What Shapes The Gut Microbiome of Lizards From Different Habitats?

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Abstract

Host-gut microbiota interactions are complex and can have a profound impact on the ecology and evolution of both counterparts. Several host traits such as taxonomy, diet and social behavior, and external factors such as prey availability and local environment are known to influence the composition and diversity of the gut microbiota. In this study, we investigated the influence of taxonomy, sex, host size, locality/habitat on gut microbiota diversity in five lizard species from two different sites in Portugal. We also analyzed the potential levels of microbial transmission between species that live in sympatry and syntopy. We studied *Podarcis bocagei* and *Podarcis lusitanicus* from northern Portugal (Moledo); and two invasive species, *Podarcis siculus* and *Teira dugesii*, and the native *Podarcis virescens* from Lisbon. We used a metabarcoding approach to characterize the bacterial communities from the cloaca of lizards, sequencing the V4 region of the 16S rRNA. Habitat/locality was found to be the main driver of the differences in composition and structure of gut bacterial communities of the studied lizards, with host effects more evident at finer taxonomic scales. Additionally, lizards from urbanized environments had higher microbiome diversity than lizards from rural areas. We detected a significant positive correlation between size and gut bacterial alpha-diversity in the invasive species *P. siculus*, which could be due to higher exploratory behaviours. Moreover, estimates of bacterial transmission indicate that *P. siculus* may have acquired a high proportion of local microbiota. These findings indicate that a diverse array of host and environmental factors can influence lizards gut microbiota.

Introduction

A myriad of microorganisms can be found living in the gastrointestinal tract of all animals. These microorganisms have a significant impact on host biology and can influence a variety of processes that affect host fitness [1, 2]. While certain variations in the composition of gut microbial communities can cause disease [3, 4], gut microbiome may also increase resistance to pathogens, besides being important for xenobiotics metabolism, nutrient uptake and energy acquisition [e.g. 5, 6]. Moreover, gut microbiota may also contribute towards host adaptation to environmental changes by enabling a response to new challenges, such as exploitation of novel food sources [7, 8]. Ultimately, gut microbiome can have major impacts on host development, behaviour and fitness, with cascading effects to the dynamics of ecosystems [2]. In turn, it can also be modulated by several host traits, such as host taxonomy, sex and size, as well as the external environmental, such as habitat or prey availability [e.g. 9, 10). In addition, social interactions between hosts can also influence the gut microbiome in many animal species, although these mechanisms remain less studied [see review by 11].

Gut microbiome dynamics has been studied in many mammals [e.g., 2], birds [e.g., 12], fishes [e.g., 13] and amphibians [e.g., 14]. Comparatively, fewer studies have been performed in reptiles, and only a handful of these addressed lizards. Nevertheless, host taxonomy and ecology were seen to be important drivers of gut microbiota diversity in reptiles. For example, feeding habits influence the gut microbiota of the Chinese crocodile lizard, *Shinisaurus crocodilurus* Ahl 1930, with potential effects on host health due to the influence of diet on the abundances of pathogenic or opportunistic gut bacteria [15]. Diet and

habitat of the Australian water dragon, *Intellagama lesueurii* (Gray, 1831*)*, also have an effect on its gut microbiome, with lizards living in urban areas presenting higher bacterial diversity than populations living in natural habitats [16]. Moreover, host taxonomy and habitat also influence the gut microbiota of venomous snakes [17].

Here, we analyzed and compared the diversity and composition of gut bacterial communities of five phylogenetic related lacertid species captured in Portugal: *Podarcis siculus* (Rafinesque-Schmaltz, 1810), *Podarcis virescens* Geniez, Sá-Sousa, Guillaume, Cluchier and Crochet, 2014, *Podarcis bocagei* (Lopez-Seoane, 1885), *Podarcis lusitanicus* Geniez, Sá-Sousa, Guillaume, Cluchier and Crochet, 2014 and *Teira dugesi* (Milne-Edwards, 1829). *Podarcis* species are considered as model organisms to study ecotoxicology, immune/histochemical reactions, among other processes [e.g. 18,19]; however, microbiome studies are still largely lacking, with a single study on the Balearic *Podarcis lilfordi* (Günther, 1874) showing that islet and time since islet separation from mainland are significant factors contributing to gut microbiome structure [20]. Our main objective was to determine whether locality (which also corresponded to different habitats) and host factors such as species, size and sex modulate the gut bacterial diversity of these five lizards. To achieve this, we used non-invasive sampling (cloacal swabs) to obtain a proxy for gut bacterial communities which were characterized by sequencing the V4 region of the 16S rRNA gene.

Methods

A total of 103 adult lizards from five different species were sampled in September 2020: *Podarcis bocagei* (n = 33), *Podarcis lusitanicus* (n = 8), *Podarcis siculus* (n = 20), *Podarcis virescens* (n = 22) and *Teira dugesii* (n = 20). All these lacertid species are small-sized, diurnal, mostly insectivorous, and exhibit sexual dimorphism, with males usually being larger than females.

Podarcis bocagei and *P. lusitanicus* were collected from a semi-natural habitat in Moledo, northern Portugal (Fig. 1d) (41°50'19.2"N 8°52'24.5"W), where they live in syntopy (i.e., occurrence of two species in the same habitat at the same time). This location has limited human disturbance and has lots of vegetation with natural and artificial shelters (e.g., walls of agricultural properties) that can be used by lizards. Ecological adaptation is considered a major factor favoring the isolation between these two species; *P. lusitanicus* lives more on rocks, while *P. bocagei* is ground-dwelling [21]. The diet of these two species is mainly composed by prey belonging to Hemiptera, Coleoptera, Diptera, Hymenoptera and Araneae, with minimal differences between species or sexes [22]. *Podarcis siculus* and *P. virescens* were collected in Lisbon, at Parque das Nações (Fig. 1a, b) (38°76'22.4"N, 9°09'44.3 W), where both live in sympatry (sharing habitat type). This is a highly urbanized area near the Tejo river, characterized by large residential and commercial areas, with considerable daily human disturbance. While *P. virescens* is native to this location, *P. siculus* is an invasive species introduced about two decades ago [23]. Its plasticity in spatial use of habitat, morphology, behaviour, and versatile diet explains its successful colonization of multiple locations outside its native range [24-27]. This invasive species can present a more versatile diet, as it can also consume fruits and nectar [28] and have a more herbivorous diet [e.g. 24], while *P. virescens*

is known to be insectivorous and to feed mainly on individuals of the class Arachnida and the orders Hymenoptera, Hemiptera, Coleoptera and Diptera [29]. Finally, we collected *Teira dugesii* in a nearby area in Lisbon, in the Alcantara docks, close to the city port area (38°70'33.8"N, 9°16'54.1"W). Similar to the other *Podarcis* spp. captured in Lisbon, *T. dugesii* occupies an anthropogenic area, although less busy, close to railway tracks with limited vegetation cover (Fig. 1c). This species is thought to have been accidentally introduced via transport ships from Madeira Island three decades ago, in 1992 [30]. *Teira dugesii* feeds preferentially on insects but also on small fruits [31].

All individuals were captured using nooses. Lizards were carefully immobilized, avoiding any human contact with the cloaca. We quickly inserted a sterile cotton swab into the entrance of the cloaca to obtain individual microbial samples. The tips of the swabs were cut into individual tubes and stored in ice boxes in the field, and then stored at -80°C upon arrival in the laboratory. After the microbial sampling, each lizard was sexed, and the snout-vent length was measured (SVL; from head to cloaca) using a digital caliper (± 0.01mm error).

In the laboratory, we extracted the DNA from the swabs using the DNeasy® PowerSoil® Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA concentration and quality were measured with the Epoch™ Microplate Spectrophotometer (BioTek Instruments, Inc.; United States of America). DNA was shipped in dry ice to the Centre for Microbial Systems at the University of Michigan Medical School (USA) where the V4 region of the 16S rRNA gene (~ 250 bp) of the bacterial communities was amplified for each sample, along with the extraction blanks and PCR controls, following the protocol of Kozich et al. [32]. Amplicons were sequenced in a single Illumina MiSeq run.

All analysis were performed using the R Software v.4.1.1 [33]. Raw FASTQ files were denoised using the DADA2 pipeline [34] in R with the parameters for filtering and trimming: trimLeft = 20, truncLen = c(220,200), maxN = 0, maxEE = c(2,2), truncQ = 2; and the SILVA 138 database [35, 36] was chosen for taxonomic assignment. After quality control and taxonomic assignment, sequences identified as Archaea, Eukaryota, Mitochondria, Chloroplast, as well as sequences unassigned to bacteria were removed from the dataset. An Amplicon Sequence Variant (ASV) frequency table was constructed using the R package *phyloseq* [37] and normalized read counts were obtained using the negative binomial distribution implemented in DESeq2 [38]. ASVs with a count of less than 0.001% of the total number of reads (3586752 [total number of reads]x 0.001% = 36) and that were present in a single sample were also removed.

Bacterial taxonomic alpha-diversity (intra-sample) and beta-diversity (inter-sample) were estimated using the *phyloseq* package. Alpha-diversity was estimated using the number of observed ASVs, and the Shannon, Faith's Phylogenetic Diversity (PD). Beta-diversity was measured using the Bray-Curtis index and the Unifrac phylogenetic weighted and unweighted distances. Principal Coordinate Analysis (PCoA) were used to visually assess dissimilarity among groups.

Statistical differences in alpha-diversity between locality/habitat, species and sex were analyzed using a linear model (lm(alpha-diversity ~ locality + species + sex)). Given the significant effect of locality (which

also corresponded to semi-natural and urbanized habitats) on alpha-diversity (see results section), differences in the proportions of the most abundant taxa at the phyla and genera level (represented by ≥ 1% on average of all sequences) were assessed between species and sex for each locality/habitat separately using a linear model (lm (bacterial phyla/genus ~ species * sex)). The effects of locality, species and sex on microbial beta-diversity were assessed using permutational analysis of variance (PERMANOVA) with 10000 permutations, implemented using the *adonis* function of the R *vegan* package [39] (adonis(beta-diversity ~ local + species + sex)). Correlations between individual size and bacterial alpha-diversity were also tested using the Pearson correlation test for each species, using the *ggpubr* package [40].

Bacterial transmission between each pair of species from sympatric populations living in Moledo and Parque das Nações was estimated using the FEAST software [41], by testing the contribution of each species (source) to the microbial diversity to its sympatric congener (sink). To this end, the non-normalized ASV frequency table was used, and due to differences in the number of samples between *P. bocagei* and *P. lusitanicus*, only a fraction of the individuals of *P. bocagei* was included (with the most similar sex and SVL ratios to the *P. lusitanicus* samples as possible), following the FEAST developers' recommendations to avoid overestimation of transmission.

Results

After filtering, the final ASV table encompassed 3923 unique ASVs, included in a total of 39 bacteria phyla. The most abundant phyla were Firmicutes, Bacteroidota, Actinobacteroidota, Proteobacteroidota and Campylobacterota (Fig. 2)

Gut bacterial alpha-diversity was significantly different between Moledo populations (*P. bocagei* and *P. lusitanicus*) and Lisbon populations (*P. siculus*, *P. virescens* and *T. dugesii*) (Table 1). Lisbon populations, those from an urbanized habitat, showed consistently higher alpha-diversity indices, with *P. siculus* having higher diversity than the native *P. virescens.* (Fig. 3). Microbial beta-diversity was also significantly different between localities when considering the Bray-Curtis (R^2 = 0.186, p < 0.001) and the Unifrac unweighted (R^2 = 0.180, p < 0.001) indices (Fig. 4 and Table 1).

Table 1
Results from the linear models. Effect of locality, species and sex in gut microbial alpha-diversity (F-statistics and respective p-values) and beta-diversity estimates (R^2 and respective p-values). Significant results are depicted in bold.

Alpha- and Beta-diversity measures	Locality	Species	Sex
Shannon	**5.9115**	0.3343	0.0098
	(<0.001)	(0.5645)	(0.9212)
Observed	**19.4695**	0.4168	0.0354
	(<0.001)	(0.5201)	(0.8512)
PD	**18.0354**	0.4897	0.1599
	(<0.001)	(0.4858)	(0.6902)
Bray–Curtis	**0.18602**	0.00731	0.00811
	(<0.001)	(0.7213)	(0.5372)
Unifrac phylogenetic unweighted	**0.18007**	0.00809	0.00730
	(<0.001)	(0.5690)	(0.7898)
Unifrac phylogenetic weighted	**0.10178**	0.00822	0.00632
	(0.0056)	(0.3571)	(0.4612)

Although no differences were found in the proportion of the most abundant phyla between *P. bocagei* and P. *lusitanicus*, significant differences were found in the proportion of the genus *Corynebacterium* (F-statistics = 6.823, p = 0.013) (online resource 1). Differences in the proportion of the most abundant taxa between *P. siculus* and *P. virescens* were found at genus levels for an unidentified genus belonging to the order Gastranaerophilales (F-statistics = 6.324, p = 0.003), *Corynebacterium* (F-statistics = 6.887, p = 0.002), *Kocuria* (F-statistics = 4.639, p = 0.0138), *Staphylococcus* (F-statistics = 6.767, p = 0.002), and *Odoribacter* (F-statistics = 11.609, p = 6.398e-05) (online resource Fig S2). Additionally, for *P. siculus* and *P. virescens*, both species and sex significantly affected the abundance of *Akkermansia* (sex: F-statistics = 5.191, p = 0.026; species: F-statistics = 3.467, p = 0.038) (online resource 2 and 3) and the interaction between species and sex (species*sex) significantly affected the proportion of *Romboutsia* (F-statistics = 3.475, p = 0.038) and *Pseudomonas* (F-statistics = 3.412, p = 0.040) (online resource 3).

Pearson correlation test only showed significantly positive correlations between SVL and bacterial alpha-diversity (for Shannon indice) for males of the in the invasive species *P. siculus* (online resource 4).

Results from FEAST software indicate that the level of bacterial transmission between sympatric species in both populations (Parque das Nações and Moledo) was high. Nevertheless, while between the syntopic *P. lusitanicus* and *P. bocagei* bacterial transmission was balanced in both directions (*P. bocagei* ◊ *P. lusitanicus* ~ 71% on average, and *P. lusitanicus* ◊ *P. bocagei* ~ 69% on average), the other two sympatric

species showed a more biased transmission, with *P. virescens* having a higher contribution towards *P. siculus* (*P. virescens* ◊ *P. siculus* of 72% on average, and *P. siculus* ◊ *P. virescens* of about 55% on average).

Discussion

In this study, we characterized the gut bacterial microbiota of five lizard species from Portugal (the native *Podarcis virescens*, *P. bocagei* and *P. lusitanicus*, and the introduced *P. siculus* and *Teira dugesii*) using a metabarcoding approach. Our results showed that locality was the main predictor of microbial diversity, significantly influencing microbiota composition and structure. Moreover, host sex and size also had an effect, albeit more discrete, in gut bacterial communities. All lizards shared the same most abundant bacteria phyla with results being in accordance with what has been found in other studies in lizards [e.g. 42, 43].

The two habitats in which lizards were captured are very different, with lizards from Lisbon living in an urbanized and artificial habitat, with greater environmental disturbance, compared to lizards from Moledo, which live in a semi-natural habitat. Plausibly, differences in habitat may lead to differences in the composition and diversity of the gut microbiome [44]. We detected higher microbiota diversity in the more urbanized environment which could be explained by the higher variety of diet items, which may also include human food waste. Additionally, environmental microbiota, which could be horizontally transferred to lizards, may also be more diverse in urban habitat than in semi-natural ones [16]. We hypothesize that habitat disturbance, the co-existence with humans and urban animals (such as cats, dogs and rats), may influence dietary behaviour and contribute to a higher bacterial load in the environment, which can then be acquired by lizards. These results agree with those on the Australian water dragon, where the gut microbiome of lizards in urban areas was more diverse compared to those residing in semi-natural habitats [16].

The proportion of some of the most abundant bacterial phyla and genera found in our study differed between lizard species at each locality. Additionally, we found that the species and sex also had an effect in the abundance of some of the gut microbiota components in *P. virescens* and *P. siculus*. The influence of host taxonomy in gut microbiota, which is a proxy not only for host genetics but also its general ecology, has been reported in many animals [45, 46] including reptiles [17]. The influence of sex in the abundance of major bacterial groups, has also been reported in striped Plateau lizards (*Sceloporus virgatus* Smith, 1938), with sex-specific cloaca microbiomes being related to different hormone levels [47]. In our study, the influence of sex was small when compared to the effect of habitat and host taxonomy. Nonetheless, the influence it exerts could also be linked to differences in the ecology of the two sexes. Importantly, the difference in size between female and male lizards may lead to slight differences in feeding behavior, and consequently on the gut microbial diversity.

Interestingly, we found a positive correlation between lizard lenght and alpha bacterial diversity in males from *P. siculus*. This lizard is larger than the other studied species and is also a very successful invasive

species [25, 26]. Indeed, *P. siculus* can be more aggressive than native *Podarcis* species [48], and also more exploratory, bolder, and better at exploiting food resources when compared to the native *P. virescens* in our study location [26, 27]. These behaviours can be associated with the displacement of *P. virescens* from gardens now inhabited by *P. siculus* [49] and can also be leading to a wider ecological and trophic niche, and consequentially to a higher microbiome diversity in *P. siculus*.

Finally, our analysis of potential bacterial transmission between the lizards living in sympatry, indicates a balanced transmission between species at Moledo and unbalanced transmission between species in Lisbon, with the invasive *P. siculus* estimated to receive a higher proportion of bacteria from the native *P. virescens*. These differences in estimates of bacterial transmission may reflect different non-exclusive aspects of the ecology of the hosts, such as the similarity in dietary niches and habitat occupancy (i.e., horizontal bacterial transmission from the environment). Differences in the microbiome of *P. siculus* and *P. virescens* could be related to an increased habitat occupancy and successful adaptation to the environment by the invasive species, which facilitated the acquisition of a higher quantity of local microbiota upon its arrival. These results could also be reflecting an increased ability to exploit a variety of food resources, or most likely a combination of both. The populations of *P. siculus* and *P. virescens* live in sympatry, occupying roughly the same area, but rarely in syntopy, although sightings of these two species within 50 m of each other have been recorded [49, pers. obs.]. On the other hand, both *Podarcis* species from Moledo are considered syntopic and may have greater overlap and similarity in their habitat occupancy. Moreover, is very likely they consume the same or very similar prey items [22], and also encounter each other more frequently. All these factors may explain the much more balanced transmission we found between these two species.

The present study contributes to the existing knowledge on the effects of environmental and host factors on the dynamics of the gut microbiome of lizards. Our results also set the stage for future research exploring the influence of other factors on the microbiome, particularly diet, as well as the use of sympatric *Podarcis* lizards as models to test the effects of behaviour on lizard microbial composition.

Statements & Declarations

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Data Availability: Data will be deposited in NCBI's Short Read Archive (SRA) upon acceptance.

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Figures

Figure 1

Aerial photographs of sampling sites for A) *P. siculus,* B) *P. viridiscens,* C) *Teira dugesii* and D) *P. bocagei* and *P. lusitanicus.* Specific collection areas are delimited by yellow lines). Map data ©2021 Google

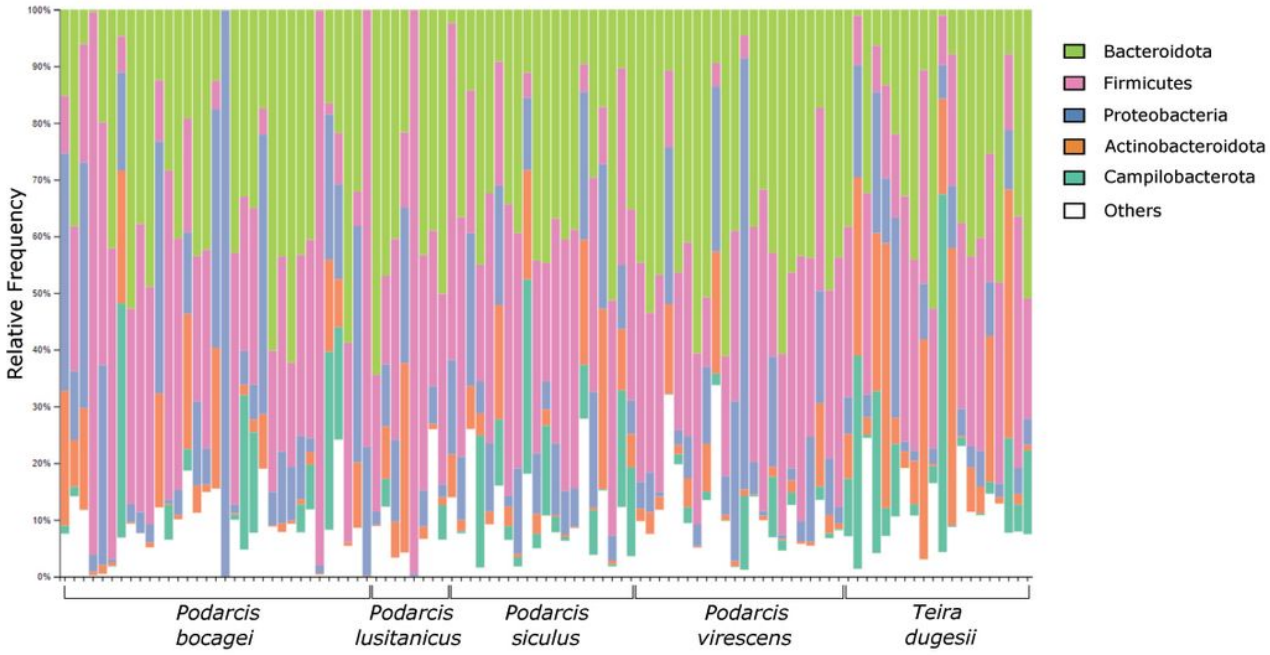

Figure 2

Relative frequency of the most abundant bacterial phyla in the gut microbiome of the studied lizard species

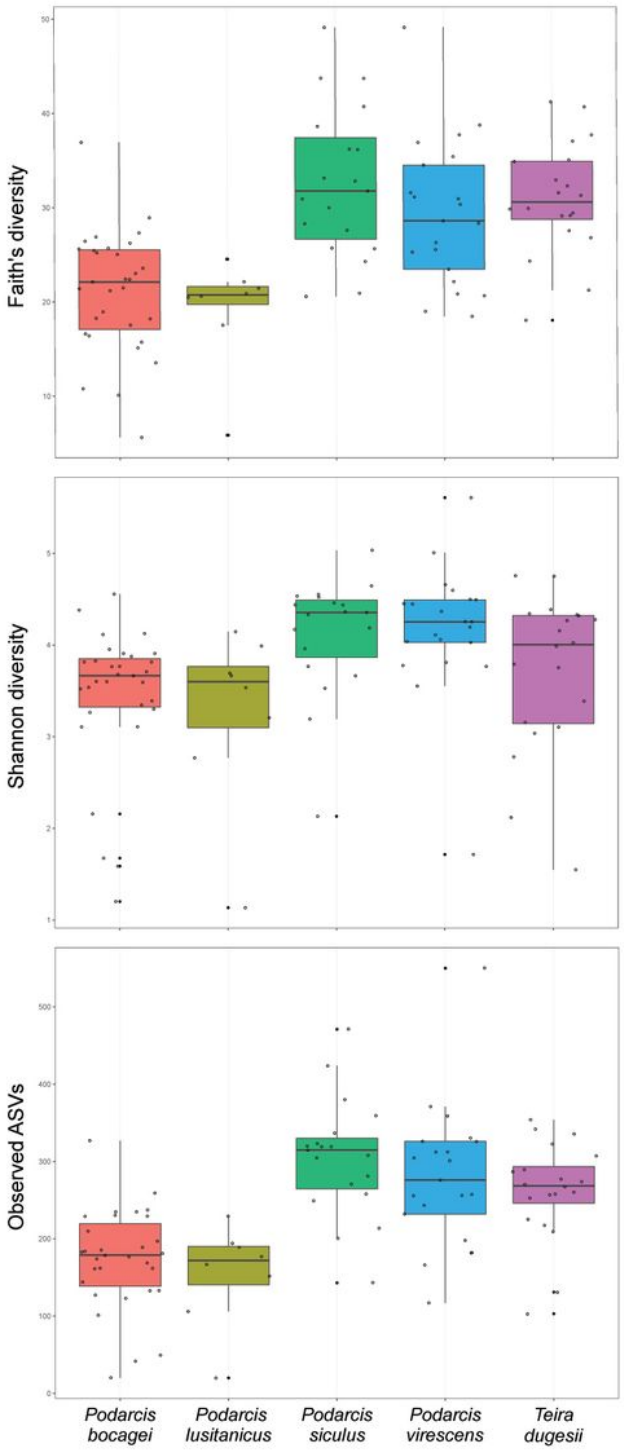

Figure 3

Boxplots of the alpha-diversity indices (Faith's phylogenetic diversity, Shannon diversity and the number of observed ASVs) for the gut microbiome of the studied lizards.

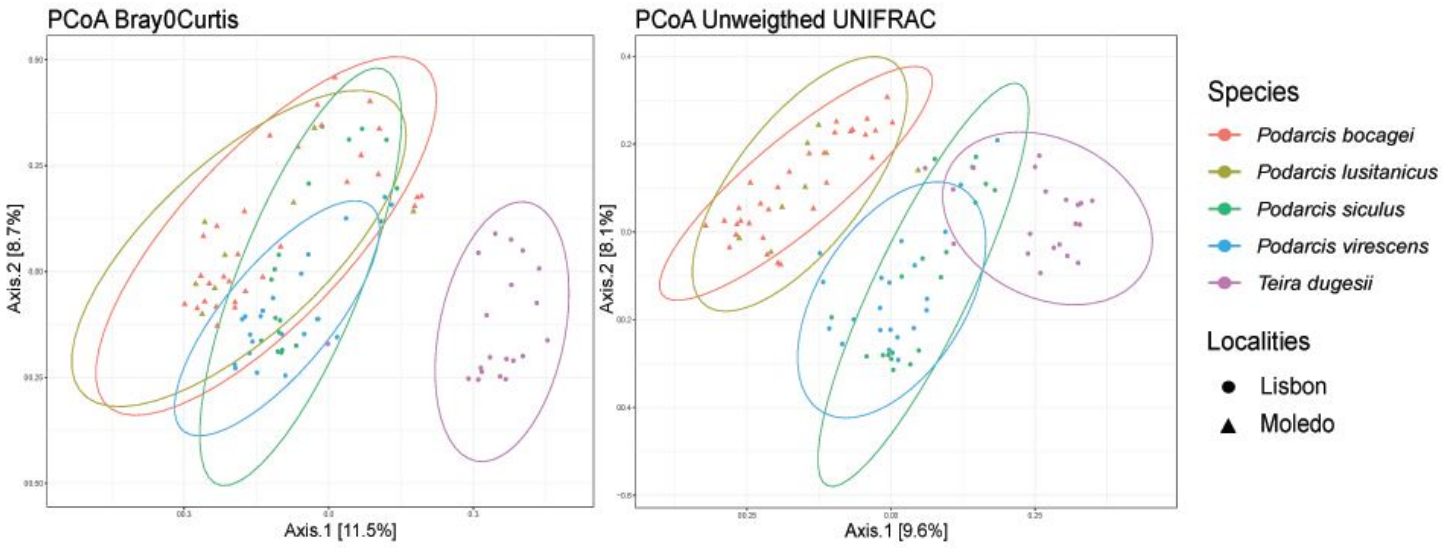

Figure 4

PCoA plots representing Bray–Curtis and Unweighted Unifrac distances, grouped by species with 95% confidence interval ellipse.

Supplementary Files

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