Salmonella Strains Isolated from Galápagos Iguanas Show Spatial Structuring of Serovar and Genomic Diversity

Emily W. Lankau¹, Lenin Cruz Bedon², Roderick I. Mackie³*

¹ Department of Animal Sciences and College of Veterinary Medicine, University of Illinois at Urbana-Champaign, Urbana, Illinois, United States of America, ² Armador del Pirata, Puerto Ayora, Isla Santa Cruz, Galápagos Islands, Ecuador, ³ Department of Animal Sciences and Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois, United States of America

Abstract

It is thought that dispersal limitation primarily structures host-associated bacterial populations because host distributions inherently limit transmission opportunities. However, enteric bacteria may disperse great distances during food-borne outbreaks. It is unclear if such rapid long-distance dispersal events happen regularly in natural systems or if these events represent an anthropogenic exception. We characterized Salmonella enterica isolates from the feces of free-living Galápagos land and marine iguanas from five sites on four islands using serotyping and genomic fingerprinting. Each site hosted unique and nearly exclusive serovar assemblages. Genomic fingerprint analysis offered a more complex model of S. enterica biogeography, with evidence of both unique strain pools and of spatial population structuring along a geographic gradient. These findings suggest that even relatively generalist enteric bacteria may be strongly dispersed limited in a natural system with strong barriers, such as oceanic divides. Yet, these differing results seen on two typing methods also suggests that genomic variation is less dispersal limited, allowing for different ecological processes to shape biogeographical patterns of the core and flexible portions of this bacterial species’ genome.

Introduction

Recent bacterial biogeography studies demonstrate that both niche selection and dispersal limitation influence bacterial distribution patterns [1–5]. Dispersal limitation can result in a genetic distance-decay pattern (or isolation-by-distance relationship). Distance-decay is a negative correlation between geographic distance and genetic similarity that presumably results from higher gene flow between populations that are closer together spatially due to more frequent dispersal events (i.e. the converse of gene flow is isolation-by-distance) [2,5–9].

Host-associated bacterial populations could be strongly dispersal limited if host distributions constrain microbial migration potential [3,10–13]. It is not clear if this restriction might possibly relax when host-associated microbes can survive in non-host environments or can associate with multiple host species. For example, gastrointestinal bacteria responsible for food-borne outbreaks can persist for days to months in a variety of environments, include sea water [14–20], resulting in wide and rapid dispersal by agricultural distribution systems [21–24]. A single strain may quickly travel long distances and thus, would not necessarily be expected to demonstrate clear genetic-by-distance relationships. However, it is unclear how common rapid long-distance dispersal events are in natural host-bacterial systems, or if such events are an artifact of agricultural production systems.

Salmonella enterica is a Gram negative proteobacterium that primarily resides in animal gastrointestinal tracts, but can also survive in the environment, food, and water [25]. While S. enterica is generally pathogenic to warm-blooded animals, it is rarely reported to cause illness in reptiles [26–27]. Salmonella enterica ecology in reptile populations is not entirely understood. Field studies suggest that S. enterica diversity in reptile populations is dependent on local exposures, with geographically proximate heterospecific populations sharing S. enterica strains [28–29]. In contrast, in warm-blooded species, S. enterica is primarily associated with food-borne outbreaks that may affect large geographic areas [e.g. 22–23], demonstrating a potential for rapid, long-distance dispersal.

Island systems have long contributed to understanding ecological and evolutionary effects of geographic isolation [30–36]. The Galápagos Islands, approximately 1,000 km off the west coast of Ecuador, have famously contributed to such studies. These islands are host to two ecologically distinct types of large herbivorous lizard, the land and marine iguanas (Conolophus species and Amblyrhynchus cristatus, respectively). Populations of both genera are present throughout the island chain, with overlapping distributions at some locations [37]. Genetic studies show historical patterns of...
sequential colonization as the island chain developed, with little or no evidence of significant contemporary gene flow among established populations [30-39]. Galápagos iguanas commonly carry *S. enterica* in their digestive tracts and land and marine iguana populations from the same location share site-specific bacterial strains [29]. These findings suggest that this system is a suitable model for exploring the effect of strong host isolation on bacterial population structure in a natural system.

In this study, we ask the question: How does geographic isolation shape *S. enterica* strain diversity and population similarity across host populations? We applied phenotypic strain typing (i.e., serotyping) and genomic fingerprinting to fecal-derived *S. enterica* isolates from Galápagos land and marine iguanas (*Conolophus* species and *Amblyrhynchus cristatus*, respectively) from five sampling sites on four islands. We hypothesized that if geographic isolation is a barrier to enteric bacterial transmission, then each island should be host to a unique strain pool. However, if this barrier is not absolute, then we would expect that bacterial dispersal would be more likely between proximate sites (i.e., distance-decay).

**Methods**

**Study design and sampling sites**

Iguana fecal samples were collected at five sites on four islands of the Galápagos chain (Figure 1, Table 1). Specimens were collected from the ground after deposition using a sterile wooden applicator. We selected fecal samples from different areas of each site and collected samples over a short period of time (24–48 hours) to avoid repeated sampling of the same individual. Fecal specimens were obtained from both iguana types on Isla Plaza Sur (Nland = 11, Nmarine = 12), Isla Fernandina (Nland = 6, Nmarine = 12), and Isla Santa Fe (Nland = 12, Nmarine = 2) and from marine iguanas on Isla San Cristobal (Lobemia N = 12, Punta Carola N = 12).

Samples were placed in sterile plastic tubes, were stored at 4°C during travel and were transported to Urbana, Illinois for storage at −20°C. Sample collection was performed under an approved animal use protocol (University of Illinois protocol # 00410). Samples were collected with permits from the Galapagos National Park (PNG Autorización de Proyecto PC-21-06 Ext 01-09) and were exported under CITES permit (007-09/PNG).

**Isolation and identification of *S. enterica***

*Salmonebella enterica* was isolated from frozen fecal samples using previously published protocols [29,40]. Briefly, 0.5 g of feces was pre-enriched in buffered peptone water (BPW) at 37°C for 24 hours followed by enrichment in a 1:9 ml dilution of turbid BPW-Rappaport-Vassiliadis broth (RVP) at 37°C for another 24 hours. Turbid RVP was then streak plated on selective-differential media (Xylose lysine deoxycholate). Plates demonstrating no *Salmonella*-characteristic growth at 24 hours were re-incubated for an additional 24 hours before being considered negative. These samples were retested once from the original fecal samples before being considered negative for *S. enterica*.

Presumptive *Salmonella* isolates were then tested on lysine iron agar and triple sugar agar to confirm membership in the genus. Up to three confirmed *Salmonella* isolates were frozen at −70°C in 40% glycerol for further analysis.

**Serotyping and genomic fingerprinting**

Traditional serotyping and genomic fingerprinting have been previously used to describe *S. enterica* strain patterns in this system [29]. Serotyping is a phenotypic bacterial typing approach which identifies O (outer membrane) and H (flagellar) antigenic variation among strains [41]. Observed concordance between sequence-based genotyping and serotype identity suggests that serotyping is a strong phenotypic marker for genetic strain relationships [29,42–43]. One isolate per sample was submitted for serotyping at the National Veterinary Service Laboratory, Ames, IA, USA.

While serotyping is often sufficient for drawing epidemiological links, higher resolution typing of *S. enterica* strains is often desirable to better understand outbreak dynamics. Genotyping techniques which target genomic-level variation have proven most successful at differentiating very closely related strains of *S. enterica* [44–46]. Repetitive extragenic palindrome PCR (Rep-PCR) produces genomic fingerprints through amplification of intervening sequences between repetitive elements of the bacterial genome, resulting in numerous bands of varied size that can be separated by agarose gel electrophoresis. Rep-PCR has been shown to be both highly repeatable and highly reliable for differentiation of *Salmonella* isolates [46].

Up to three isolates per sample were analyzed by Rep-PCR. DNA was extracted from colonies grown on tryptic soy agar using a modification of the manufacturer’s protocol for Instacute gene chexel resin (Biorad, Hercules, CA, USA). Two to four large, well-isolated colonies were placed in 100 µl of 6% chexel 100 resin and lyzed at 95°C for 10 minutes. Lysates were centrifuged for 5 minutes at 14,000 g and 80 µl of supernatant were transferred to a new storage tube. Rep-PCR was performed as in previously described protocols using primers ERIC1R (5′-ATG TAA GCT CCT GGG GAT TCA-3′) and ERIC2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′), targeting enterobacterial repetitive intergenic consensus (ERIC) repetitive motifs [47–48]. PCR was performed on a TGradient thermocycler (Biometra, Germany) with an initial denaturation at 95°C for 2 minutes, followed by 30 cycles each of 94°C for 3 seconds, 92°C for 30 seconds, 50°C for 1 minute and 65°C for 8 minutes, and a final extension at 65°C for 8 minutes. Polymerase chain reaction amplification mixtures (25 µl) included 1.75 U of Takara Taq polymerase (Takara Bio Inc., Japan), 1× Takara PCR Buffer with 2.5 mM (final concentration) of MgCl2, 2.0 mM Takara dNTP mixture (0.5 mM each), 1 µM each of the forward and reverse primers, and approximately 50 ng of template. PCR products were separated on a 2% Agarose gel in 0.75% TAE (Tris-Acetate EDTA) run at 80 V for 12 hours. Three lanes of a 1 kb plus DNA ladder (Novartis, Carlsbad, CA) were included to allow for standardization of molecular weight assignments to DNA fragments, with one lane at each end of the gel and one located in the approximate middle to allow for correction of irregular gel runs. Gels were stained with ethidium bromide and digitally photographed using an Alphalmagger™ 2200 (Alpha Innotec Co., San Leandro, CA, USA).

**Statistical analysis**

Rep-PCR band assignment and sizing from gel images was done using BioNumerics version 4.0 (Applied Maths, Belgium). Band assignments were exported as a binomial presence-absence matrix for statistical analysis. To control for non-independence (i.e., pseudoreplication) resulting from inclusion of multiple isolates per individual iguana fecal sample, fingerprint profiles of isolates from the same individual iguana were nested by averaging presence/absence within an individual host for analysis (see online supplemental information for [29]).

To evaluate explanatory variable effects on bacterial population structure (sampling site, host genus, and geographical coordinates of site), we used the adonis function in the vegan package in R [49] to perform a series of permutation (non-parametric) multivariate analysis of variance tests on genomic fingerprint patterns. These models are similar to more traditional multivariate analysis of
Figure 1. Sampling locations where fecal specimens were collected. Samples were collected from marine iguanas (Amblyrhynchus cristatus) on Isla San Cristóbal sites Punta Carola, La Lobería, Isla Santa Fe, Isla Plaza Sur and Isla Fernandina and from two species of land iguanas on three islands (Conolophus pallidus on Isla Santa Fe and Conolophus subcristatus on Isla Plaza Sur and Isla Fernandina). Black stars indicate the three major port towns of the Galápagos Islands, Puerto Baquerizo Moreno on Isla San Cristóbal, Puerto Ayora on Isla Santa Cruz, and Puerto Villamil on Isla Isabela.

doi:10.1371/journal.pone.0037302.g001

Table 1. Sampling site characteristics and Salmonella enterica prevalence in Galápagos iguana populations.

| Site (Abbr.) | Island (A km²)** | Iguana Species | N   | % Pos. (N Pos) | N Serovar | Nserovar/Npos |
|--------------|------------------|----------------|-----|---------------|-----------|--------------|
| Punta Carola (C) | San Cristóbal (558) | Marine         | 12  | 75.0% (9)     | 5         | 0.56         |
| La Lobería (L)  | San Cristóbal (558) | Marine         | 12  | 75.0% (9)     | 5         | 0.56         |
| (whole island) (P) | Plaza Sur (0.13) | All samples    | 23  | 65.3% (15)    | 7         | 0.47         |
|               |                  | Marine         | 12  | 75.0% (9)     |           |              |
|               |                  | Land¹          | 11  | 54.5% (6)     |           |              |
| El Miedo (S)   | Santa Fe (24)    | All samples    | 14  | 42.9% (6)     | 4         | 0.67         |
|               |                  | Marine         | 2   | 50.0% (1)     |           |              |
|               |                  | Land¹          | 12  | 45.5% (5)     |           |              |
| Cape Douglas (F) | Fernandina (642) | All samples    | 18  | 44.4% (8)     | 7         | 0.88         |
|               |                  | Marine         | 12  | 50.0% (6)     |           |              |
|               |                  | Land¹          | 6   | 33.3% (2)     |           |              |

*Abbr. = Abbreviation; % Pos. = % of samples positive for S. enterica; Npos = Number of samples positive for S. enterica; N serovar = Number of unique serovars identified in each site; Nserovar/Npos = serovar richness for each site adjusted for the number of positive samples, in units of number of serovars/positive sample.

**Land area estimates from [37];

¹Conolophus pallidus;

²Conolophus subcristatus.

doi:10.1371/journal.pone.0037302.t001
variability (MANOVA), but they partition the sums of squares of distance matrices among treatments and have relaxed assumptions relative to traditional MANOVA. For categorical variables, a significant p-value indicates that isolates within a group are more similar to each other than they are to isolates from other groups. For a continuous variable, such as geographic coordinates, a significant p-value indicates that more proximate isolates are more similar (i.e. is analogous on a multivariate level to distance-decay). Significance in the permutation tests was determined by comparing the observed effects against 5000 random permutations of the data for each model run independently. Interaction terms were not included as land iguanas were only present for sampling at 3/5 sites.

Geographical location was included in subsequent permutation MANOVA models as the latitude and longitude coordinates of each site or as a rotated axis derived from the principal component scores of the sites’ latitudes and longitudes. This ordination produced two orthogonal axes oriented along a southeastern to northwestern axis, PC1, and along a northeastern to southwestern axis, PC2. Many plant and animal species in the Galápagos demonstrate a southeast-to-northwest genetic diversity gradient, with the most divergent and isolated populations located on the older islands in the eastern and central islands and younger, more genetically recent populations in the northwestern islands (i.e., the “progression hypothesis”) [38–39,50]. This, the principal components rotation of the primary latitudinal and longitudinal axes has the potential to more fully capture the primary geographical pattern in this system by capturing the majority of the variation present in the geographical axis into a single variable (PC1).

We then performed multivariate ordinations on the genomic fingerprint patterns to visualize the significant effects detected by permutation MANOVA, using both an unconstrained and a constrained correspondence analysis (CA and CCA, respectively, both performed with the cca function in the vegan package) [49]. Correspondence analysis is a statistical ordination technique that summarizes multivariate data, such as banding patterns, into two-dimensional space for visualization of patterns in the data. When unconstrained, CA can provide a visual demonstration of dominant patterns in a dataset, independent of other variables of interest. When constrained by a variable of interest, CCA can provide a visual summary of patterns in the data that specifically relate to that variable. CCA was performed using sampling site as the constraining variable, as this factor explained the largest amount of variation (i.e., had the highest R²) in the various permutation MANOVA analyses. The CCA isolates those aspects of the genomic information most strongly contributing to geographic structuring. We then performed an environmental fitting of the previously described PC1 and PC2 location axes on the CA and CCA patterns using the envfit function in the vegan package of R [49]. A significant p-value on environmental fitting indicates that there is a statistically significant association between the variable of interest and the summary of the data provided by the correspondence analysis ordination; the environmental fit can be plotted on the ordination graph to demonstrate the nature of this relationship visually.

Basic statistical and graphing procedures were performed in either JMP 8 (SAS Institute, Cary, NC) or R statistical language [51].

Results
Prevalence and serovar patterns
Prevalence of S. enterica in Galápagos iguana populations ranged from 33% to 75%. Marine iguanas tended to have higher prevalence (68%, n = 50) than land iguanas (45%, n = 29) but this difference was only marginally statistically significant (Fisher exact test p = 0.058, Table 1). Salmonella enterica prevalence was not significantly different among sites within a host type (Fisher exact tests: marine iguana among sites p = 0.544, land iguana among sites p = 0.702).

We isolated 23 unique serovars from 47 iguanas distributed among the five sampling sites (Table 2, serogroups as in [52]). A number of serovars detected in this study were also noted in a previous sampling of a subset of these sites in 2005 (Table 2). Serovar richness controlled for the number of positive samples varied among sites, ranging from 0.47–0.88 serovars/positive sample (Table 1).

Genomic fingerprint patterns
Genomic fingerprint patterns on unconstrained ordination reflected serogroup level differences similar to those seen on serotyping (Figure 2a, CA). Grouping of isolates by sampling site is not notable in this unconstrained analysis (Figure 2b, CA). However, sampling site was a significant factor on permutation MANOVA analysis of genomic fingerprints (Table 3, site R² = 0.137, p = 0.004). In models including sampling site, geographic factors latitude, longitude and PC2 were not significant. However, PC1 was a significant factor explaining genomic similarity among isolates (Table 3). In a model including site, PC1, and PC2, both site and PC1 significantly explained aspects of genomic variation. This suggests that while sites differed uniquely from each other (as seen also in the serotyping results in Table 2), there was also a portion of genomic variation structured along this geographical axis. PC1 was also a significant environmental fit to the sampling site-constrained canonical correspondence analysis, aligning directionally in a southeast to northwest direction across sampling sites (Figure 2c; PC1 environmental fitting R² = 0.678, p < 0.001).

Discussion
Wild reptile populations commonly harbor diverse S. enterica assemblages. In this study, adjusted serovar richness (i.e., N serovars/positive sample) was comparable to that seen in previous studies (the average for 8 published studies = 0.44 serovars/positive sample with a range of 0.19–0.75 serovars/positive sample) [28–29,40,53–57]. Salmonella enterica is a common and diverse member of the enteric biota of Galápagos land and marine iguanas on multiple islands [29,57–59]. This diversity presented a unique opportunity to more fully explore the effects of host population isolation on strain and genetic diversity patterns of this host-associated enteric bacterium.

The observed pattern of distinct serovar assemblages at each site might suggest that rare and stochastic strain dispersal among sites is a driving force for determining S. enterica population structure. When dispersal limitation is a primary driver of population or community composition, considerable site-to-site variation occurs as species (or strains) arrive and establish by chance at each site independently [60–61].

In contrast, when assembly of diversity is driven by niche selection, similar communities should be found in similar selective environments [62–64]. While land and marine iguanas represent quite dissimilar selective environments for enteric microbial communities due to host dietary differences [65], S. enterica populations of sympatric land and marine environments have been shown to overlap considerably [29]. This suggests that despite host-level differences in enteric environments, local exposures are key for serovar diversity and supports the contention
that isolation rather than selection primarily determines the serovar distributions seen in this study.

Also notable is the degree of overlap in serovars detected in 2005 and 2009 iguana fecal samples collected at Isla Plaza Sur and Isla Santa Fe, including Manhattan, Panama, Poona and Sandiego for Isla Plaza Sur and Pomona and Sandiego for Isla Santa Fe. In addition, a local *Salmonella enterica* prevalence study of land iguanas from cloacal swabs collected in 2003 from Isla Santa Cruz, quite near Isla Plaza Sur, also detected serovars Manhattan, Pomona and Poona [57]. This strain stability within geographic locations over a relatively long time period (for ecological processes to influence microbial strain turnover, that is) might suggest that within-site strain selection may also play an important role in maintaining these unique strain pools within local reptile host communities or that immigration of new serovars is rare, resulting in slow serovar turn-over.

While the presence of unique serovar assemblages at each site suggests dispersal is quite rare among sites, genomic patterns were more complicated. On unconstrained ordination (correspondence analysis) of genomic fingerprint patterns, isolates from the same serovar or O-antigen serogroup are generally located in proximate ordination space. A quantitative test of this hypothesis would be difficult with this diverse strain assemblage, but qualitatively this might suggest that genetic relationships among these serovars represent a deeper evolutionary strain history, rather than a more recent ecological distribution of strains among these sites. Sequence-based genotyping methods such as multi-locus sequence typing are generally concordant with phenotypic serotyping but

| Sub-species | Serotype | Serogroup* | Total | C | L | P | S | F | Total | P | S |
|-------------|----------|------------|-------|---|---|---|---|---|-------|---|---|
| **2009 & 2005** | | | | | | | | | | | |
| I | Muenchen | O:8 (C2–C3) | 2 | 1 | 1 | | | | | | |
| I | Manhattan | O:8 (C2–C3) | 2 | 2 | 2 | | | | | | |
| I | Sandiego | O:4 (B) | 6 | 6 | 10 | 7 | 3 | | | | |
| I | Poona | O:13 (G) | 3 | 3 | 2 | 2 | | | | | |
| IV | 53x4:23- | O:53 | 1 | 1 | | | | | | | |
| IV | 44x36- | O:44 | 1 | 1 | | | | | | | |
| I | Pomona | O:28 (M) | 4 | 1 | 3 | 3 | 2 | 1 | | | |
| I | Berta | O:9 (D1) | 4 | 3 | 1 | | | | | | |
| I | Treforest | O:51 | 1 | 1 | | | | | | | |
| I | Rough O:1:L,V:1,7 | R | 2 | 1 | 1 | 3 | 3 | | | | |
| I | Montevideo | O:7 (C1) | 2 | 2 | 1 | 1 | | | | | |
| I | Bredeney | O:4 (B) | 1 | 1 | | | | | | | |
| I | Newport | O:8 (C2–C3) | 2 | 2 | | | | | | | |
| II | II 47:b:1,5 | O:47 (K) | 3 | 3 | | | | | | | |
| I | Mjordan | O:30 (N) | 2 | 2 | | | | | | | |
| I | Saintpaul | O:4 (B) | 1 | 1 | | | | | | | |
| I | Reading | O:4 (B) | 2 | 2 | | | | | | | |
| I | Rubislaw | O:11 (F) | 1 | 1 | | | | | | | |
| I | Rough O:8:1,7 | R | 1 | 1 | | | | | | | |
| I | 57:b:- | O:57 | 2 | 2 | | | | | | | |
| I | Rough O:2:enz15 | R | 1 | 1 | | | | | | | |
| I | Wedding | O:28 (M) | 1 | 1 | | | | | | | |
| IV | Rough O:9:51:- | R | 1 | 1 | | | | | | | |
| Untyped\ | Untyped | Untyped | 1 | 1 | | | | | | | |
| **2005 only** | | | | | | | | | | | |
| I | Panama | O:9 (D1) | 6 | 6 | | | | | | | |
| I | Oranienburg | O:7 (C1) | 2 | 2 | | | | | | | |
| I | Rough O:2:enx | R | 1 | 1 | | | | | | | |
| I | Rough O:2:enz15 | R | 3 | 3 | | | | | | | |
| I | SSI 28:v:- | O:28 (M) | 1 | 1 | | | | | | | |
| Total N | 47 | 9 | 9 | 15 | 6 | 8 | 34 | 21 | 13 | | |

*Serogroups as in [52].

**C = Punta Carola, Isla San Cristóbal, L = La Loberia, Isla San Cristóbal, P = Isla Plaza Sur, S = Isla Santa Fe, F = Isla Fernandina.

\untyped isolate established as presumptive *Salmonella* spp. by metabolic assay but submission to the National Veterinary Services Laboratory was found to be contaminated and was not typed further.

*S. enterica* isolates from 2005 were part of a previous study and are described further in [29].

doi:10.1371/journal.pone.0037302.t002
cannot distinguish variation within a serovar [66–67]. Thus, the lack of clustering by site in unconstrained analysis is still compatible with conclusions drawn from serovar patterns and is consistent with the significant site effects noted on permutation analysis that were independent of geographical location (i.e. independent of latitude and longitude, or PC1 and PC2 axes).

Yet we also detected a significant southeast to northwest geographic relationship among S. enterica populations, even when controlling for these site effects. This suggests that not only are sites significantly unique in strain composition (as detected on both serovar analysis and in the significant site effect on genomic variation), but also that a separate portion of the genomic variation is specifically oriented along this geographical axis. These findings together suggest that while dispersal of specific strains of S. enterica among sites is rare, some aspect of S. enterica genetic diversity does move among sites and this dispersal is more frequent among proximate sites. Given the considerable horizontal gene transfer often detected in bacterial communities, both generally (as reviewed in [68–70]), and within marine iguana intestinal communities [71], it is possible that these directional geographical relationships among S. enterica populations are driven by movement of genetic elements among strains. Thus, strains within and between sites may share certain genomic elements, despite not sharing a recent strain history.

This potential for disconnect between evolutionary and ecological history based on different typing methods was hinted at in a previous work in this system [29]. Further, this finding is not inconsistent with current knowledge of S. enterica genetic diversity. Salmonella enterica shares a large portion of its genome with related bacteria such as Escherichia coli, but has in addition the tendency to flexibly gain and lose large, mobile pathogenicity islands [72]. Full genome sequence comparisons of diverse S. enterica serovars reveals that genetic differences among strains is primarily based on insertions and deletions of these chromosomal elements, rather than on coding region sequence divergence of the types of genes typically targeted for sequence analysis [73]. Such discordance between genetic markers has also been observed for Escherichia coli, where multilocus enzyme electrophoresis of E. coli reference strains demonstrated six distinct phylogenetic groups while rep-PCR based analysis grouped these same isolates primarily by species of origin [74].

If serovar (as a proxy for genetic strain history) and genomic fingerprint marker variation operate under different ecological and evolutionary pressures, then this would explain the observed

Table 3. Permutation MANOVA analysis of rep-PCR fingerprint patterns, evaluating the influence of site, and geographic location on Salmonella similarities.

| Source          | F     | R²   | p    |
|-----------------|-------|------|------|
| Site            | 1.687 | 0.137| 0.004** |
| Host            | 1.163 | 0.024| 0.306 |
| Site            | 1.666 | 0.137| 0.004** |
| Latitude        | 1.181 | 0.024| 0.323 |
| Longitude       | 1.053 | 0.022| 0.371 |
| Site            | 1.698 | 0.070| 0.018** |
| PC1 (S.E.-N.W.) | 2.176 | 0.045| 0.010** |
| PC2 (S.W.-N.E.) | 1.072 | 0.022| 0.381 |

*Significant at α = 0.10; **Significant at α = 0.05.

doi:10.1371/journal.pone.0037302.t003
disjunction in biogeographical patterns seen in this study. Given this tendency to flexibly acquire genomic elements, gains and losses of these elements may contribute to differences in genomic fingerprint patterns on a site-to-site basis. These mobile elements may represent S. enterica- or Enterobacteriaceae-specific genetic elements or more broadly disseminated transposable elements (e.g. transposons, viruses etc.) horizontally acquired from a wide diversity of bacterial interactions within the enteric or environmental habitat.

Even with this explanation, what is particularly striking about the detected site differences by genomic fingerprinting is the geographical directionality of the site similarity pattern, which correspond to ecological forces unique to this island chain. The Cromwell and Humboldt currents and associated trade winds sweep across the Galápagos Islands in a primarily east-west or southeast-northwest direction for much of the year, and may potentially carry with them a variety of bacterial elements. Thus, the expectation of simple isolation-by-distance patterns may not be the most appropriate test for dispersal-limitation in other similarly structured systems where dispersal capacity may differ by direction.

Visualizing the ecological complexity of S. enterica biogeography in this system was dependent on using more than one measure of diversity. Traditionally, biogeographical studies of plants and animals have relied on such combined approaches to fully explore how migration and selection combine to shape geographic patterns. In particular, it is often important to evaluate both neutral and trait-based markers as their geographic patterns will reflect different driving forces (stochastic versus deterministic) [75–79]. Given the growing understanding of how horizontal gene transfer influences bacterial genetics, it is a reasonable parallel to suggest that bacterial biogeography might also benefit from simultaneous measures of core and mobile genetic diversity [80–82].

These results also suggest that diversity patterns arising from dispersal limitation may be more subtle than simple isolation-by-distance if dispersal potential is asymmetrical [83]. However, simple isolation-by-distance certainly remains a reasonable initial model for consideration (as reviewed in [84]). Only a few studies to date have begun to incorporate potential asymmetries in dispersal potentials into understanding microbial population or community biogeography [85–86].

Acknowledgments

We acknowledge Martin Wikelski; the Galápagos National Park, Washington Tapia, Charles Darwin Research Station, Sonja Cisneros, Paulina Couenbench, Augusto G. Haz Beltran and the crew of the Pirata for facilitating sample collection; R. Ryan Lash for GIS support; Nancy Henry for administrative support; Richard Lankau for statistical support. Thanks to Isaac Cann, Rex Gaskins, Richard Lankau, Ken Paige, Patrick Weatherhead and two anonymous reviewers for constructive comments.

Author Contributions

Conceived and designed the experiments: RIM. Performed the experiments: EWL LCB RIM. Analyzed the data: EWL. Wrote the paper: EWL. Critically reviewed the manuscript: EWL LCB RIM.

References

1. Finlay BJ (2002) Global dispersal of free-living microbial eukaryote species. Science 296: 1061–1063.
2. Whitaker RJ, Grodan DW, Taylor JW (2003) Geographic barriers isolate endemic populations of hyperthermophilic archaea. Science 301: 976–978.
3. Finlay BJ, Fenichel T (2004) Cosmopolitan metapopulations of free-living microbial eukaryotes. Protist 155: 237–244.
4. Fontaneto D, Barracloch TG, Chen K, Ricci C, Herniou EA (2008) Molecular evidence for broad-scale distributions in bdelloid rotifers: everything is not everywhere but most things are very widespread. Mol Ecol 17: 3136–3140.
5. Takacs-Vesbach C, Mitchell K, Jackson-Weaver O, Reyensbach AL (2008) Volcanic calderas delineate biogeographic provinces among Yellowstone thermophiles. Environ Microbiol 10: 1681–1689.
6. Wright S (1943) Isolation by distance. Genetics 28: 114–138.
7. Choo JC, Cote Ellis JM (2000) Biogeography and degree of endemism of fluorescent Pseudomonas strains in soil. Appl Environ Microbiol 66: 5448–5456.
8. Papke RT, Ramsing NB, Bateson MM, Ward DM (2003) Geographical barriers isolate endemic populations of hyperthermophilic archea. Science 301: 976–978.
9. Vos M, Velmec G (2000) Isolation by distance in the spore-forming soil bacterium Mucoraceous salmon. Curr Biol 18: 396–391.
10. Falush D, Wirth T, Linz B, Prichard JK, Stephens M, et al. (2003) Traces of human migrations in H. sapiens populations. Science 299: 1582–1585.
11. Hedlund BP, Staley JT (2003) Microbial endoum and biogeography. In: Bull AT, ed. Microbial Diversity and Bioprospecting, American Society of Microbiology: Washington, DC.
12. Zhang N, Blackwell M (2002) Population structure of dogwood anthracnose fungus. Phytopathology 92: 1276–1283.
13. Bala A, Murphy P, Giller KE (2003) Distribution and diversity of rhizobia nodulating agroforestry legumes in soils from three continents in the tropics. Mol Ecol 12: 917–929.
14. Rhodes MW, Kator H (1980) Survival of Escherichia coli and Salmonella spp. in estuarine environments. Appl Environ Microbiol 41: 2902–2907.
15. Rozen Y, Belkin S (2001) Survival of enteric bacteria in seawater. FEMS Microbiol Rev 25: 513–529.
16. Guan TV, Holley RA (2003) Pathogen survival in swine manure environments and transmission of human enteric illness – a review. J Environ Qual 32: 383–392.
17. Harris Ld, Farber JN, Beuchat LR, Parish ME, Suidow TV, et al. (2003) Outbreaks associated with fresh produce: incidence, growth, and survival of pathogens in fresh and fresh-cut produce. Compr Rev Food Sci Food Safety 2: 78–141.
18. Brandt MT (2006) Fitness of human enteric pathogens on plants and implications for food safety. Annu Rev Phytopathol 44: 367–392.
19. Alegre I, Abasias M, Anguera M, Usal J, Vivas I (2010) Fate of Escherichia coli O157:H7, Salmonella, and Listeria innocua on minimally processed peaches under different storage conditions. Food Microbiol 27: 862–868.
20. Semenov AM, Kuprianov AA, van Bruggen AHC (2010) Transfer of enteric pathogens to successive habitats as part of microbial cycles. Microb Ecol 60: 239–249.
21. Centers for Disease Control and Prevention [CDC] (2006) Ongoing multistate outbreak of Escherichia coli O157:H7 infections associated with consumption of fresh spinach – United States, September 2006. Morb Mortal Wkly Rep 55: 1–2.
22. Centers for Disease Control and Prevention [CDC] (2007) Multistate outbreak of Salmonella serotype Saintpaul infections associated with eating alfalfa sprouts – United States, 2006–2007. Morb Mortal Wkly Rep 57: 521–524.
23. Centers for Disease Control and Prevention [CDC] (2009) Outbreak of Salmonella serotype Saintpaul infections associated with eating alfalfa sprouts – United States, 2009. Morb Mortal Wkly Rep 58: 500–503.
24. Centers for Disease Control and Prevention [CDC]. (2010). Two multistate outbreaks of shiga toxin-producing Escherichia coli infections linked to beef from a single slaughter facility – United States, 2008. Morb Mortal Wkly Rep 59: 557–560.
25. Foster JW, Spector MP (1995) How Salmonella survive against the odds. Ann Rev Microbiol 49: 145–174.
26. Scherer CA, Miller SI (2001) Molecular pathogenesis of Salmonella. In: Principles of Bacterial Pathogenesis, Academic Press, San Diego, CA. pp 265–333.
27. Geue L, Loshner U (2002) Salmonella enterita in reptiles of German and Austrian origin. Vet Microbiol 84: 79–91.
28. Briones V, Teles S, Goyache J, Ballesteros C, Lanzarot MD, et al. (2004) Salmonella diversity associated with wild reptiles and amphilians in Spain. Environ Microbiol 6: 868–871.
29. Wheeler E, Cann IKO, Mackie RI (2011) Genomic fingerprinting and serotyping of Salmonella from Galápagos iguanas demonstrates island differences in strain carriage. Environ Microbiol Reports 3: 166–173. doi:10.1111/j.1758-2229.2010.00203.x.
30. Darwin C (1859) On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life. John Murray: London.
31. Mayr E (1963) Animal Species and Evolution. Harvard University Press: Cambridge, MA.
32. MacArthur RH, Wilson EO (1967) The Theory of Island Biogeography. Princeton University Press: Princeton, NJ.
33. Diamond JM, May RM (1976) Island biogeography and the design of national reserves. Pages 153–186. In: May RM, ed. Theoretical Ecology: Principles and Applications, Blackwell Scientific: Oxford, UK.
34. Krebs CJ (1994) Ecology: The Experimental Analysis of Distribution and Abundance. Harper-Collins: New York, NY.
35. van der Gast CJ (2006) Chapter 4: Islands shaping thought in microbial ecology. Adv Appl Microbiol 64: 167–182.
36. Losos JB, Ricklefs RE (2009) Adaptation and diversification on islands. Nature 457: 830–836.
37. Jackson MH (2007) Galápagos: A Natural History (9th ed.). University of Calgary Press: Calgary, Alberta, Canada.
38. Tizka AC, Rosa SFP, Fabiani A, Snell HL, Snell HM, et al. (2008) Population genetics of Galápagos land iguanas (genus Conolophus) remnant populations. Mol Ecol 17: 4943–4952.
39. Steinfartz S, Gläsermann S, Lanterbeq D, Russell MA, Rosa S, et al. (2009) Progressive colonization and restricted gene flow shape island-dependent population structures in Galápagos marine iguanas (Conolophus subcristatus). BMC Evol Biol 9: 297.
40. Corrente M, Madjo A, Friedrich KG, Greco G, Dario C, et al. (2004) Isolation of Salmonella strains from reptile faeces and comparison of different culture methods. Adv Appl Microbiol 96: 709–715.
41. Popoff MY, Le Minor L (1997) Antigenic formulas of the Salmonella serovars (7th revision). WHO Collaborating Centre for Reference and Research on Salmonella In Vitro: Paris, France.
42. Tankou-Sunday P, Seslicht A, Liebana E, Kornschbocher C, Allerberger F, et al. (2007) MS-PCR, multilocus sequence typing based on virulence genes for molecular typing of Salmonella enterica subsp. enterica serovars. J Microbiol Methods 69: 23–36.
43. Ben-David E, De Pina E, Threlfall EJ, Bolton EJ, Upton M, et al. (2010) Comparison of semi-automated rep-PCR system and multilocus sequence typing for differentiation of Salmonella enterica isolates. J Microbiol Meth 81: 11–16.
44. Olen J, Skov MN, Threlfall EJ, Brown DJ (1994) Clonal lines of Salmonella enterica serotype Enteritidis documented by IS200-, ribo-, pulsed-field gel-electrophoresis and RFLP typing. J Med Microbiol 40: 15–22.
45. Beyer W, Makundi FM, Kinung P, Bohn R (1998) Suitability of repetitive DNA-sequence-based PCR fingerprinting for characterizing epidemic isolates of Salmonella enterica serovar Saintpaul. J Clin Microbiol 36: 1549–1554.
46. Wurzler R, Cattaneo A, Hla, T, Debernard-Beyh, C, Ribaud P, et al. (2004) Comparison of pulsed field gel electrophoresis and repetitive sequence polymerease chain reactions as genotyping methods for detection of genetic diversity and inferring transmission of Salmonella. Vet Microbiol 100: 205–217.
47. Verdouw MPJ, van Duin J, Lapuente J (1991) Distribution of repetitive DNA sequences in bacteria and application to fingerprinting of bacterial genomes. Nucl Acids Res 19: 6825–6831.
48. Rademaker J, Louws W, Louws EJ, De Bruijn FJ (1998) Characterization of the diversity of ecologically important microbes by rep-PCR genomic fingerprinting. In: Molecular Microbial Ecology Manual. Kluwer Academic: Dordrecht, the Netherlands. pp 113–136.
49. Oksanen J, Kindt R, Legendre P, O’Hara RB, Simpson GL, et al. (2010) vegan: Community Ecology Package. R package version 1.17–4. http://cran.r-project.org/.
50. Parent CE, Baccone A, Petten K (2008) Colonisation and diversification of Galápagos terrestrial fauna: a phylogenetic and biogeographical synthesis. Philos Trans R Soc Lond B Biol Sci 363: 1349–1354.
51. Wurzler R, Cattaneo A, Hla, T, Debernard-Beyh, C, Ribaud P, et al. (2004) Comparison of pulsed field gel electrophoresis and repetitive sequence polymerease chain reactions as genotyping methods for detection of genetic diversity and inferring transmission of Salmonella. Vet Microbiol 100: 205–217.
52. Verdouw MPJ, van Duin J, Lapuente J (1991) Distribution of repetitive DNA sequences in bacteria and application to fingerprinting of bacterial genomes. Nucl Acids Res 19: 6825–6831.
53. Rademaker J, Louws W, Louws EJ, De Bruijn FJ (1998) Characterization of the diversity of ecologically important microbes by rep-PCR genomic fingerprinting. In: Molecular Microbial Ecology Manual. Kluwer Academic: Dordrecht, the Netherlands. pp 113–136.
54. Oksanen J, Kindt R, Legendre P, O’Hara RB, Simpson GL, et al. (2010) vegan: Community Ecology Package. R package version 1.17–4. http://cran.r-project.org/.
55. Parent CE, Baccone A, Petten K (2008) Colonisation and diversification of Galápagos terrestrial fauna: a phylogenetic and biogeographical synthesis. Philos Trans R Soc Lond B Biol Sci 363: 1349–1354.
56. Verdouw MPJ, van Duin J, Lapuente J (1991) Distribution of repetitive DNA sequences in bacteria and application to fingerprinting of bacterial genomes. Nucl Acids Res 19: 6825–6831.
57. Rademaker J, Louws W, Louws EJ, De Bruijn FJ (1998) Characterization of the diversity of ecologically important microbes by rep-PCR genomic fingerprinting. In: Molecular Microbial Ecology Manual. Kluwer Academic: Dordrecht, the Netherlands. pp 113–136.
58. Oksanen J, Kindt R, Legendre P, O’Hara RB, Simpson GL, et al. (2010) vegan: Community Ecology Package. R package version 1.17–4. http://cran.r-project.org/.
59. Parent CE, Baccone A, Petten K (2008) Colonisation and diversification of Galápagos terrestrial fauna: a phylogenetic and biogeographical synthesis. Philos Trans R Soc Lond B Biol Sci 363: 1349–1354.
60. Verdouw MPJ, van Duin J, Lapuente J (1991) Distribution of repetitive DNA sequences in bacteria and application to fingerprinting of bacterial genomes. Nucl Acids Res 19: 6825–6831.
61. Rademaker J, Louws W, Louws EJ, De Bruijn FJ (1998) Characterization of the diversity of ecologically important microbes by rep-PCR genomic fingerprinting. In: Molecular Microbial Ecology Manual. Kluwer Academic: Dordrecht, the Netherlands. pp 113–136.
62. Oksanen J, Kindt R, Legendre P, O’Hara RB, Simpson GL, et al. (2010) vegan: Community Ecology Package. R package version 1.17–4. http://cran.r-project.org/.
63. Parent CE, Baccone A, Petten K (2008) Colonisation and diversification of Galápagos terrestrial fauna: a phylogenetic and biogeographical synthesis. Philos Trans R Soc Lond B Biol Sci 363: 1349–1354.
64. Verdouw MPJ, van Duin J, Lapuente J (1991) Distribution of repetitive DNA sequences in bacteria and application to fingerprinting of bacterial genomes. Nucl Acids Res 19: 6825–6831.