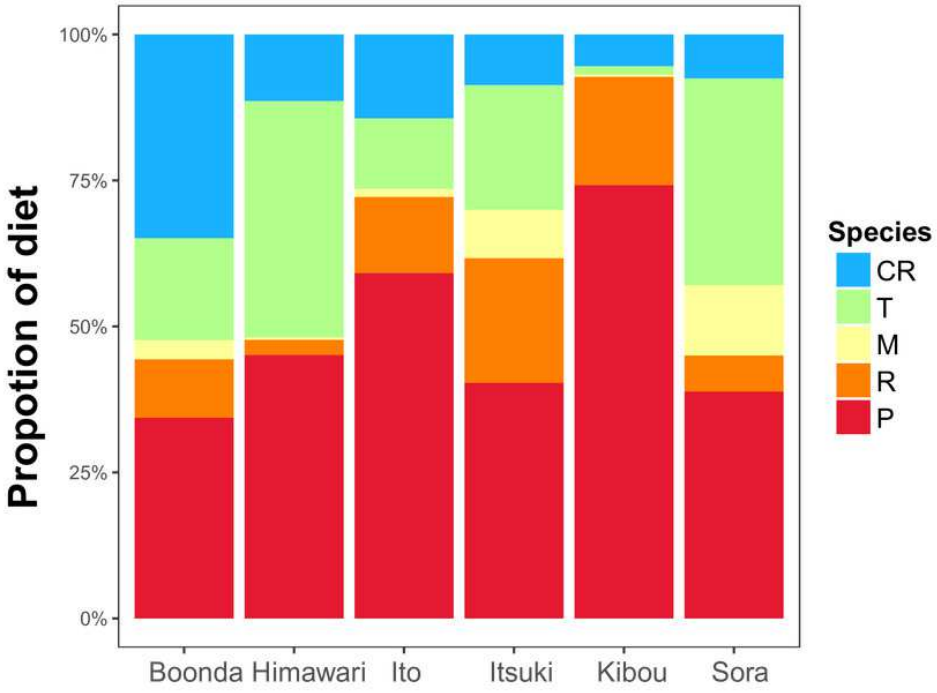
Propotion of diet
100%
75%
50%
25%
0%
Boonda
Himawari
Ito
Itsuki
Kibou
Sora
Species
CR
T
M
R
P

Fig. 5

Figure 6

Results of NMDS using foraging data.

Mitochondrial lineages significantly influenced the foraging pattern (F = 5.88; R^2 = 0.677; P = 0.014; number of permutations = 719).

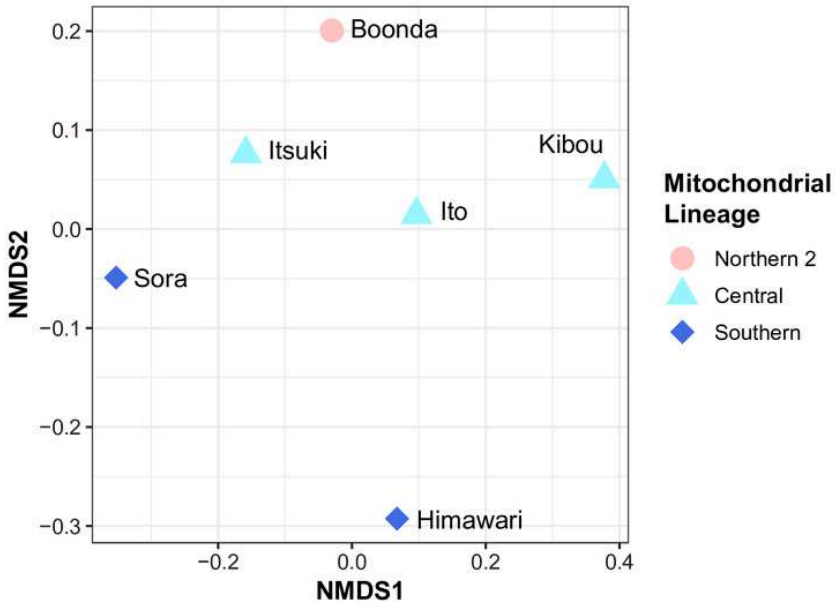
Boonda
Itsuki
Kibou
Ito
Sora
Himawari
NMDS1
NMDS2
0.2
0.1
0.0
−0.1
−0.2
−0.3
−0.2
0.0
0.2
0.4
Mitochondrial Lineage
Northern 2
Central
Southern

Fig. 6

Figure 7

Correlation between the foraging diversity and gut microbiome diversity (Shannon index).

A significant correlation was observed between foraging diversity and gut microbiome diversity (Spearman's rank correlation; $\rho = 0.886$, $P = 0.009$).

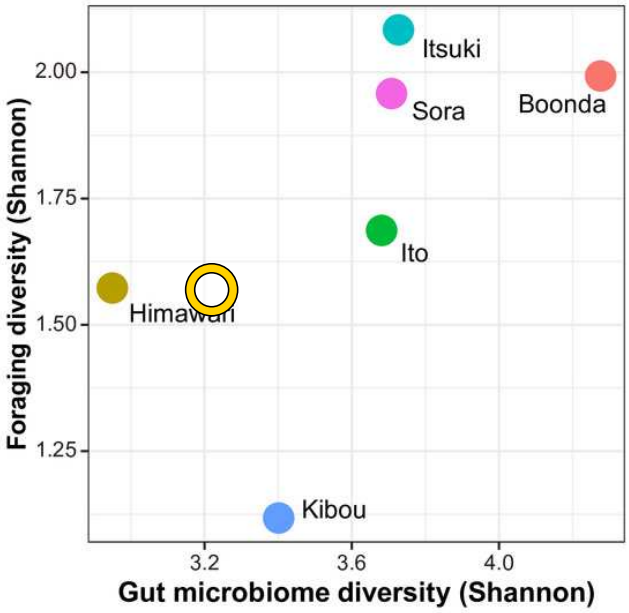
Itsuki
Sora
Boonda
Ito
Himawari
Kibou
2.00
1.75
1.50
1.25
3.2
3.6
4.0
Foraging diversity (Shannon)
Gut microbiome diversity (Shannon)

Fig. 7

Figure 8

The heat map shows changes in the relative abundance of gut bacteria because of changes in the proportion of eucalypt species in the diet.

Spearman's rank correlation was conducted with Benjamini–Hochberg correction, and bacteria with significant correlations are indicated with an asterisk ($q < 0.05$; See Table S7 for individual values.). The genera with the correlation coefficient $|\rho|$ of > 0.7 are shown.

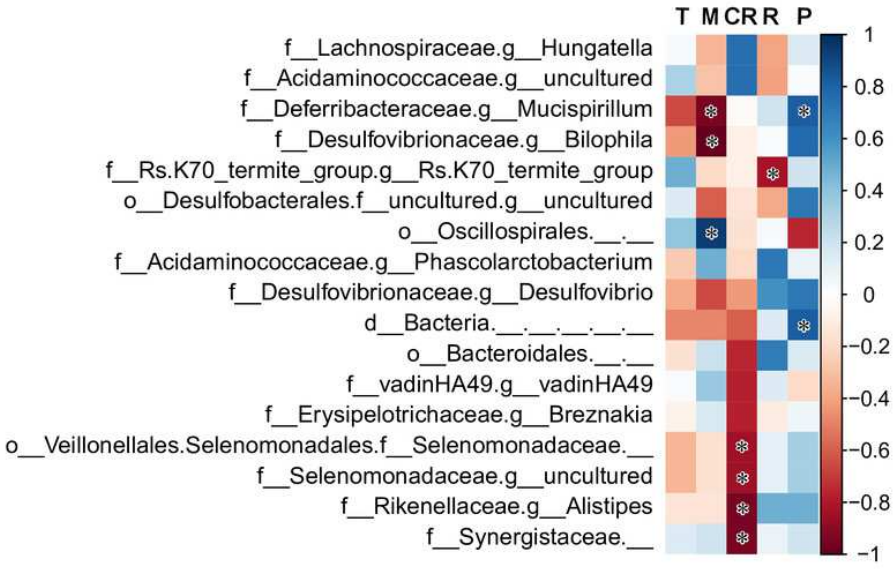
T M CR R P
f__Lachnospiraceae.g__Hungatella
f__Acidaminococcaceae.g__uncultured
f__Deferribacteraceae.g__Mucispirillum
f__Desulfovibrionaceae.g__Bilophila
f__Rs.K70_termite_group.g__Rs.K70_termite_group
o__Desulfobacterales.f__uncultured.g__uncultured
o__Oscillospirales.__.__
f__Acidaminococcaceae.g__Phascolarctobacterium
f__Desulfovibrionaceae.g__Desulfovibrio
d__Bacteria.__.__.__.__.__
o__Bacteroidales.__.__
f__vadinHA49.g__vadinHA49
f__Erysipelotrichaceae.g__Breznakia
o__Veillonellales.Selenomonadales.f__Selenomonadaceae.__
f__Selenomonadaceae.g__uncultured
f__Rikenellaceae.g__Alistipes
f__Synergistaceae.__
1
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1

Fig. 8

Table 1(on next page)

Data on koalas included in this study.

Table 1 Data on koalas included in this study.

Zoo	Name	Sex	Age	Foraging	mtDNA	Management	Number of fecal samples
Hirakawa	Boonda	M	11	+	Northern 2	Northern	3
	Himawari	F	2	+	Southern	Northern	2
	Itsuki	M	1	+	Central	Northern	3
	Ito	F	4	+	Central	Northern	4
	Kibou	F	2	+	Central	Northern	4
	Sora	M	1	+	Southern	Northern	3
	Indeco	F	2	−	Southern	Northern	5
	Archer	M	3	−	Southern	Northern	5
	Peace	F	1	−	Central	Northern	3
Awaji	Yuki	M	13	−	Southern	Southern	5
	Daichi	M	8	−	Southern	Southern	5
	Nozomi	F	14	−	Southern	Southern	5
	Midori	F	25	−	Southern	Southern	5
	Umi	F	8	−	Northern 2	Northern	5
	Peter	M	6	−	Southern	Northern	5

1