Gut microbiome communities demonstrate fine-scale spatial variation in a closed, island bird population

Short title: Microbiomes vary at fine spatial scales

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Abstract

Environmental variation is a key factor shaping microbiome communities in wild animals. However, most studies have focussed on separate populations distributed over large spatial scales. How ecological factors shape inter-individual microbiome variation within a single landscape and host population remains poorly understood. Here, we use dense sampling of individuals in a natural, closed population of Seychelles warblers on Cousin Island (<0.7 km diameter, 0.34 km^2 total area) to determine whether gut microbiome communities exhibit high-resolution spatial variation over fine scales (average territory area is 0.0023 km^2). We identified a strong quadratic relationship between geographic distance and gut microbiome beta diversity across the island. Microbiome composition initially diverged with increasing geographic distance between territories. However, after > *ca* 300 m microbiome composition became increasingly similar among individuals situated on different sides of the island. This relationship was robust to the effects of host relatedness, age, and sex. Further analysis showed that microbiome composition differed between individuals inhabiting coastal and inland territories. Warblers in coastal territories harboured greater abundances of marine bacteria and lower abundances of anaerobic taxa commonly linked to host metabolic health, suggesting that exposure to different environmental microbes and variation in host condition (which is lower in coastal territories) could drive spatial patterns of gut microbiome variation across the island. This work demonstrates that host-microbe interactions can be extremely plastic even at very fine spatial scales. Such plasticity may have implications for how species respond to anthropogenic disturbance in wild habitats.

Keywords: gut microbiome, biogeography, microbial ecology, environmental gradients, *Acrocephalus sechellensis*

Introduction

The vertebrate gut microbiome plays an important role in host health by contributing to processes such as host digestion, behaviour, and immunity [1, 2]. However, in wild populations, gut microbiome composition can be extremely variable, even amongst individuals living in the same natural population [3–5]. In some cases, such variation has been associated with differences in host fitness components, including survival [6, 7], disease resistance [8, 9], and reproductive performance [10]. Thus, determining the drivers of inter-individual gut microbiome variation has important implications for understanding how host-microbe interactions shape the health and evolutionary trajectory of their hosts.

Various ecological factors have been proposed as drivers of gut microbiome variation in wild animal species. For example, variation in habitat type [11], anthropogenic disturbance [12], climatic variables (e.g. rainfall) and food availability [13] have all been associated with differences in microbiome composition. Such factors could have a direct impact on host-microbe interactions because variation in biotic and abiotic factors, coupled with microbial dispersal limitation, can lead to spatial heterogeneity in the pool of microbes able to colonise a host from the environment [14, 15]. Conversely, indirect effects could arise if the environment influences factors such as host condition, stress, and behaviour, all of which can alter the gut microbiome [16, 17].

The impact of environmental factors on the gut microbiome has primarily been demonstrated using host groups or populations that are distributed over large spatial scales (often separated by several to hundreds of kilometres) [e.g. 14, 18–20]. How ecological factors shape inter-individual gut microbiome variation at a much finer scale within a landscape (e.g. across territories that are metres apart), and within a single host population, is much less well understood. Studying the role of environmental factors in shaping the microbiome at different spatial scales will not only shed light on the plasticity of host-microbe interactions but is especially urgent given the increasing influence of anthropogenic disturbance on wild habitats.

Here, we use an isolated population of Seychelles warblers (*Acrocephalus sechellensis*) on Cousin Island to understand how environmental factors influence the gut microbiome of individuals at fine spatial scales. Cousin Island measures < 0.7 km in diameter (0.34 km^2 total

area) and is inhabited by *ca* 320 adult Seychelles warblers, distributed across *ca* 115 territories (average territory area 0.0023 km^2) which are defended year-round [21, 22]. Territories differ in terms of their size, density, proximity to standing water, and quality (quantified in terms of insect abundance) across the island [23]. In particular, the prevailing wind direction, which differs between the two monsoonal seasons, can affect coastal territories as trees become defoliated by salt spray [23]. This can result in reduced insect abundances which have, in turn, been associated with reduced reproductive success in these territories [23].

Previous research has shown that individual Seychelles warbler gut microbiome composition varies according to season, average yearly territory quality, and host factors such as immunogenetic variation [5, 24, 25]. Gut microbiome variation has also been linked to differential survival [7, 25]. However, it is unknown whether spatial stratification of gut microbiome differences can be detected across the island. Here, we use dense sampling of individuals across the island to address this question. First, we investigate whether gut microbiome diversity and composition differ according to the distance between territories on the island. We hypothesise that gut microbiome similarity will decrease at greater geographical distances due to spatial heterogeneity in habitat types and the abundances of environmentally-derived microbial species, as has been observed at larger spatial scales [15, 19, 26]. Second, we investigate whether specific habitat features influence the gut microbiome. In particular, does habitat type (territories located on the exposed and sheltered coastline, or inland), distance to standing water, and/or territory density impact gut microbiome characteristics? We expect microbiome differences between birds inhabiting coastal and inland territories due to differential exposure to marine microbes and potentially via indirect effects of inhabiting territories of differing quality (e.g. greater host stress and lower condition in coastal territories exposed to prevailing winds) [23, 27]. Distance to standing water may also impact the microbiome by influencing the availability of insect prey. Similarly, territory density (the number of territories bordering a focal territory) may impact gut microbiome diversity via territory quality effects, but also due to the greater number of social interactions expected amongst birds living at greater density (e.g. via boundary disputes and extra-pair reproductive attempts) [28, 29].

Materials and methods

Study system

The Seychelles warbler is a small insectivorous passerine endemic to the Seychelles archipelago. Samples were collected from the closed population of warblers inhabiting Cousin Island (0.34 km^2; 04°20′S, 55°40′ E). Here, virtually all individuals are uniquely marked with a combination of a British Trust for Ornithology (BTO) metal ring and three plastic colour rings, enabling identification and monitoring throughout their lives [30, 31]. Population monitoring is carried out biannually in the minor (January-March) and major (June-September) breeding seasons, respectively.

Territories consist of a dominant breeding pair which may also be accompanied by independent subordinates, some of which help in reproductive attempts [21, 32]. Foraging and reproduction occurs within the territory and defensive behaviours, including physical fights, are observed at territory boundaries [33, 34]. Territory boundaries are identified each breeding season by observing foraging behaviours and boundary disputes once every two weeks. Digital territory maps are subsequently generated using ArcGIS PRO software. The average territory size on Cousin Island is 0.0023 km^2 [22]

Habitat classification

Territories were classified into habitat categories using ArcGIS PRO software. The prevailing wind direction has a profound effect on coastal territories, whereby salt spray leads to the defoliation of vegetation and a subsequent reduction in insect abundances [23, 27, 35]. Prevailing winds come from the south-east (SE) in April – September, or from the north-west (NW) in October – March. Thus, territories with a direct boundary on the SE coast were categorised as “exposed coast” during the major breeding season, whilst the remaining coastal territories were categorised as “sheltered coast” (Figure 1). The opposite was the case for the minor season. All territories with no coastal border were classified as “inland” (Figure 1).

Two areas that are permanently marshy and can contain standing water are found on Cousin Island; one is a mangrove swamp dominated by *Avicennia maritime*, the other is a freshwater marsh area (Figure 1). Although the size of the freshwater marsh fluctuates greatly with rainfall, it is always marshy and normally contains some standing water year-round (Figure 1). The distance of each territory to the freshwater marsh was calculated as the distance (in

metres) from the marsh edge to the centre of each territory. Distances ranged from 0 m (where the territory overlapped with the marsh) to 301 m.

The density of territories also differs across the island; local density is lower in the central elevated area (Figure 1, >10 m elevation) which is rocky and has sparser vegetation compared to the central lowland plateau [23]. Local territory density was calculated as the number of territories sharing a physical border with the focal territory. Local density varied between 0 and 9.

Sample collection

Faecal samples were collected across nine breeding seasons between 2017-2022. Each season, birds were caught in mist-nets and then placed into a disposable, flat-bottomed paper bag containing a sterilised weigh boat protected by a metal grate [24, 36]. This allows faecal matter to be collected from the tray whilst reducing contamination from the bird's surface. Birds were removed from the bag after defecation or after 30 minutes. Faecal samples were collected using a sterile flocked swab and placed into a microcentrifuge tube containing 1 ml of absolute ethanol. Control swabs from fieldworker hands and collection bags were also taken at time of sampling. All samples were stored at 4°C for the remainder of the season (0-3.8 months) before being transferred to −80°C for long-term storage. A blood sample was also taken from each bird via brachial venipuncture and stored in absolute ethanol at 4°C. DNA was extracted from blood samples using the DNeasy Blood and Tissue kit (Qiagen, Crawley, UK); DNA was used for molecular sexing via a PCR-based method [31, 37] as well as genotyping at up to 30 polymorphic microsatellite loci [see 30, 31].

Fieldwork was carried out in accordance with local ethical regulations and agreements (University of East Anglia ethics approval ID ETH2223-0665). The Seychelles Department of Environment and the Seychelles Bureau of Standards approved the fieldwork (permit number A0157).

Microbiome sequencing and bioinformatics

Genomic DNA was extracted from all faecal samples and collection controls using the DNeasy PowerSoil kit (Qiagen), according to a modified version of the manufacturer's instructions [see 24]. Extracted DNA was submitted for 16S rRNA gene amplicon sequencing at the NEOF Centre for Genomic Research (Liverpool, UK). Amplicon sequencing libraries

were generated using the V4 primers 515F and 806R [see 24] and underwent 2 × 250 bp, paired-end sequencing on an Illumina MiSeq platform. Negative extraction blanks (*ca* 1 per 50 samples) and a ZymoBIOMICS microbial mock community standard (D6300) were also sequenced to identify contaminants, check for batch effects, and assess sequencing success [as described in 5].

Sequencing reads were processed using QIIME2 2019.10 [38]. Briefly, forward and reverse reads were truncated at 240 bp and low-quality base calls were trimmed from the 5′ end using the DADA2 plugin [39]. Amplicon sequencing variants (ASVs) were then inferred for each sample, followed by dereplication and pair-end joining, as well as the removal of putative chimeras and singleton reads. ASVs were then taxonomically classified by training a naïve-Bayes classifier on the SILVA 132 reference database for 16S rRNA gene sequences. ASVs classified as chloroplast or mitochondria were subsequently removed. A mid-point rooted phylogeny was constructed using MAFFT [40] and Fast Tree [41]. The final ASV, taxonomy, and tree files were exported from QIIME2 into R 4.2.2 for use in all subsequent analysis [42].

Files were further processed using *phyloseq* 1.42.0 [43]. ASVs were filtered to remove non-bacterial sequences and those unassigned at phylum level. Potential contaminants were also identified and removed from faecal samples using the prevalence method in *decontam* 1.18.0 [44]. Additionally, ASVs with fewer than 50 reads across all samples were removed prior to downstream analysis as these may represent sequencing errors (filtered ASVs accounted for ~1% of all reads).

In total, 691 samples from 390 individuals were included in downstream analysis. These were individuals classified as old fledglings (3-5 months of age), sub-adults (5 months to 1 year), or adults (≥ 1 year). We excluded samples from chicks and fledglings due to small sample sizes; these individuals are also still dependent on their parents and harbour an immature gut microbiome [7]. A total of 21,053 ASVs were identified across the 691 samples (mean per sample 225.85 ± 6.11 SE).

Statistical analysis

Alpha and Beta diversity metrics

Samples were rarefied to 8,000 reads, based on rarefaction curves and samples reaching >95% completeness at this depth, prior to calculating alpha diversity metrics. Observed ASV richness and Shannon diversity were calculated for each sample using *phyloseq* 1.42.0 [43]. Unrarefied reads were used to calculate gut microbiome beta diversity (i.e. compositional differences amongst samples). Unrarefied reads were filtered to remove rare taxa present in <5% of samples as these can disproportionately influence beta diversity metrics and may represent environmental transients (78% of reads were retained). ASV abundances were then transformed using a centred log ratio (CLR) transform in *microbiome* 1.20.0 [45] to control for compositionality [46]. Finally, a pairwise Aitchison distance matrix of CLR transformed ASV abundances (i.e. beta diversity) was constructed using *vegan* 2.6.8 [47].

Geographic distance and gut microbiome similarity

To establish whether warbler gut microbiome characteristics varied with geographic distances between territories a series of distance matrices was constructed. A Euclidean distance matrix of sample alpha diversity (either Shannon diversity or observed ASV richness) was constructed using *vegan* 2.6.8 [47]. A matrix of geographic distances between territory centroids was calculated using the st_distance() function in *sf* 1.0.16 [48, 49]. Geographic distances ranged from 0 m (individuals in the same territory) to 698 m (average distance 283.43 ± 0.86 SE). The minimum distance between adjacent territory centroids was 25.81 m. Pairwise alpha diversity or Aitchison (beta diversity) gut microbiome distances were then used as the response variable in separate Multiple Regression on distance Matrices (MRM) models. Alpha diversity distances were right-skewed and therefore square root transformed prior to analysis. In MRMs, tests of significance are performed using a randomised permutation procedure which controls for the non-independence of pairwise comparisons involving the same sample [50, 51]. MRMs were conducted using the MMRR function [implemented by 52] using 999 permutations.

Geographic distance was included as an independent variable in models. We also controlled for differences in sex (1= same sex, 0 = different sex), age class (1= same age class, 0= different age class), and genetic relatedness between individuals. Pairwise genetic relatedness was calculated using *related* 1.0 [53] based on data from genotyping at up to 30 microsatellite loci [30, 31] and the Queller and Goodnight's estimation of relatedness [54]. We tested for quadratic relationships between geographic distance, as well as relatedness, and gut microbiome distances, but quadratic terms were removed sequentially if not significant (in

order of least significant) to enable interpretation of the main effects. To simplify models and avoid the confounding effect of temporal environmental variation across sampling periods (which we know has a considerable effect on the GM [5]) we only included comparisons of samples taken from different individuals within the same sampling period (i.e. excluding between-sampling period comparisons). In total, 27,330 pairwise comparisons were included in the full model. To test whether coastal territories were having a disproportionate impact on the relationship between geographic and gut microbiome distances, we also re-ran the model using only inland-inland pairwise territory comparisons (i.e. excluding coastal territories, 11,522 pairwise comparisons). All variance inflation factors were < 2, indicating no issues with collinearity.

Landscape features and gut microbiome differences

To further investigate the importance of habitat type and landscape features in driving variation in gut microbiome alpha diversity across individuals, generalised linear mixed models were constructed with either a Gaussian (for Shannon diversity) or negative binomial (for observed ASV richness) distribution using *lme4* 1.1.34 [55]. Habitat type (exposed coast, sheltered coast, or inland), distance to marsh, local territory density, age, and sex (male/female) were included as predictors. We also controlled for the time of day at which samples were collected (minutes since sunrise) and the number of days samples were stored at 4°C in the field, both previously shown to impact the warbler gut microbiome [5]. Bird ID and sample year were included as random effects. We tested for quadratic relationships between distance to marsh, as well as local territory density, and gut microbiome alpha diversity but these quadratic terms were not significant and so were removed to enable interpretation of the main effects. We tested for residual spatial autocorrelation using the Moran's I test embedded within *DHARMa* 0.4.6 [56], however this was not significant for any models, indicating that spatial variation had already been adequately explained by independent terms.

A marginal permutational analysis of variance (PERMANOVA) was used to test whether variation in gut microbiome beta diversity was associated with habitat type and landscape features. This was performed on pairwise Aitchison distances using the *adonis2()* function within *vegan* 2.6.8 [47]. The same predictors were used as for alpha diversity analysis. Bird ID was included as a blocking factor to control for repeated sampling. Differences in beta diversity were visualised using a principal components analysis (PCA) in *vegan* 2.6.8 [47].

To test whether the abundances of specific bacterial ASVs differed according to habitat type an Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) was performed using *ancombc2* 2.1.4 [57]. Only landscape factors that were significantly associated with gut microbiome beta diversity were included as predictors in the model. However, host age, sex, time of day, storage time in the field, and sample year were controlled for in all analyses. Bird ID was included as a random effect. As part of ANCOM-BC, the Holm method was used to correct *P*-values for multiple testing. Example maps showing the average abundance per territory of the most differentially abundant ASVs were generated using *sf* 1.0.16 [48, 49] and *tmap* 3.3.4 [58] using the Cousin Island 2021 major breeding season territory map as a base layer.

Results

Geographic distance and gut microbiome similarity

Alpha diversity distances based on observed ASV richness were significantly lower among individuals within the same (versus different) age class ($P = 0.009$, Table S1). None of the other variables (geographic distance, host genetic relatedness, or sex differences) were associated with ASV richness distances between individuals ($P > 0.05$, Table S1). Gut microbiome distances based on sample Shannon diversity were also not associated with any of the predictors in the model ($P > 0.05$, Table S1).

There was a significant, quadratic, relationship between geographic distance and gut microbiome beta diversity on Cousin Island ($P = 0.001$, Figure 2, Table 1). Specifically, gut microbiome composition was most similar (i.e. lowest Aitchison distances) amongst individuals sampled in the same territory (0 m geographic distance) but gradually diverged as the geographic distance between territories increased (Figure 2). However, after *ca* 300 m, gut microbiome composition became increasingly similar again as geographic distances increased (Figure 2). This relationship was robust, even after controlling for the relatedness of individuals (see below), which is likely to be higher amongst individuals in the same territory.

There was also a significant, linear negative relationship between gut microbiome distances and the pairwise relatedness of individuals ($P = 0.001$, Figure 2, Table 1). Similarly, gut

microbiome compositional distances were significantly lower amongst individuals in the same age class and of the same sex (P = 0.001 and 0.024, respectively, Table 1).

The greatest geographic distances on Cousin Island are between coastal territories on different sides of the island; as such, the quadratic relationship identified between gut microbiome and geographic distances (Table 1, Figure 2) may partly reflect similarity in habitat type along the coast. To test whether coastal territories were having a disproportionate impact on the relationship between geographic and gut microbiome distances, we re-ran the model using only inland-inland pairwise territory comparisons (i.e. excluding coastal territories). This revealed a significant linear relationship, whereby gut microbiome distances increased with increasing geographic distance between territories (P= 0.001, Table S2, Figure S1).

Landscape features and gut microbiome differences

We further tested whether specific habitat types/territory features were associated with gut microbiome characteristics. Neither gut microbiome Shannon diversity nor observed ASV richness varied according to differences in territory habitat type (exposed coast, sheltered coast, or inland territories), distance to marsh, or with territory density (P > 0.05, Table S3).

Gut microbiome composition varied significantly according to habitat type (P = 0.004 in a PERMANOVA, Table 2). A PCA plot showed that the gut microbiome of individuals inhabiting coastal territories (both on the exposed and sheltered coast) tended to cluster away from those in territories situated inland (Figure 3). Conversely, neither distance to marsh nor territory density were significant predictors of gut microbiome composition (P > 0.05, Table 2). Bird age and sex were also not associated with gut microbiome composition (P > 0.05, Table 2). However, the season and year of sampling, the time-of-day samples were collected, and the time stored at 4°C were significantly associated with variation in gut microbiome composition (P < 0.01, Table 2).

Differential abundance analysis

We next tested if specific ASVs differed in abundance between territory habitat types. In total, 19 ASVs were significantly differentially abundant between exposed coast and inland territories (P_{adj} < 0.05, Figure 4, Table S4). Of these, 8 ASVs were more abundant in exposed coast territories (Figure 4, Table S4); three were in the phylum Proteobacteria (in the families

Enterobacteriaceae, *Rhodobacteraceae*, *Rhizobiaceae*) and five were in the phylum Actinobacteria (two ASVs in the genus *Rubrobacter*, one in the genus *Pseudokineococcus*, one in the genus *Marmoricola*, and one in the genus *Nocardioides*). The remaining 11 ASVs were more abundant in inland (versus exposed coast) territories (Figure 4, Table S4). Of these, one ASV was in the phylum *Verrucomicrobia* (genus *Akkermansia*), four were in the phylum *Proteobacteria* (two in the genus *Methylobacterium*, one in the family *Rhizobiaceae*, one in the family *Enterobacteriaceae*), two in the phylum *Firmicutes* (one in the genus *Lachnoclostridium*, and one in the family *Christensenellaceae*), and four in the phylum *Actinobacteria* (one in each of the genera *Actinomycetospora*, *Microbacterium, Williamsia, and Pseudonocardia*). The abundances of the most differentially abundant taxa - one *Rubrobacter* ASV that was more abundant in exposed and sheltered coastal territories and one *Christensenellaceae* ASV that was more abundant inland - are plotted on territory maps as an example of these relationships (Figure 5).

Only one ASV was differentially abundant between sheltered and exposed coastal territories; an ASV in the genus *Microbacterium* was more abundant in sheltered coastal territories ($P_{adj} < 0.05$, Table S4). Five ASVs were differentially abundant between sheltered coastal and inland territories ($P_{adj} < 0.05$, Table S4); two ASVs classified as the genera *Marmoricola* and *Rubrobacter,* respectively were more abundant in sheltered coast territories, whereas three ASVs in the genera *Actinomycetospora, Williamsia* and *Lachnoclostridium* were more abundant in inland territories.

Discussion

In this study, we evaluated fine-scale spatial heterogeneity in vertebrate host-gut microbe interactions across a landscape using a small island population of the Seychelles warbler. We identified a strong quadratic relationship between geographic distance and gut microbiome beta diversity which emerged over very small spatial scales (geographic distances of < 0.7 km in total). Gut microbiome composition was more similar amongst individuals in the same territory but gradually diverged with increasing geographic distance. However, individuals sampled in territories that were > *ca* 300 m apart increasingly converged on a more similar gut microbiome. This relationship was robust even when controlling for host relatedness, age, and sex differences, and was likely to be partly driven by variation in habitat types across the island. Indeed, habitat type had a strong effect on the warbler gut microbiome, whereby the gut microbiome of individuals sampled in coastal territories diverged from those inhabiting

inland territories, both in terms of overall composition and the abundance of specific bacterial ASVs.

Biogeographic patterns in gut microbiome diversity have been noted previously in other systems. For example, in humans, gut microbiome similarity generally decreases with geographic distance within and between populations [59] and individuals living in shared spaces tend to harbour more similar microbial communities [60]. There is also some evidence of spatial microbiome variation in wild animals, with greater gut microbiome similarities occurring amongst sympatric versus allopatric species [61, 62], populations [19], and individuals [26]. However, such studies have been conducted over the scale of several, or even thousands of kilometres. We now show that spatial gut microbiome patterns can also be detected at much finer spatial scales (where territories are tens of metres apart) within a single species.

Patterns of spatial gut microbiome variation may be partly driven by an increase in genetic relatedness amongst individuals living in familial groups and/or near one another. Indeed, studies in humans [63] and wild animals [64–66] have shown that related individuals tend to harbour more similar microbiome communities than pairs of unrelated individuals. These studies highlight the importance of host genetic differences in regulating microbiome composition. Pairwise genetic relatedness was a significant predictor of microbiome similarity between Seychelles warblers. Relatedness also tends to be higher amongst warblers living in the same territory, as some offspring remain as subordinates to help with future breeding attempts [32, 67]. Thus, increased genetic similarity may partly drive the pattern of increased gut microbiome similarity for individuals living in the same breeding group. However, there was a strong negative quadratic relationship between geographic and gut microbiome distances even after controlling for relatedness in models. In this relationship, microbiome composition initially diverged with increasing geographic distance but then became more similar again amongst individuals separated by distances of > *ca* 300 m. This suggests that factors other than relatedness are likely to be important in driving spatial patterns of gut microbiome variation in this species.

Recent research has also shown that microbial taxa can be shared amongst individuals via social interactions [28, 29, 68]. Microbial sharing can occur via direct interaction or through host microbial shedding to a shared environment [28]. Such processes could partly explain

the increase in gut microbiome similarity amongst Seychelles warblers inhabiting the same territory. However, it is difficult to disentangle the relative importance of greater social transmission, versus increased relatedness and shared environmental conditions, when species are highly territorial. In the warbler, local territory density may represent a proxy for the number of opportunities to interact with birds from neighbouring territories. For example, these interactions may occur via boundary disputes [33, 34] and extra-pair copulations which are normally with males from adjacent territories [30, 69]. However, local territory density was not associated with gut microbiome composition in the Seychelles warbler. Furthermore, it is unlikely that social transmission dynamics are driving the gradual convergence of gut microbiome communities at geographic distances > 300 m. Thus, although social interactions may play a role in structuring spatial gut microbiome variation, particularly amongst adjacent territories, other factors are also likely to be important.

Fine-scale habitat differences are likely to be a key factor driving spatial patterns of gut microbiome variation within a landscape. Indeed, environmental variation has been shown to be an important factor shaping the gut microbiome in captive cross-foster experiments [70, 71] and among wild animal populations and individuals [11, 18, 72]. Whilst environmental differences could influence the microbiome indirectly, for example via effects on host stress and condition [17, 73], abiotic/biotic variation can also determine the type of microbes that can survive and be acquired by hosts horizontally from the external environment [15, 74]. Such acquisition may occur through direct interaction with the local environment, or indirectly, for example via variation in microbes derived from the host's diet. The gut microbiome of passerines may be particularly responsive to microbial variation in the external environment since their short intestinal tracts (an adaptation to flight) make them more susceptible to acquiring transient microbial species which can persist during gut transit [75, 76].

On Cousin Island, the greatest geographic distances between Seychelles warbler territories arise between coastal locations (i.e. territories on different sides of the island). Thus, the gradual increase in microbiome similarity at distances > *ca* 300 m suggests that habitat similarity may be a key driver of compositional microbiome similarity. Indeed, habitat type was significantly associated with gut microbiome composition, whereby individuals in coastal territories harboured significantly different microbial communities compared to those inland. Comparisons between individuals in exposed coast versus inland territories yielded

the greatest number of differentially abundant ASVs. However, there was also overlap in significant taxa when comparisons were made between sheltered coast and inland territories suggesting a general effect of coastal conditions on the gut microbiome that was made more extreme by the prevailing wind direction.

Individuals inhabiting coastal territories generally harboured greater abundances of aerobic, marine-associated or extremophile bacterial taxa. For instance, members of the genus *Rubrobacter* are frequently isolated from marine environments and are tolerant of high levels of radiation, temperature, and salinity [77, 78]. Similarly, members of the *Rhodobacteraceae* and *Pseudokineococcus* have also been isolated from marine and hyper-saline environments [79, 80]. By contrast, individuals inhabiting inland territories tended to harbour greater abundances of bacterial taxa commonly found in soil and terrestrial habitats such as *Methylobacterium*, *Pseudonocardia*, and members of the *Rhizobiaceae* [81–83]. This suggests that differential exposure to, and acquisition of, environmental microbes may be driving some of the gut microbiome differences observed between inland and coastal birds. However, several anaerobic bacterial taxa that are commonly found in the gut microbiome of other vertebrate species were also more abundant in warblers inhabiting inland territories. This included ASVs in the *Lachnoclostridium*, *Enterobacteriaceae*, *Akkermansia*, and *Christensenellaceae*. Commensal members of the *Lachnoclostridium* (recently reclassified as *Clostridium*) and the *Enterobacteriaceae* play an important role in producing short chain fatty acids such as butyrate and lactic acid; such molecules have been shown to contribute to gut epithelial tissue maintenance and are beneficial to host health [84–86]. Similarly, *Christensenallaceae* is one of the most heritable members of the human gut microbiome and the abundance of this family, as well as the genus *Akkermansia*, is positively associated with various aspects of metabolic health in mammals [87, 88]. Whilst the function of these taxa has not been assessed in passerines it is possible that differences in their abundance are linked to variation in the condition of Seychelles warblers living in inland versus exposed coast territories. Tree defoliation and reduced insect abundances in coastal areas has been associated with reduced reproductive success in the Seychelles warbler [23]. Whilst it is extremely difficult to disentangle cause from effect in wild systems, changes to dietary quality, increased stress, and reduced host condition could result in disruption to key components of the gut microbiome that contribute to the maintenance of metabolic health; this could, in turn, feedback to further influence host condition and fitness components. However, further functional characterisation of gut microbes in avian species, for example

using metagenomics, and experimental disruption of the gut microbiome would be needed to understand whether this is the case [89].

In conclusion, our study demonstrates that gut microbiome communities can vary at extremely fine spatial scales within a landscape and that at least some of this variation is likely to be driven by differences in local environmental conditions. Further work is needed to understand the mechanisms by which the environment shapes the microbiome and the impact of spatial gut microbiome variation on host fitness, but this work suggests that host-microbe interactions can be extremely plastic even among individuals of the same species living in close proximity. Given the importance of the gut microbiome to host health, such plasticity may have implications for the resilience of species to anthropogenic disturbance in wild habitats. This may be especially important in restricted, small island populations that have no emi- or immigration.

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Data Availability Statement

All sequencing reads have been uploaded to the European Nucleotide Archive (ENA) under the following accession numbers: PRJEB45408 (samples taken in 2017 and 2018), PRJEB47095 (samples taken in 2019 and 2020), and PRJEB67634 (samples taken in 2021 and 2022). The scripts and metadata to reproduce all analyses and figures can be accessed via

the GitHub repository https://github.com/Seychelle-Warbler-Project, and will be archived in the Dryad repository.

Authors' contributions

The study was conceived by SFW and DSR. SFW, CZL and DSR performed the fieldwork. SFW conducted the microbiome laboratory work. SFW performed the bioinformatics and statistical analyses and drafted the manuscript with input from DSR. DSR, HLD, JK and TB managed the Seychelles Warbler Project. All authors read and approved the final manuscript.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Tables and Figures

Table 1. The results of a Multiple Regression on distance Matrices (MRM) model investigating the relationship between geographic distance and gut microbiome beta diversity in Seychelles warblers. A total of 27,330 pairwise comparisons of 691 samples were included in the model. Reference categories were different sex (0) and different age class (0) for sex and age similarity variables, respectively. Tests of significance were performed using a randomised permutation procedure (999 permutations) to control for the non-independence of pairwise comparisons involving the same sample. Significant predictors are shown in bold.

Predictor	Estimate	*t*	Permuted *P*
Intercept	82.726	258.115	0.721
Geographic distance	**0.012**	**5.733**	**0.001**
Geographic distance^2	**<-0.001**	**-6.171**	**0.001**
Sex similarity	**-0.367**	**-2.240**	**0.024**
Age similarity	**-1.394**	**-8.491**	**0.001**
Relatedness	**-1.693**	**-3.837**	**0.001**

Table 2. A PERMANOVA analysis of the relationship between gut microbiome compositional differences and habitat features in Seychelles warblers. The analysis was performed using Aitchison distances calculated using centred log ratio (CLR)-transformed amplicon sequencing variant (ASV) abundances. Significant predictors ($P < 0.05$) are shown in bold. N = 691 samples from 390 individuals. Bird ID was included as blocking factor to control for repeated measures.

Predictor	df	R^2	F	*P*
Habitat type	**2**	**0.008**	**2.980**	**0.004**
Distance to marsh	1	0.003	1.881	0.457
Territory density	1	0.002	1.721	0.525
Age	1	0.002	1.246	0.979
Sex	1	0.002	1.543	0.552
Season	**1**	**0.004**	**2.678**	**<0.001**
Sample year	**5**	**0.023**	**3.300**	**<0.001**
Time of day	**1**	**0.006**	**4.384**	**<0.001**
Storage time	**1**	**0.004**	**2.719**	**0.001**

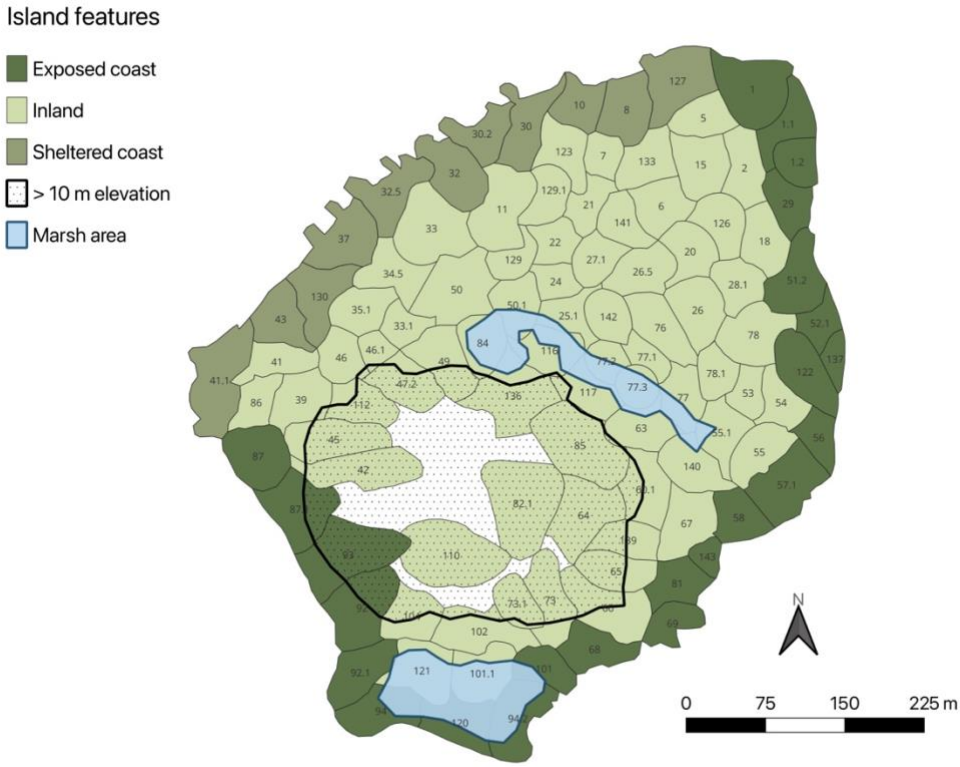

Figure 1. Seychelles warbler territories on Cousin Island. The 2021 major breeding season map is shown as an example. Territories have been coloured according to their habitat type. Prevailing winds come from the south east in the major breeding season; thus, exposed coastal territories are those with a direct boundary on the south-east coast (note that the opposite is true in minor breeding seasons). Two marshy areas (where open water sometimes exists) have been marked in blue. An area >10 m elevation above sea level is also shown (dotted area).

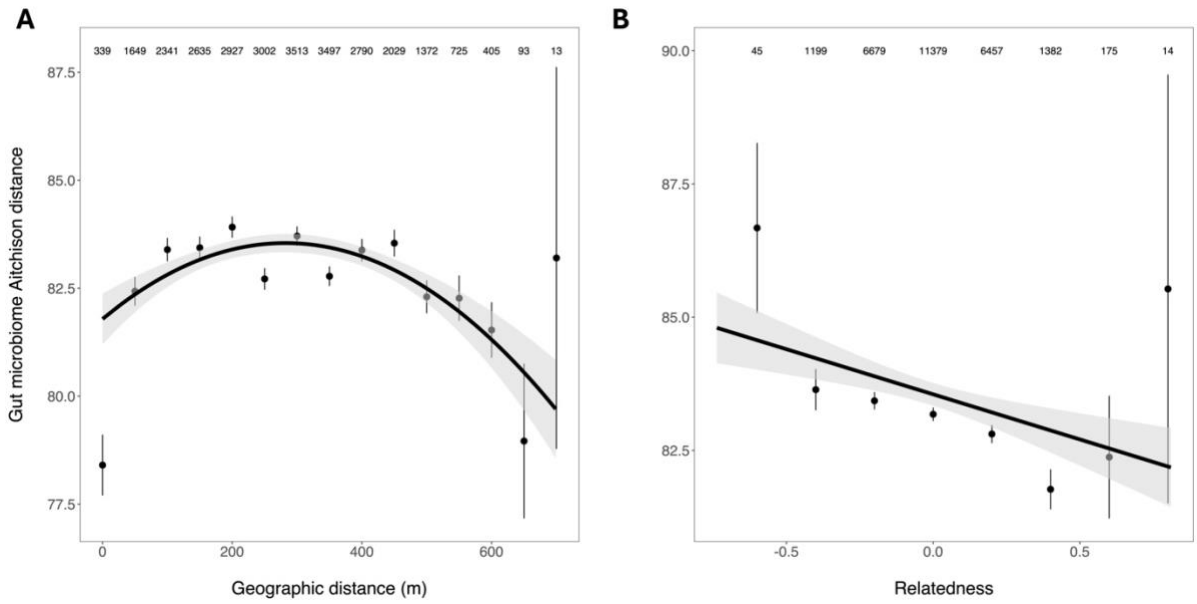

Figure 2. The relationship between A) geographic distance (in metres) or B) pairwise genetic relatedness and gut microbiome Aitchison distance (beta diversity) in Seychelles warblers. Points represent the mean (± SE) Aitchison distance per A) 50 m or B) 0.2 relatedness score and are calculated from the raw data. Numbers at the top of each panel represent the number of pairwise comparisons contributing to each mean. Total N = 27,330 pairwise comparisons between 691 samples (from 390 individuals) were included in models. Black lines are the model predicted slopes ± 95% CI from a Multiple Regression on distance Matrices (MRM) model (permuted *P*-values < 0.05).

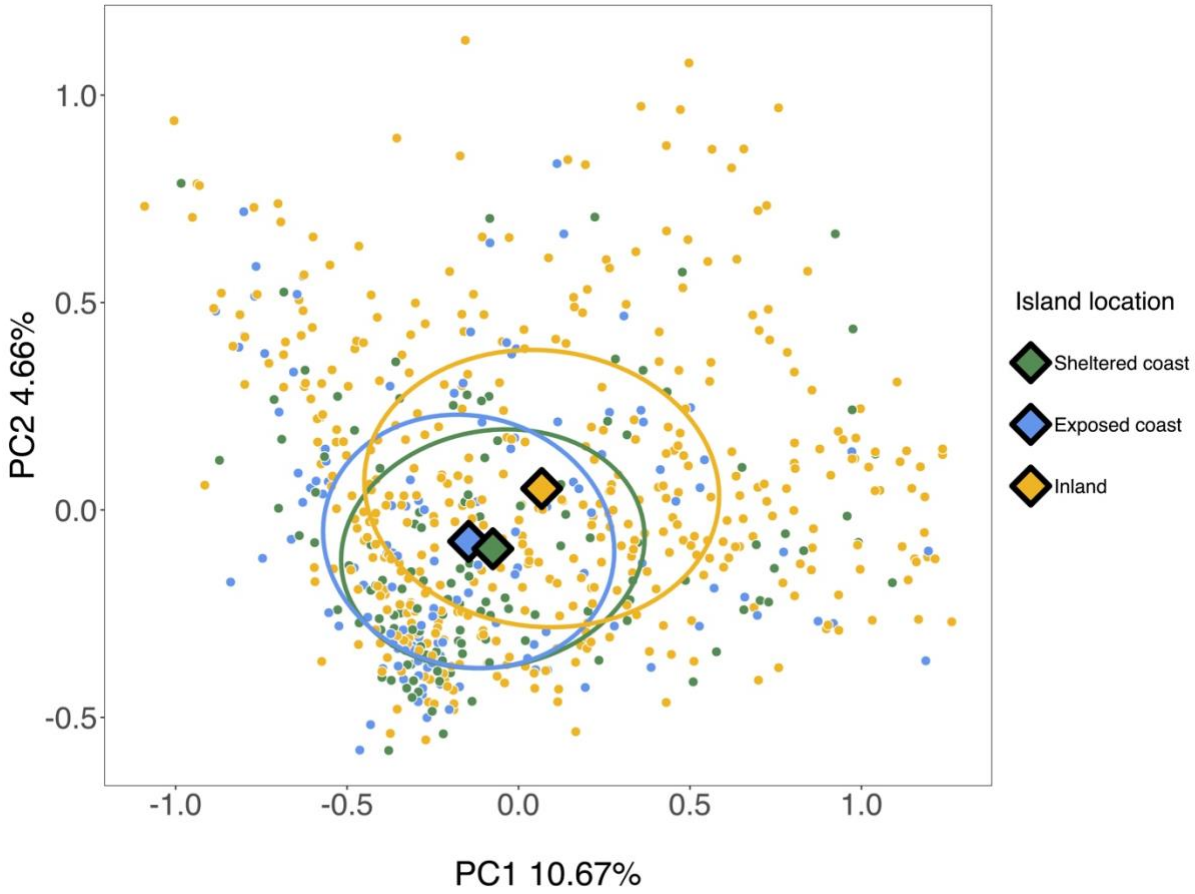

Figure 3. Variation in Seychelles warbler gut microbiome composition according to the habitat type of the individual's territory on Cousin Island. PCA ordination was carried out using Aitchison distances calculated on Centred Log Ratio (CLR)- transformed amplicon sequencing variant (ASV) abundances. Each point represents a unique gut microbiome sample (N = 691 samples from 390 individuals). Large diamonds represent the group centroids. Principal components 1 and 2 explained 10.67% and 4.66% of the variation in gut microbiome composition, respectively.

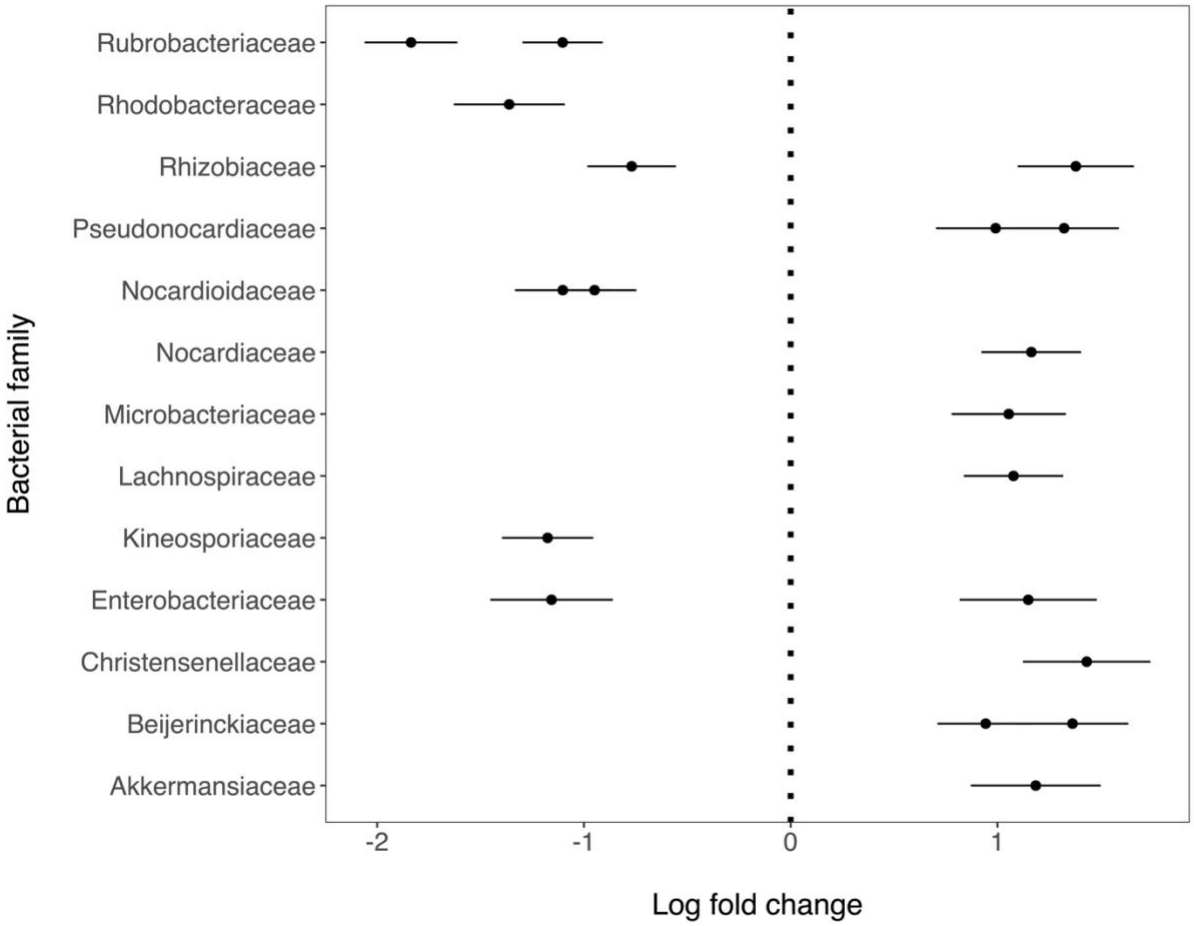

Figure 4. Differentially abundant Amplicon Sequencing Variants (ASVs) in the gut microbiome of Seychelles warblers inhabiting exposed coastal versus inland territories on Cousin Island. N = 691 samples from 390 individuals were included in the analysis. Points represent the log fold change (effect size ± SE) of individual bacterial ASVs calculated using an ANCOM-BC model; only those with significant effect sizes ($P_{adj} < 0.05$) are shown. A positive log fold change indicates that an ASV is more abundant in individuals inhabiting inland territories (right), and a negative log fold change indicates a higher abundance in individuals in exposed coast territories (left). Results of differential abundance tests and full ASV taxonomies are presented in Table S4.

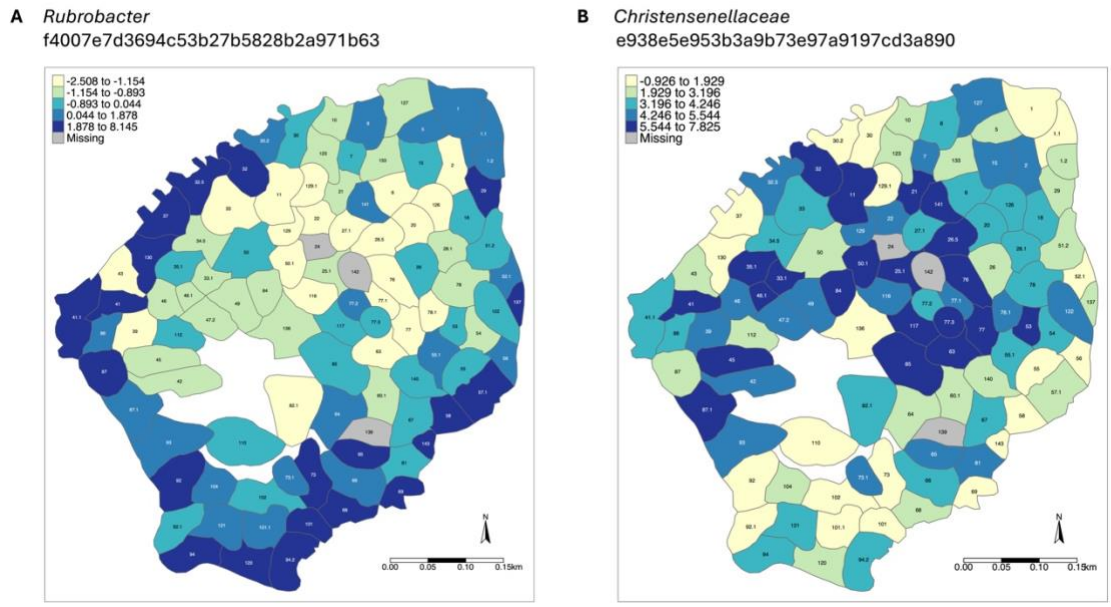

Figure 5. The average abundance of Seychelles warbler gut bacterial ASVs classified as A) *Rubrobacter (*ASV ID: f4007e7d3694c53b27b5828b2a971b63) or B) *Christensenallaceae* (ASV ID: e938e5e953b3a9b73e97a9197cd3a890) across different territories on Cousin Island. Territories are coloured according to the mean abundance of each ASV per territory across all sampling periods (2017-2022). A centred log ratio transformation was applied to ASV abundances prior to averaging. The 2021 major season territory map is used as a base layer; numbers represent unique territory IDs. Territories with no gut microbiome samples are shown in grey ("Missing"). Plotted ASVs were identified as having significantly greater abundances ($P < 0.05$) in A) exposed and sheltered coast territories and B) inland territories, respectively (see Table S4 for full results of differential abundance tests using ANCOM-BC).

Supplementary Material

Table S1. The results of a Multiple Regression on distance Matrices (MRM) analysis investigating the relationship between geographic and gut microbiome alpha diversity distances in Seychelles warblers. Two metrics of alpha diversity – A) Shannon and B) observed ASV richness – were used to calculate Euclidean distance matrices that were included as the response variable in separate models. A total of 27,330 pairwise comparisons of 691 samples were included in each model. Reference categories were different sex (0) and different age class (0) for sex and age similarity variables, respectively. Tests of significance were performed using a randomized permutation procedure (999 permutations) to control for the non-independence of pairwise comparisons involving the same sample. Significant predictors are shown in bold and underlined.

Predictor	**Estimate**		***t***		**Permuted *P***	
	A) Shannon	**B) Richness**	**A) Shannon**	**B) Richness**	**A) Shannon**	**B) Richness**
Intercept	1.070	10.467	145.672	131.552	0.590	0.097
Geographic distance	<-0.001	<-0.001	-0.556	-0.146	0.638	0.876
Sex similarity	-0.002	-0.006	0.317	-0.104	0.748	0.935
Age similarity	0.007	**-0.162**	1.273	**-2.715**	0.212	**0.009**
Relatedness	<0.001	-0.299	0.030	-1.876	0.978	0.059

Table S2. The results of a Multiple Regression on distance Matrices (MRM) model investigating the relationship between geographic distance and gut microbiome beta diversity across inland territories of the Seychelles warblers. A total of 11,522 pairwise comparisons between inland territories were included in the model. Reference categories were different sex (0) and different age class (0) for sex and age similarity variables, respectively. Tests of significance were performed using a randomized permutation procedure (999 permutations) to control for the non-independence of pairwise comparisons involving the same sample. Significant predictors are shown in bold and underlined.

Predictor	Estimate	*t*	Permuted *P*
Intercept	85.004	275.814	0.139
Geographic distance	**0.003**	**3.154**	**0.001**
Sex similarity	-0.268	-1.145	0.255
Age similarity	**-1.398**	**-5.980**	**0.001**
Relatedness	**-1.423**	**-2.298**	**0.022**

Table S3. Variation in gut microbiome A) Shannon diversity and B) observed ASV richness according to territory habitat type and landscape features in Seychelles warblers. Estimates are derived from (generalised) linear mixed models with a gaussian or negative binomial distribution, respectively. A total of 691 samples from 380 individuals were included in each analysis. Significant predictors ($P < 0.05$) are shown in bold and underlined. The reference categories for categorical variables are as follows: exposed coast (habitat type), female (sex), major (season).

A) Shannon diversity				
Predictor	**Estimate**	**SE**	***t***	***P***
Intercept	3.196	0.141	22.676	<0.001
Habitat type				
Inland	0.037	0.143	0.261	0.794
ShelteredCoast	-0.016	0.157	-0.103	0.918
Distance to marsh	0.010	0.104	0.093	0.926
Territory density	0.090	0.113	0.795	0.427
Age	-0.125	0.096	-1.305	0.193
Sex	-0.159	0.094	-1.683	0.093
Season	-0.071	0.120	-0.590	0.558
Time of day	-0.082	0.096	-0.861	0.390
Storage time at 4°C	**-0.300**	**0.101**	**-2.954**	**0.003**
Random effects	**691 Observations**		**Variance**	
Bird ID	390 individuals		0.058	
Sample Year	6 years		0.023	
B) Observed ASV richness				
Predictor	**Estimate**	**SE**	***z***	***P***
Intercept	5.234	0.115	45.641	<0.001
Habitat type				
Inland	0.154	0.081	1.907	0.057
Sheltered coast	0.092	0.089	1.036	0.300
Distance to marsh	-0.015	0.057	-0.269	0.788
Territory density	0.065	0.064	1.010	0.312
Age	-0.042	0.055	-0.762	0.446
Sex	-0.100	0.053	-1.912	0.056
Season	-0.038	0.079	-0.482	0.630

Time of day	-0.002	0.054	-0.044	0.965
Storage time at 4°C	**-0.123**	**0.060**	**-2.052**	**0.040**
Random effects	**691 Observations**		**Variance**	
Bird ID	390 individuals		0.010	
Sample Year	6 years		0.046	

Table S4. The results of an ANCOM-BC analysis investigating differences in gut microbiome amplicon sequencing variant (ASV) abundance according to territory habitat types in the Seychelles warbler. Amplicon sequencing variants (ASVs) that were significantly, differentially abundant ($P_{adj} < 0.05$) between two habitat categories are shown. Effect sizes (log fold change- "LFC") are shown with standard errors (SE). All *P*-values were adjusted with the Holm correction for multiple testing. A positive log fold change indicates that an ASV is more abundant in individuals inhabiting A) inland (versus exposed coast) territories B) Sheltered (versus exposed) coast territories and C) Sheltered (versus inland) territories. ASV taxonomic classifications are shown to bacterial genus level (or the highest resolution classification if unclassified at genus level).

ASV ID	LFC	SE	P_{adj}	Phylum	Family	Genus
A) Exposed coast versus inland territories						
f4007e7d3694c53b27b5828b2a971b63	-1.836	0.225	<0.001	*Actinobacteria*	*Rubrobacteriaceae*	*Rubrobacter*
2e5c86b8abc011cf866470d59d4e783f	-1.361	0.269	<0.001	*Proteobacteria*	*Rhodobacteraceae*	
144d2b8f94ec382e34460e8217d6a750	-1.176	0.221	<0.001	*Actinobacteria*	*Kineosporiaceae*	*Pseudokineococcus*
aee9f354c80ca7baa872c3da2fe462c2	-1.157	0.296	0.006	*Proteobacteria*	*Enterobacteriaceae*	
ac01fd659628399d0265775786cad38e	-1.103	0.194	<0.001	*Actinobacteria*	*Rubrobacteriaceae*	*Rubrobacter*

fac1e2799f556a919b52ebfb8ab31e5a	-1.102	0.231	<0.001	*Actinobacteria*	*Nocardioidaceae*	*Marmoricola*
5df7f6da7fe2d484098b5119cb19028b	-0.949	0.202	<0.001	*Actinobacteria*	*Nocardioidaceae*	*Nocardioides*
664765dce1a593784085345685a67650	-0.769	0.214	0.020	*Proteobacteria*	*Rhizobiaceae*	
e0f50c5adf537a0a3a63e61720b38ed5	0.943	0.233	0.003	*Proteobacteria*	*Beijerinckiaceae*	*Methylobacterium*
1c7a5248b18573f28b2901a63298dbc7	0.991	0.289	0.035	*Actinobacteria*	*Pseudonocardiaceae*	*Actinomycetospora*
e4c616e0e34cf5f3837203933c18e498	1.055	0.276	0.008	*Actinobacteria*	*Microbacteriaceae*	*Microbacterium*
09a261cd2ba6d5db1a38bfe0ef012286	1.078	0.240	<0.001	*Firmicutes*	*Lachnospiraceae*	*Lachnoclostridium*
3b7cb4615c07aeaa9415acee19c6db7c	1.149	0.332	0.032	*Proteobacteria*	*Enterobacteriaceae*	
749906e6079c81c5b2979a147e503684	1.164	0.241	<0.001	*Actinobacteria*	*Nocardiaceae*	*Williamsia*
574d387c22a18447c5c5375cf1f1b98d	1.185	0.314	0.010	*Verrucomicrobia*	*Akkermansiaceae*	*Akkermansia*
ff933fa75ad5cc076102ec14b504da6c	1.323	0.265	<0.001	*Actinobacteria*	*Pseudonocardiaceae*	*Pseudonocardia*

04ecfad5772d2e09a84a0f5ef460536c	1.363	0.270	<0.001	*Proteobacteria*	*Beijerinckiaceae*	*Methylobacterium*
d4ed8e95671bb076cf81536b9db1e1be	1.379	0.281	<0.001	*Proteobacteria*	*Rhizobiaceae*	
e938e5e953b3a9b73e97a9197cd3a890	1.432	0.308	<0.001	*Firmicutes*	*Christensenellaceae*	
B) Sheltered versus exposed coast territories						
e4c616e0e34cf5f3837203933c18e498	1.315	0.345	0.008	*Actinobacteria*	*Microbacteriaceae*	*Microbacterium*
C) Sheltered coast versus inland territories						
1c7a5248b18573f28b2901a63298dbc7	-1.423	0.337	0.002	*Actinobacteria*	*Pseudonocardiaceae*	*Actinomycetospora*
749906e6079c81c5b2979a147e503684	-1.326	0.296	<0.001	*Actinobacteria*	*Nocardiaceae*	*Williamsia*
09a261cd2ba6d5db1a38bfe0ef012286	-1.115	0.296	0.001	*Firmicutes*	*Lachnospiraceae*	*Lachnoclostridium*
fac1e2799f556a919b52ebfb8ab31e5a	1.021	0.287	0.023	*Actinobacteria*	*Nocardioidaceae*	*Marmoricola*
f4007e7d3694c53b27b5828b2a971b63	1.243	0.282	0.001	*Actinobacteria*	*Rubrobacteriaceae*	*Rubrobacter*

Figure S1. The relationship between geographic distance (in metres) and gut microbiome Aitchison distance (beta diversity) across inland territories of the Seychelles warblers. Points represent the mean (± SE) Aitchison distance per 50 m and are calculated from the raw data. Numbers at the top of each panel represent the number of pairwise comparisons contributing to each mean. Total N = 11,522 pairwise comparisons between inland territories. Black lines are the model predicted slopes ± 95% CI from a Multiple Regression on distance Matrices (MRM) model (permuted *P*-value = 0.001).