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Does adaptive radiation of a host lineage promote ecological diversity of its bacterial communities? A test using gut microbiota of *Anolis* lizards

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Abstract

Adaptive radiations provide unique opportunities to test whether and how recent ecological and evolutionary diversification of host species structures the composition of entire bacterial communities. We used 16S rRNA gene sequencing of faecal samples to test for differences in the gut microbiota of six species of Puerto Rican *Anolis* lizards characterized by the evolution of distinct 'ecomorphs' related to differences in habitat use. We found substantial variation in the composition of the microbiota within each species and ecomorph (trunk-crown, trunk-ground, grass-bush), but no differences in bacterial alpha diversity among species or ecomorphs. Beta diversity analyses revealed subtle but significant differences in bacterial composition related to host phylogeny and species, but these differences were not consistently associated with *Anolis* ecomorph. Comparison of a trunk-ground species from this clade (*A. cristatellus*) with a distantly related member of the same ecomorph class (*A. sagrei*) where the two species have been introduced and are now sympatric in Florida revealed pronounced differences in the alpha diversity and beta diversity of their microbiota despite their ecological similarity. Comparisons of these populations with allopatric conspecifics also revealed geographic differences in bacterial alpha diversity and beta diversity within each species. Finally, we observed high intraindividual variation over time and strong effects of a simplified laboratory diet on the microbiota of *A. sagrei*. Collectively, our results indicate that bacterial communities are only weakly shaped by the diversification of their lizard hosts due to the strikingly high levels of bacterial diversity and variation observed within *Anolis* species.

Keywords: alpha diversity, beta diversity, ecomorph, molecular OTUs, species diversity

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Introduction

Gut microbiota are essential to the health and development of their hosts (Turnbaugh *et al.* 2006; Qin *et al.* 2010; Hooper *et al.* 2012), yet we know relatively little about the ecological and evolutionary processes that structure these important bacterial communities. Recent comparative studies show that the similarity of gut microbiota among host species often mirrors the host phylogeny, suggesting that hosts and their microbiota co-evolve together (Ley *et al.* 2008; Degnan *et al.* 2012; Amato 2013; Sanders *et al.* 2014). This association between host phylogeny and microbiota could be due to the vertical transmission of bacteria, as observed in the case of codiversification of *Helicobacter pylori* with humans (Falush *et al.* 2003), as well as phylogenetic conservatism in factors such as diet and habitat, which can strongly influence gut microbial communities (Degnan *et al.* 2012; Sanders *et al.* 2014). Adaptive radiations may provide an informative framework in which to simultaneously explore the ecological and evolutionary factors that shape bacterial communities because they

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are comprised of closely related host species that have diverged to fill different ecological niches.

Lizards in the genus *Anolis* represent a classic example of adaptive radiation, having diversified into nearly 400 morphologically and ecologically diverse species that occur throughout Central and South America and islands of the Caribbean (Losos 2009). On the four major islands of the Greater Antilles (Cuba, Jamaica, Hispaniola and Puerto Rico), a key feature of this radiation is the convergent evolution of the same 'ecomorphs' (morphologically and behaviourally similar species that share similar ecological niches, but are not necessarily close phyletically; Williams 1972, 1983) across different islands (Losos *et al.* 1998; Losos 2009). These ecomorphs are classified according to their partitioning of the spatial habitat, and each of the four Greater Antillean islands contains independently derived representatives from most of the six major ecomorph classes: crown-giant, trunk-crown, trunk, trunk-ground, twig and grass-bush (Fig. 1A). Although most anoles are insectivorous dietary generalists, ecomorphs may differ in foraging mode (Losos 2009), and partitioning of trophic resources has been observed both within and among sympatric *Anolis* species (Schoener 1967, 1968; Stamps *et al.* 1997). This, along with the fundamental differences in habitat use that characterize ecomorphs, suggests that anoles may provide an intriguing test of the extent to which the evolutionary and ecological diversification of a host lineage structures the biodiversity of entire bacterial communities.

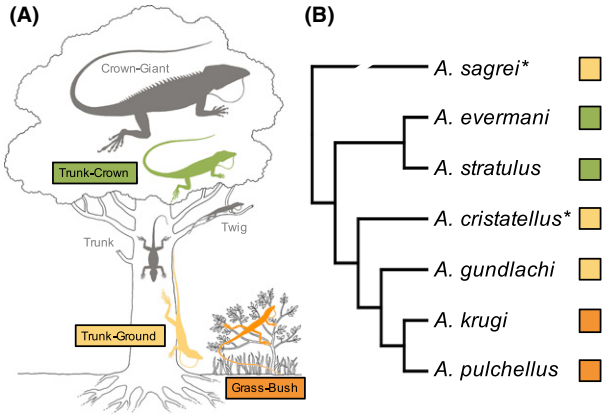

Fig. 1 (A) Illustration of the six *Anolis* ecomorphs, depicting habitat partitioning and major morphological differences (modified from Williams 1983; Losos 2009). (B) Phylogenetic relationships and ecomorph classifications for the seven *Anolis* species sampled in this study, including the 'outgroup' *A. sagrei* (native to Cuba and the Bahamas) and six species from a clade that evolved on Puerto Rico (phylogeny based on Rabosky & Glor 2010). Asterisks indicate the two trunk-ground ecomorphs that have invaded southern Florida from Cuba (*A. sagrei*) and Puerto Rico (*A. cristatellus*).

To explore this idea, we sequenced bacterial 16S rRNA genes from anole faecal samples to compare microbiota across six sympatric *Anolis* species on Puerto Rico. These species are members of a single clade that radiated on Puerto Rico and consist of two sister species classified as trunk-crown ecomorphs (*A. evermanni* and *A. stratulus*), two sister species classified as grass-bush ecomorphs (*A. krugi* and *A. pulchellus*) and two species classified as trunk-ground ecomorphs (*A. cristatellus* and *A. gundlachi*, Fig. 1B). Hence, we predicted that bacterial communities would be more similar within than among these three ecomorph pairs, a difference that could reflect the phylogenetic affinity and/or ecological similarity of each pair. Next, to test whether phylogenetically distant but ecologically similar species differ in their microbiota when compared in sympatry, we capitalized on the invasion of southern Florida by two trunk-ground ecomorphs from different sources in the Greater Antilles: *A. sagrei*, which has been repeatedly introduced to southern Florida from Cuba beginning about 75 years ago (Lee 1985; Kolbe *et al.* 2004), and *A. cristatellus*, which was introduced from Puerto Rico about 40 years ago (Kolbe *et al.* 2012). We also tested for the effects of local environment by comparing these two southern Florida populations with allopatric conspecifics in northern Florida (*A. sagrei*) and Puerto Rico (*A. cristatellus*). Finally, to assess the possible sources of intraspecific variation in microbiota, we conducted two further studies on *A. sagrei*. First, we tested for temporal variation in bacterial communities of free-living individuals by sampling, releasing and resampling the same animals within a week of initial capture. Second, we compared the microbiota of free-living *A. sagrei* to those of individuals that we maintained in captivity for over a year on a simplified diet of domestic crickets. This allowed us to assess both overall dietary effects on the microbiota and the extent of variation among individuals when controlling for any differences in diet.

Materials and methods

Sample collection

Dates, locations and sample sizes for individuals of each species are reported in Table 1. Only samples from adult male anoles were included in our study. We captured wild anoles by hand or noose and immediately placed each individual into an unused plastic sandwich bag, where it was held overnight at ambient temperature. The following day, we transferred a faecal pellet from the bag of each individual into a microcentrifuge tube, immediately froze each pellet at −20 °C and kept samples on ice (during transportation) or at −20 °C

Table 1 Collection dates and localities for wild anoles from Puerto Rico and Florida. Sample sizes are reported for the total number of faecal samples before and after rarefaction to exclude samples with low sequencing coverage (i.e. <3000 reads per sample)

Location	Species	Number of samples	Number after rarefaction	Collection date	Comparison
Puerto Rico	*A. stratulus*	17	17	June 2014	Ecomorph
Puerto Rico	*A. evermani*	15	15	June 2014	Ecomorph
Puerto Rico	*A. krugi*	12	12	June 2014	Ecomorph
Puerto Rico	*A. pulchellus*	10	9	June 2014	Ecomorph
Puerto Rico	*A. gundlachi*	13	13	June 2014	Ecomorph
Puerto Rico	*A. cristatellus*	16	15	June 2014	Ecomorph, allopatry
Miami, FL (southern FL)	*A. cristatellus*	13	8	May 2014	Sympatry, allopatry
Miami, FL (southern FL)	*A. sagrei*	11	8	May 2014	Sympatry, allopatry, captive/wild
Palm Coast, FL (northern FL)	*A. sagrei*	17	12	July 2015	Allopatry, repeatability, captive/wild
Laboratory	*A. sagrei*	12	12		Captive/wild
Laboratory	Cricket	1	1		Captive/wild

until DNA extraction. Because we did not collect faecal pellets immediately upon defecation, it is possible that bacterial composition may have changed prior to preservation and that the variation among samples (but not among species or populations, which were sampled in identical fashion) may reflect unmeasured variation in the time between defecation and preservation. Bacterial communities sampled from faeces are often qualitatively similar to those sampled directly from the gut or cloaca, although they may differ quantitatively (Colston *et al.* 2015; Stanley *et al.* 2015), so we use faecal samples as a proxy for gut microbiota while acknowledging these caveats.

For *A. sagrei* individuals that we recaptured to assess individual repeatability of the microbiota, we used toe clips to identify each individual between captures. For *A. sagrei* individuals that we maintained on a controlled diet in captivity, we initially collected wild adults from Great Exuma, Bahamas (23°29′N, 75°45′W), and transported them to the University of Virginia. We maintained these anoles individually in small, plastic terraria (40 × 23 × 32 cm; Lee's Kritter Keeper, San Marcos, CA) containing a potted plant, carpet substrate and PVC tube for perching and hiding. We maintained constant 29 °C diurnal temperature and 65% relative humidity, 13L:11D (breeding season) or 12L:12D (non-breeding season) photoperiod and placed each cage under two ReptiSun 10.0 UVB bulbs (ZooMed, San Luis Obispo, CA) that were illuminated during the light phase. Three times per week, we offered captive anoles 5–7 crickets (*Gryllodes sigillatus*; Ghann's Cricket Farm, Augusta, GA), dusted weekly with Fluker's Reptile Vitamin and Calcium supplements (Fluker's Cricket Farms, Port Allen, LA) and maintained on a diet of carrots, kale, sweet potatoes and apples. We maintained these wild-caught anoles in captivity on this diet for over a year before collecting the faecal samples. To assess the bacterial communities associated with crickets as a food source, we froze and homogenized 15 whole crickets, then extracted DNA from this homogenate.

DNA extraction and 16S rRNA gene sequencing

We extracted DNA in a 96-well format using ZR-96 Fecal DNA Kits (Zymo Research, Orange, CA) following the manufacturer's protocol, then amplified the V1-V3 hypervariable regions of the 16S rRNA gene using two primers containing the universal sequences 27F (5′-AGRGTTTGATCMTGGCTCAG-3′) and 534R (5′-TTACCGCGGCTGCTGGCAC-3′). We added a unique 8-bp barcode to each primer to tag the samples and used a 50-μL reaction mixture for each PCR amplification by QIAGEN Taq polymerase (Qiagen Inc, CA). PCR conditions consisted of 94 °C for 3 min, followed by 25 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 60 s, with a final extension of 5 min at 72 °C. We quantified 16S rRNA amplicons from different samples, pooled them in equal molar ratios, then gel-purified and sequenced them on an Illumina MiSeq platform using the 300-bp paired-end (PE) protocol. We performed all liquid transfer steps on a Biomek NXp liquid handling station (Beckman-Coulter Inc., Fullerton, USA).

Sequence processing, quality control and OTU classification

We filtered the resulting sequences according to base quality using TRIMMOMATIC 0.32 with settings of LEADING = 3, SLIDING WINDOW = 10:20 and MINLEN = 50 (Bolger *et al.* 2014). Paired-end reads passing the quality filter were merged using FLASH (-r 301 -f 447 -s 45 -x 0.05) (Magoč & Salzberg 2011). The successfully merged

reads were assigned to samples by barcodes and processed using the QIIME pipeline (Caporaso *et al.* 2010). We identified chimeric sequences using usearch (Edgar *et al.* 2011) implemented in QIIME with both de novo and reference-based detection algorithms, retaining only those sequences that were flagged as nonchimeras with both detection methods. We removed non-16S rRNA sequences using hmmsearch (Eddy 1998) against a custom-made 16S rRNA gene model and removed mitochondrial and chloroplast sequences using the Ribosomal Database Project (RDP) classifier (Wang *et al.* 2007). We clustered the remaining reads to operational taxonomic units (OTUs) using the centroid-based UCLUST algorithm (Edgar 2010) with a 97% identity threshold, then selected the most abundant sequence of each OTU as the representative sequence using the RDP classifier.

Analysis of microbiota

To remove heterogeneity due to sequencing effort, we rarefied samples to 3000 reads. This resulted in the removal of 15 samples and produced a final data set of 121 samples (Table 1). Rarefaction curves for each species or population are presented in Fig. S1 (Supporting information). Good's coverage estimates averaged 0.97 ± 0.02 (range: 0.84–0.99) for all samples (species means ranged from 0.96 to 0.98) following rarefaction to 3000 reads, indicating that the majority of the bacterial community was captured at this level of rarefaction. We rarefied the Florida subset (*A. cristatellus* and *A. sagrei* from northern and southern Florida) to 1000 reads to increase the number of samples in the analysis, which added seven samples that were excluded from the 3000-read data set (mean Good's coverage estimate = 0.94 ± 0.03). A Mantel test correlating distance matrices for the 1000 and 3000-read data sets revealed a high level of congruence (Jaccard distance: $r = 0.90$, $P = 0.001$; unweighted UniFrac: $r = 0.85$, $P = 0.001$). We calculated descriptive and comparative statistics for microbiota using QIIME 1.9.1, unless otherwise specified.

We calculated two measures of alpha diversity (Chao1 and Shannon indices) for each sample using rarefied OTU tables. The Chao1 index estimates species richness based on the number of observed OTUs in each sample using the formula:

$$S_{\text{chao1}} = S_{\text{obs}} + \frac{n_1(n_1 - 1)}{2(n_2 + 1)}$$

where S_{chao1} is the estimated richness, S_{obs} is the observed number of species, n_1 is the number of singleton taxa (taxa represented by a single read in that community), and n_2 is the number of doubleton taxa. If a sample contains many singletons, it is likely that more undetected OTUs exist, and the Chao 1 index will estimate greater species richness than it would for a sample without rare OTUs. The Shannon index combines species richness (the number of OTUs) and evenness (the relative abundance of different OTUs) to produce a summary measure of species diversity. We tested for the differences in Chao1 and Shannon indices among Puerto Rican anoles using two-way ANOVA with the measure of alpha diversity as the dependent variable and ecomorph and species (nested within ecomorph) as independent variables. Depending on the distribution of data, we used both ANOVA and nonparametric Kruskal–Wallis tests with post hoc comparisons to assess the differences in alpha diversity among sympatric and allopatric populations of *A. cristatellus* and *A. sagrei*. We used *t*-tests to assess the differences in mean alpha diversity and Brown–Forsythe tests to compare the variance in alpha diversity between *A. sagrei* maintained in captivity on a controlled diet vs. those from wild populations.

To compare beta diversity among samples, we first excluded any OTUs that were only represented by a single sequence read. We then performed principal coordinate analysis (PCoA) on Jaccard, unweighted UniFrac and weighted UniFrac distances (Lozupone & Knight 2005) calculated using 97%-similarity OTUs. Jaccard distance measures the dissimilarity of two communities based on bivariate classifications of the presence or absence of microbial OTUs. UniFrac distance takes phylogenetic relationships among OTUs into account, and weighted UniFrac distance further considers the relative abundance of OTUs. For each of these metrics, we calculated pairwise distances between all individual samples and then classified each pairwise distance as occurring within a given group (species, ecomorph, population) or between two groups. We then (i) tested for the differences in within-group distances among different groups, (ii) tested for the differences in between-group distances among different pairwise combinations and (iii) compared within- and between-group distance to one another using nonparametric Mann–Whitney tests or Kruskal–Wallis tests with post hoc Dunn's multiple comparisons tests. To test whether the host phylogeny explained the variation in microbiota, we used Mantel tests implemented in QIIME to assess congruence between bacterial community dissimilarities and host genetic distances, which we calculated from branch lengths in the *Anolis* phylogeny of Rabosky & Glor (2010). Rare OTUs can confound detection of meaningful patterns in bacterial diversity (e.g. Colston *et al.* 2015), so in addition to using measures of community dissimilarity weighted by OTU abundance (e.g. weighted UniFrac distance), we repeated the analyses

described above with a data set in which we excluded the rarest 5% of OTUs from each sample.

Results

General patterns in Anolis microbiota

We analysed bacterial composition in 121 anole faecal samples (Table 1). From these samples, we generated a total of 2 662 283 high-quality reads, yielding a median of 15 157 reads per sample. Bacterial communities of anoles were complex, averaging 105 unique 97% OTUs per 1000 sequences. Species richness estimated by Chao1 varied substantially among samples from 22 to 1106 (mean = 209, SD = 137), and the Shannon index also varied substantially from 0.14 to 6.57 (mean = 3.63, SD = 1.60). We also observed considerable variation in bacterial composition, such that only eight OTUs were shared by >50% of wild anole samples (three *Bacteroides* spp., one *Citrobacter* sp., *Clostridium perfringens*, *Eubacterium dolichum* and two unclassified taxa in Peptostreptococcaceae and Lachnospiraceae). The average Jaccard distance between pairs of samples collected in the wild was 0.93, which means that, on average, any two samples only shared 7% of their bacterial OTUs. Exclusion of the rarest 5% of OTUs lowered the average Jaccard distance to 0.91. Taxonomic assignment revealed representatives from 22 bacterial phyla and 251 genera (Fig. 2; Appendix S2, Supporting information). The vast majority of sequences (95%) belonged to the bacterial phyla Firmicutes (61.1% of reads), Proteobacteria (19.1%) and Bacteroidetes (14.8%). Of the 251 genera identified, the most abundant were *Bacteroides* (10.1%), *Citrobacter* (9.6%), *Clostridium* (8.8%), *Lactococcus* (4.6%), *Parabacteroides* (3.0%), *Eubacterium* (2.5%), *Enterococcus* (2.3%), *Bacillus* (2.2%), *Dorea* (2.1%), *Blautia* (2.0%), *Staphylococcus* (1.7%) and *Enterobacter* (1.6%).

Comparison of ecomorphs on Puerto Rico

Microbiota were highly variable both within and among Puerto Rican species and ecomorphs when compared at the level of bacterial phylum, family and genus (Fig. 2). We found no differences in alpha diversity among ecomorphs or species (nested within ecomorph) when assessed using either the Chao1 index (ecomorph: $F_{2,75} = 0.64$; $P = 0.53$; species: $F_{3,75} = 0.26$, $P = 0.85$; Fig. 3A) or the Shannon index (ecomorph: $F_{2,75} = 0.15$, $P = 0.86$; species: $F_{3,75} = 0.26$; $P = 0.85$; Fig. 3B). Moreover, visual inspection of PCoA plots revealed no obvious clustering of bacterial beta diversity by ecomorph or species (Fig. 3C,D). When comparing within- and between-group Jaccard and UniFrac distances, we found slightly but significantly lower distances within species than between species (Fig. 3E,F), although distances within ecomorphs were equivalent to distances between ecomorphs (after removing all within-species comparisons; Fig. S3, Supporting information). These results were consistent irrespective of whether we included or excluded two species (*A. gundlachi* and

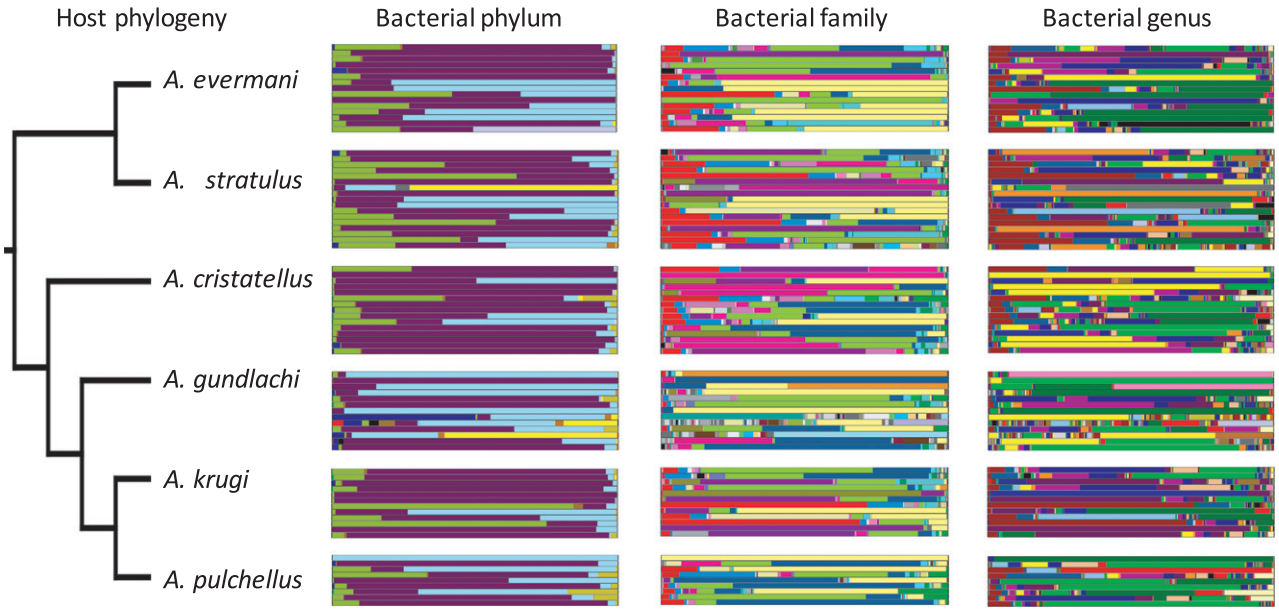

Fig. 2 Diversity of *Anolis* gut microbiota as a function of host phylogeny. Each thin horizontal bar represents an individual lizard, with bacterial diversity (the proportion of reads) coded at phylum, family and genus (see Appendix S2, Supporting information for key to microbial taxa).

A. pulchellus; Fig. S3, Supporting information) exhibiting high within-species distances that also gave rise to high between-species distances (Fig. S4, Supporting information). Accounting for OTU abundance using weighted UniFrac distances tended to homogenize within-species distances across species and reduce the difference in within- vs. between-species distances (Fig. 3F), indicating that rare OTUs were driving differences in microbiota among species. Removal of the rarest 5% of bacterial OTUs had a similar effect in reducing the difference in within- vs. between-species distances (Fig. S5, Supporting information), but all other patterns in beta diversity remained essentially unchanged when excluding rare OTUs (Fig. S5, Supporting information). Mantel tests revealed a weak but significant association between genetic distances calculated from the *Anolis* phylogeny and both Jaccard ($r = 0.1299$; $P < 0.001$; excluding rare OTUs: $r = 0.1291$; $P < 0.001$) and unweighted UniFrac distances ($r = 0.1135$; $P < 0.001$; excluding rare OTUs: $r = 0.1068$; $P < 0.001$), indicating a weak effect of host phylogeny on composition of the microbiota.

Comparison of convergent ecomorphs in sympatry and allopatry

Comparison of two distantly related trunk-ground ecomorphs (*A. cristatellus* and *A. sagrei*) in sympatry and allopatry revealed pronounced effects of species and location on alpha diversity and beta diversity of their bacterial communities. In southern Florida, where both species are sympatric, *A. cristatellus* exhibited a higher Chao1 index ($F_{1,20} = 6.92$; $P = 0.016$; Fig. 4B), but the two species did not differ in Shannon indices ($F_{1,20} = 0.14$; $P = 0.710$; Fig. 4C). PCoA plots for these sympatric populations revealed distinct clustering of beta diversity by species (Fig. 4D). Within-species distances for *A. cristatellus* were significantly lower than

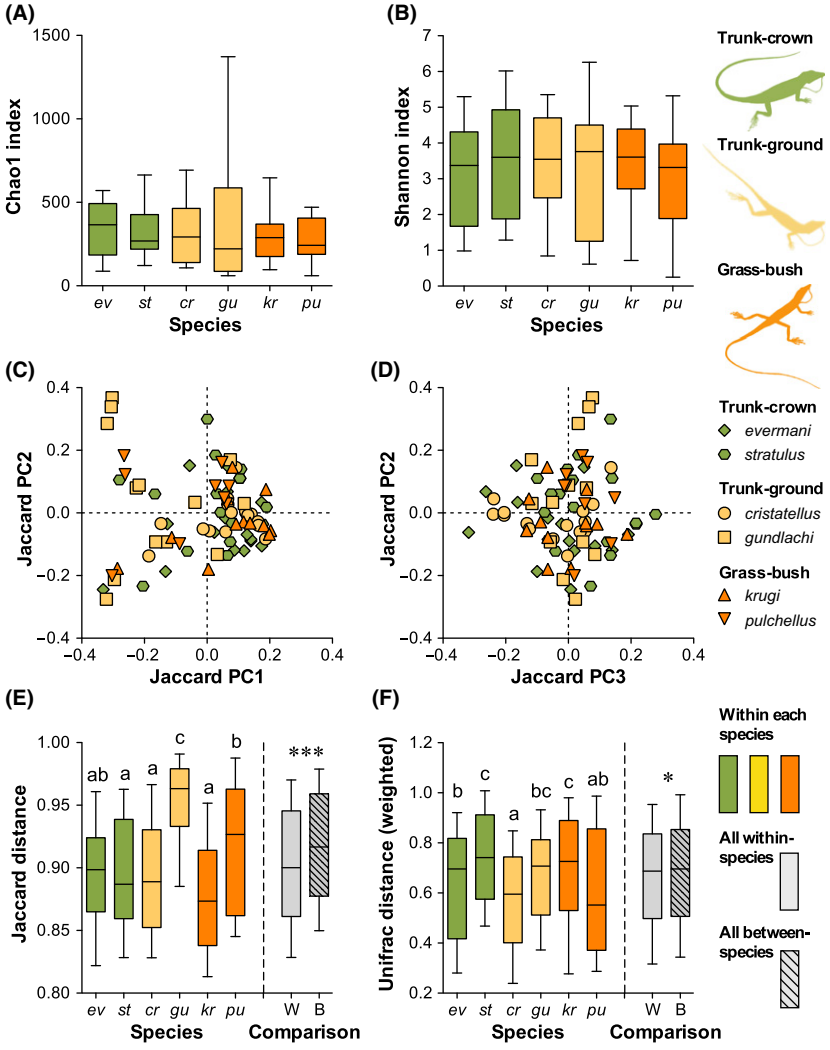

Fig. 3 (A, B) Box-and-whisker plots illustrating medians, interquartiles and ranges of bacterial alpha diversity expressed using (A) Chao1 and (B) Shannon indices for each host species, with bacterial OTUs assigned at 97% sequence similarity. (C, D) Distribution of gut microbiota across individual lizards, coded by species and ecomorph, as a function of the first three principle coordinate axes based on Jaccard distances. (E, F) Box-and-whisker plots (medians, interquartiles and 10–90% percentiles) for within-species (E) Jaccard, and (F) weighted UniFrac distances (coloured boxes) alongside comparisons of all within-species (W) to all between-species (B) distances (grey boxes). Letters denote statistical separation based on post hoc Dunn's multiple comparison tests. Asterisks indicate statistical significance at $P < 0.0001$ (***) and $P < 0.05$ (*).

those for *A. sagrei* when measured as Jaccard distances (Fig. 4G) and as unweighted UniFrac distances ($P < 0.0001$; data not shown), but not as weighted UniFrac distances (Fig. 4G), indicating that species differences were driven in part less abundant bacterial OTUs. Nonetheless, these patterns persisted even after exclusion of the rarest 5% of OTUs. Comparison of within- and between-species distances revealed that *A. cristatellus* individuals were more similar to one another than to heterospecific *A. sagrei* individuals (Fig. 4G).

In allopatric comparisons, the population of *A. cristatellus* from southern Florida exhibited a higher Chao1 index ($F_{1,27} = 10.97$; $P = 0.002$; Fig. 4B) and Shannon index ($F_{1,27} = 13.14$; $P = 0.001$; Fig. 4C) than the population from Puerto Rico. Likewise, the population of *A. sagrei* from southern Florida exhibited a marginally

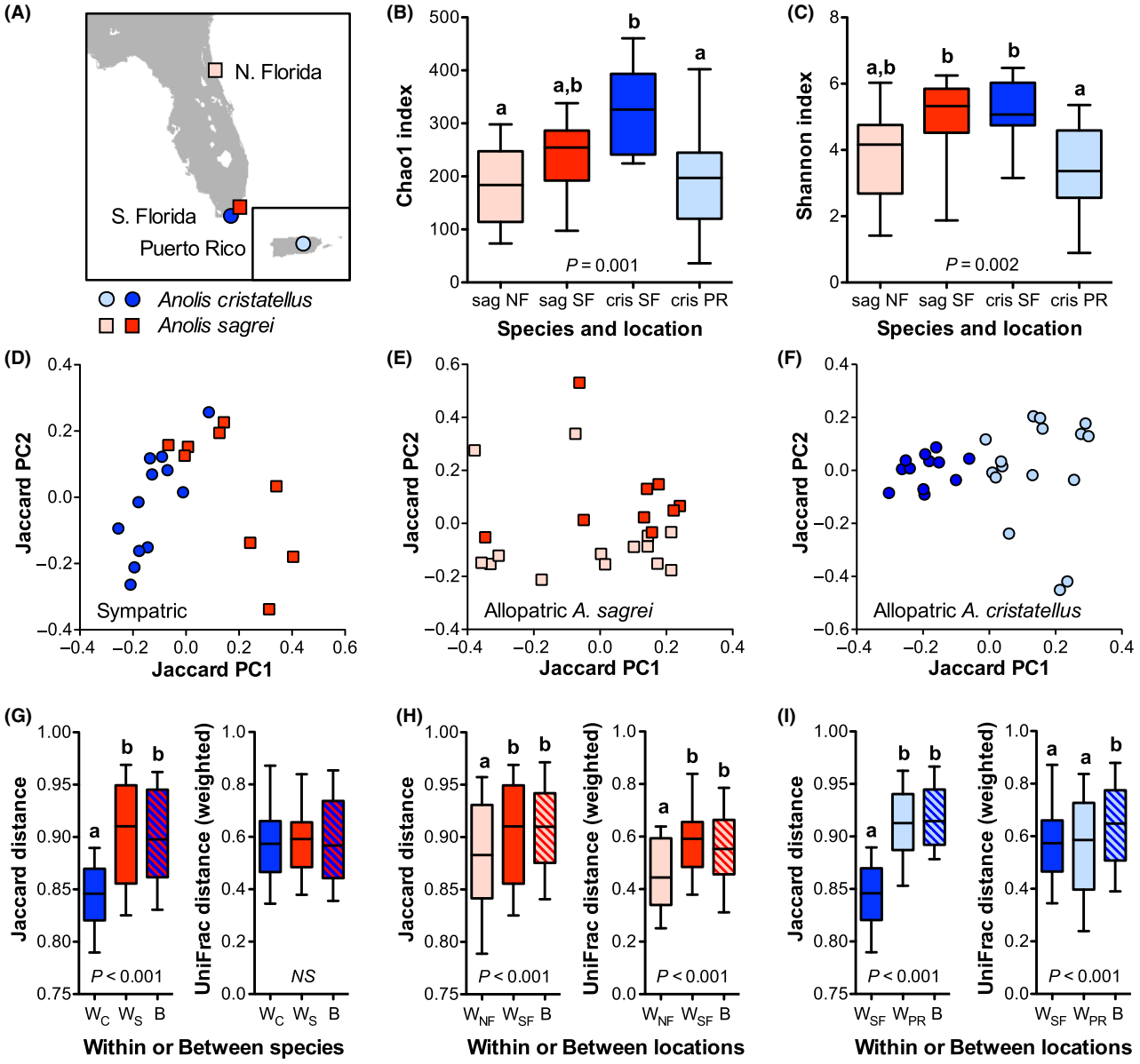

Fig. 4 (A) Locations of sympatric and allopatric populations of *Anolis cristatellus* and *A. sagrei*. (B, C) Box-and-whisker plots (medians, interquartiles, 10–90% percentiles) for two measures of alpha diversity within each population: (B) Chao1 index and (C) Shannon index. (D–F) Principal coordinate analyses for (D) sympatric populations of both species, (E) allopatric populations of *A. sagrei* and (F) allopatric populations of *A. cristatellus*. (G, H) Box-and-whisker plots (medians, interquartiles, 10–90% percentiles) for within- (solid boxes) and between-population (lined boxes) pairwise comparisons using Jaccard distance (left panels) and weighted UniFrac distance (right panels). Significance values are from nonparametric Kruskal–Wallis tests, and lowercase letters indicate post hoc separation based on Dunn's multiple comparison tests.

higher Chao1 index ($F_{1,22}$ = 4.33; P = 0.049; Fig. 4B) and Shannon index ($F_{1,22}$ = 3.44, P = 0.077; Fig. 4C) than the population from northern Florida, although these minor population differences in *A. sagrei* were not significant following correction for multiple comparisons (Fig. 4B, C). PCoA plots revealed pronounced clustering by geographic location in *A. cristatellus* (Fig. 4F), but not in *A. sagrei* (Fig. 4E). Regardless of the measure of beta diversity, *A. sagrei* individuals from northern Florida were more similar to one another than to individuals from southern Florida, whereas *A. sagrei* individuals from southern Florida were no more similar to one another than to conspecifics from northern Florida (Fig. 4H). Likewise, *A. cristatellus* individuals from southern Florida were more similar to one another than to individuals from Puerto Rico, whereas individuals from Puerto Rico were no more similar to one another than to conspecifics from southern Florida when using Jaccard distances (Fig. 4I). Only when using weighted UniFrac distances in *A. cristatellus* (Fig. 4I) did we observe a consistent tendency for conspecifics within each population to resemble one another more strongly than they resembled conspecifics from another population. Patterns in beta diversity for allopatric comparisons remained essentially unchanged when excluding the rarest 5% of OTUs.

Temporal variation in individual gut microbiota

Resampling of five *A. sagrei* individuals from northern Florida within a week of their initial capture revealed pronounced intraindividual variation in bacterial composition at the phylum and genus levels (Fig. S6, Supporting information). Analyses of beta diversity revealed that within-individual differences in bacterial OTUs were consistently lower than between-individual differences for Jaccard (mean within = 0.59, mean between = 0.88), unweighted UniFrac (0.46, 0.65) and weighted UniFrac distances (0.31, 0.48). Nonetheless, on average, only 72% of the same bacterial phyla and 53% of the same bacterial genera were present at both time points in any individual anole (average Jaccard distance = 0.28 at phylum level, 0.47 at genus level; Fig. S6, Supporting information).

Comparison of wild and captive anoles

Comparison of microbiota between wild *A. sagrei* and captive *A. sagrei* maintained in the laboratory on a simplified diet of domestic crickets revealed higher Shannon indices in wild anoles ($F_{1,23}$ = 8.82, P = 0.007), but no difference in Chao1 indices ($F_{1,23}$ = 2.186, P = 0.153). Individuals maintained in the laboratory also exhibited less variance in bacterial diversity relative to free-living *A. sagrei* (Brown–Forsythe test for unequal variances in Shannon indices: $F_{1,22}$ = 5.79: P = 0.025). PCoA revealed a clear separation of laboratory and free-living *A. sagrei* along PC1, although captive anoles still harboured microbiota that were distinct from their cricket diet (Fig. 5A). Within-group distances were lower in the laboratory than in free-living anoles, irrespective of whether they were calculated as Jaccard or UniFrac distances, and within-group distances were lower than between-group distances using Jaccard and unweighted UniFrac metrics (Fig. 5B).

Discussion

We examined 121 anoles representing seven species from Puerto Rico and Florida to test whether and how the ecological and evolutionary diversification of a host lineage influences the composition of its bacterial communities. We found that populations and species differed subtly in both alpha diversity and beta diversity of their microbiota and that species differences in beta diversity were associated with genetic distances estimated from the host phylogeny, as observed in other taxa (Yildirim *et al.* 2010; Degnan *et al.* 2012; Sanders *et al.* 2014). Nonetheless, the major finding to emerge from our study is that bacterial diversity and intraspecific variation in community composition are strikingly high for the microbiota of *Anolis* lizards. On average, any two conspecific anoles from Puerto Rico shared only 10% of their bacterial OTUs, and <1% of OTUs appeared in >50% of all individual samples. Moreover, the differences in bacterial composition between conspecific individuals were generally comparable to those between heterospecific individuals. In comparison, nestmates of turtle ants (genus *Cephalotes*) shared an average of 34% of their bacterial OTUs and this consistency in gut microbiota extended from the colony to the genus level of the host (Sanders *et al.* 2014). Accordingly, in turtle ants, there was a strong correlation between gut microbiota and the host phylogeny (Mantel test $r \approx 0.5$) (Sanders *et al.* 2014). In contrast, we found that the association between bacterial composition and host phylogeny was much weaker in a clade of *Anolis* lizards from Puerto Rico (Mantel test $r \approx 0.1$), presumably due to high bacterial variation within each species, rather than low variation across species (e.g. Fig. 2; Appendix S2, Supporting information).

In other host species, changes in habitat, such as those induced by deforestation, can significantly alter gut microbiota on relatively short ecological timescales (Amato *et al.* 2013). Over longer evolutionary timescales, broad convergence in gut microbiota has been documented in association with the evolution of

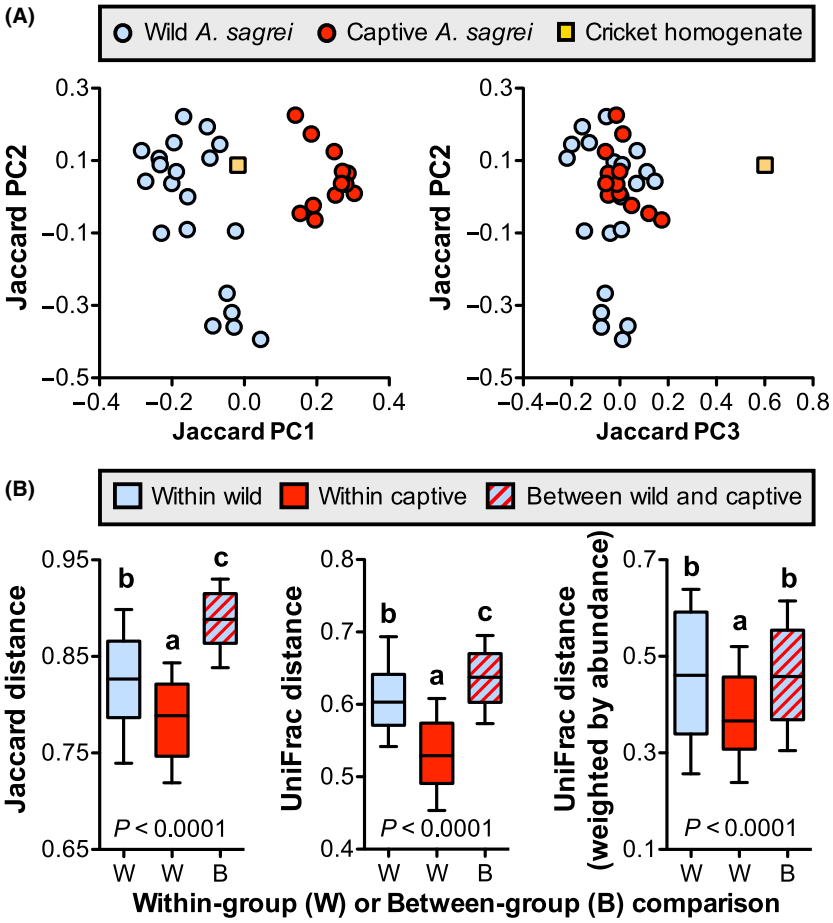

Fig. 5 (A) Principal coordinate analyses comparing gut microbiota of wild *Anolis sagrei* individuals to captive *A. sagrei* individuals maintained on a controlled diet of domestic crickets, alongside the bacterial community of a homogenate prepared from this food source. (B) Box-and-whisker plots (medians, interquartiles, 10–90% percentiles) for pairwise distances (Jaccard, unweighted UniFrac and weighted UniFrac) calculated between individual lizards and binned into within- and between-group comparisons. Significance values are from Kruskal–Wallis tests, and lowercase letters denote post hoc separation based on Dunn's multiple comparison tests.

myrmecophagy across several mammalian lineages (Delsuc *et al.* 2014), and other broad dietary classifications (e.g. carnivory, herbivory, omnivory) also explain significant variation in mammalian gut microbiota (Ley *et al.* 2008; Muegge *et al.* 2011; Delsuc *et al.* 2014). Likewise, gut microbiota of fruit-feeding *Drosophila* species differ from those of flower-feeding species (Chandler *et al.* 2011). These examples of habitat and diet shaping gut microbiota on ecological and evolutionary timescales stand in contrast to our finding that microbiota of *Anolis* lizards did not differ in any obvious fashion with respect to host ecomorph. Moreover, sympatric members of the same ecomorph class often differed in bacterial beta diversity, despite their presumed similarity in habitat use (Figs 3 and 4D,G). Major divergences in the evolution of this *Anolis* clade on Puerto Rico likely date back millions to tens of millions of years (Brandley & de Queiroz 2004; Losos 2009). Therefore, the lack of obvious separation of *Anolis* microbiota by ecomorph is unlikely to be the result of an insufficient evolutionary timescale for divergence.

We suggest that the lack of clear separation of microbiota by ecomorph is most likely due to high intraspecific variation in bacterial gut communities, which may in turn be due to a tendency for *Anolis* species to function as dietary generalists, despite their spatial segregation with respect to habitat niches (Losos 2009). Detailed analyses of stomach contents from Puerto Rican anoles reveal that most species eat a wide variety of arthropod taxa and other food items (e.g. snails, seeds), often with no particular prey item consistently dominating the diet (Wolcott 1923; Lister 1981; Losos 2009). More recently, sequencing of arthropod 16S DNA from faecal samples, which is analogous to our approach for bacterial 16S DNA, was used to characterize the diet of *Anolis sagrei* (Kartzinel & Pringle 2015). This approach revealed a diverse diet containing at least 217 molecular OTUs from nine arthropod orders, but only three of these OTUs were frequent enough to occur in >50% of the individuals sampled, whereas 180 were found in <5% of the individuals sampled (Kartzinel & Pringle 2015). This is analogous to our observation that only 19 (0.7%) of 2722 bacterial OTUs occurred in >50% of the individuals we sampled on Puerto Rico, whereas 2092 (77%) OTUs were found in <5% of individual anoles. If representative of other

Anolis species, this high degree of variation in diet (Kartzinel & Pringle 2015) may help explain why faecal microbiota appear so variable among individual anoles.

Our comparison of two convergent trunk-ground anoles (*A. cristatellus* and *A. sagrei*) that are recently sympatric (i.e. within about 40 generations, Kolbe *et al.* 2012) in southern Florida illustrates that even ecologically similar species that share the same environment can differ substantially in their microbiota. Despite the broad ecological convergence between these two species, their lineages likely diverged over ten million years ago (Brandley & de Queiroz 2004; Losos 2009). This suggests that, over longer evolutionary timescales, *Anolis* evolution could more strongly impact the diversification of their microbiota, potentially via genetic divergence in host digestive and immune physiology. However, we cannot exclude the possibility that the differences in contemporary microbiota between these two invasive species could simply reflect the persistence (over approximately 40–80 generations) of distinct bacterial communities from the different source islands (Puerto Rico and Cuba) on which these two species evolved and from which they invaded southern Florida. However, in the absence of a clear mechanism for vertical transmission of microbiota from mother to offspring, this seems unlikely. We also found that geographic location had a significant effect on microbiota when comparing conspecific populations of these same two species in allopatry (Fig. 4). This may be due to the availability of different food sources across different local environments, as well as geographic variation in numerous other environmental factors, such as biotic habitat, rainfall and microclimate.

Although we cannot directly assess the role of diet in structuring geographic variation in *Anolis* microbiota, we did observe considerable temporal variation when resampling the same free-living individuals within a few days of initial capture (Fig. S5, Supporting information). In similar fashion, pronounced temporal variation in the microbiota of individuals was also observed in faecal samples from wild baboons (Ren *et al.* 2015). In Burmese pythons, bacterial diversity and community composition in the gut changed rapidly and dramatically within hours to days of feeding, and these changes were primarily due to shifts in the abundance of endogenous gut bacteria, rather than the introduction of new bacteria from the rodent meal (Costello *et al.* 2010). This agrees with our observation that captive anoles maintained on a simplified diet of domestic crickets retained microbiota that were distinct from the bacterial composition of their prey. We also found that these same captive *A. sagrei* had higher alpha diversity and lower variance in alpha diversity and beta diversity of their bacterial communities, relative to wild conspecifics. This is similar to the differences observed between laboratory and wild populations of fruit flies (Chandler *et al.* 2011). Nonetheless, we cannot definitively attribute these differences between captive and wild anoles to diet per se, as they could reflect numerous other differences between the laboratory and natural environments.

Our analysis of 121 individuals representing seven *Anolis* species provides the most comprehensive study to date of faecal or gut bacterial diversity in any reptile lineage (Costello *et al.* 2010; Hong *et al.* 2011; Lankau *et al.* 2012; Colston *et al.* 2015; McLaughlin *et al.* 2015). *Anolis* microbiota were dominated by the phyla Firmicutes (61.1% of reads), Proteobacteria (19.1%) and Bacteroidetes (14.8%), which collectively accounted for 95% of the reads we detected (Fig. 2A; Appendix S2, Supporting information). This is broadly similar to the patterns in mammals (Ley *et al.* 2008), and with the exception of alligators (Keenan *et al.* 2013), the gut microbiota of other reptiles also appear to be consistently dominated by Firmicutes and Bacteroidetes (Colston *et al.* 2015; Wehrle 2013; Costello *et al.* 2010; Hong *et al.* 2011; Yuan *et al.* 2015), although Proteobacteria range from the dominant microbial taxon in some studies (McLaughlin *et al.* 2015; Martin *et al.* 2010) to minor components of the reptile gut in others (Costello *et al.* 2010; Hong *et al.* 2011; Yuan *et al.* 2015). It will be illuminating to see whether future studies of reptiles and other ectothermic vertebrates reveal comparably high levels of intraspecific variation in gut microbiota. Despite this variation, we found a significant correlation between bacterial diversity and *Anolis* phylogeny, suggesting that the recent adaptive radiation of this host lineage on Puerto Rico has weakly influenced the diversification of their microbiota. Nonetheless, the variation that we observed within species was comparable to that observed between species, and we found no tendency for the microbiota to vary predictably as a function of ecomorph, potentially because most *Anolis* species function as dietary generalists.

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Data accessibility

DNA sequences and metadata of individual anoles: Dryad doi:10.5061/dryad.jn400.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1. Rarefaction curves for each species and population.

Appendix S2. Key to colour coding of bacterial taxa in Figs 2 and S5.

Fig. S3. Comparison of within- and between-group distances by species and ecomorph.

Fig. S4. All pairwise between-species distances for microbiota of Puerto Rican anoles.

Fig. S5. Comparison of full data set vs. data set excluding lower 5% of rare OTUs.

Fig. S6. Short-term changes in gut microbiota of *A. sagrei* individuals from northern Florida.