The skin microbiota of the axolotl Ambystoma altamirani is highly influenced by metamorphosis and seasonality but not by pathogen infection

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Abstract

Background: Microbiomes have been increasingly recognized as major contributors to host health and survival. In amphibians, bacterial members of the skin microbiota protect their hosts by inhibiting the growth of the fungal pathogen *Batrachochytrium dendrobatidis* (Bd). Even though several studies describe the influence of biotic and abiotic factors over the skin microbiota, it remains unclear how these symbiotic bacterial communities vary across time and development. This is particularly relevant for species that undergo metamorphosis as it has been shown that host physiology and ecology drastically influence diversity of the skin microbiome.

Results: We found that the skin microbiota of the axolotl *A. altamirani* is largely influenced by the metamorphic status of the host and by seasonal variation of abiotic factors such as temperature, pH, dissolved oxygen, and conductivity. Despite high Bd prevalence in these samples, the bacterial diversity of the skin microbiota did not differ between infected and non-infected axolotls, although particular bacteria were correlated with Bd infection intensity.

Conclusions: Our work shows that metamorphosis is a crucial process that shapes skin microbiota and that axolotls under different developmental stages response differently to environmental seasonal variations. Moreover, this study greatly contributes to a better understanding of the factors that shape amphibian skin microbiota, especially in a largely underexplored group like axolotls (Mexican *Ambystoma* species).

Background

Host associated microbiomes are vital for host health and survival, as they contribute with functions related to nutrition, reproduction, behavior, defense against pathogens or predators [1–5]. Specifically, some animal associated microbiomes contribute to host health due to their ability to inhibit the growth of pathogens responsible of infectious diseases threatening diverse host species such as bats, snakes, or amphibians [6–8]. For instance, it has been shown that some members of the amphibian skin microbiome inhibit the growth of the lethal pathogens *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* [9–12], which have caused amphibian populations declines and extinctions worldwide [13].

Studies accumulated over the past two decades showed that the amphibian skin microbiome is influenced by host associated factors (host genetics and development) [14–16], microhabitat related factors (environmental microorganisms, habitat abiotic conditions and pathogen presence) [17–21], and climatic and geographical factors (seasonality, precipitation, temperature or land use) [14, 22–25].

In the case of host-associated factors, it has been shown that the skin microbiota of amphibians (specifically frogs) changes across development and particularly before and after metamorphosis [26–28]. During metamorphosis amphibians in larval stages transition to adults following a series of physiological rearrangements such as tail reabsorption, limb development and the remodeling of muscles, heart, intestine brain, and skin [29]. Metamorphosis also induces immunosuppression into response to thyroid and corticosteroid hormone signaling and eventually the immune system reorganizes and gradually matures in newly metamorphosed adults [30].

Along with physiological rearrangements, many amphibian species go through behavior and lifestyle changes, while larval stages inhabit aquatic environments, adults become terrestrial and only return to water environments in the reproductive season [31–33]. These changes in microhabitat occupancy could influence skin microbiota

composition as the main source of skin microbiota diversity is selected from the environmental pool of free-living microorganisms[16, 17].

In the case of climatic factors, temporal variation of some abiotic factors [36] such as temperature and precipitation have a strong influence over amphibian skin microbial community structure [22, 37]. For example, in tropical regions microbial diversity on the amphibian skin differs between wet and dry seasons [19, 26, 34]. In temperate regions, where the four seasons are well defined through the year, seasonal changes have been linked to the temporal dynamics of the amphibian skin microbiota [22, 38–40].

Bd influence over the amphibian skin microbiota has been described in amphibian species with contrasting Bd infection status (infected – non-infected [19] and high Bd prevalence- low Bd prevalence [41]). These studies showed that disruption of skin microbiota following Bd infection can influence host survival and that the final outcome of the infection depends on a interplay between host, microbiome and environment [21, 23, 42].

Here we analyzed the skin bacterial diversity of the axolotl *Ambystoma altamirani*, a stream dwelling salamander endemic to conifer and oak-pine forest from the central region of Mexico [43]. *A. altamirani* is a facultative paedomorphic species in which, metamorphic (without gills) and non metamorphic (with gills) individuals inhabit the same streams all year long [44, 45], allowing us to evaluate how metamorphosis and seasonality influence the skin microbiota in a species living in the same aquatic environment across time and development. In addition, we evaluated if skin microbiota differs from environmental bacterial communities and if Bd presence and infection intensity influence the skin microbiota of *A. altamirani*. We hypothesized that *A. altamirani* skin microbiota would a) differ from environmental bacterial communities, b) vary between metamorphic and non metamorphic salamanders, c) change across seasons and d) differ according to Bd infection status

Results

We sampled a total of 279 *A. altamirani* individuals (85 metamorphic and 194 non metamorphic) at four locations across four seasons. Additionally, 159 environmental samples from sediment (80) and water (79) were collected. After quality control and rarefaction at 10,000 reads, 13 samples were discarded, and 438 samples were used to perform all diversity analyses (Table 1). A final table with a total of 72,408 amplicon sequence variants (ASVs) was obtained including all samples.

Table 1
List of samples collected of each sample type by sampling period.

	Metamorphic	Non metamorphic	Sediment	Water	Total Samples (N)
Summer (July 2019)	25	41	19	20	**105**
Autumn (October 2019)	28	29	20	20	**97**
Winter (January 2020)	9	66	20	20	**115**
Spring (April 2020)	23	58	20	20	**121**
Total Samples (N)	**85**	**194**	**79**	**80**	438

Number of samples from the skin of *A. altamirani* individuals (metamorphic and non metamorphic) and environmental samples (sediment and water). Numbers in bold indicate total number of samples collected for each sample type or season.

A. altamirani skin microbiota differs from environmental bacterial communities

When comparing the number of unique and shared ASVs across sample types, we found that each sample type harbored many unique ASVs and only 2408 ASVs (3.32 % of the total) were shared among the four sample types (Figure 1A). Sediment and water samples were the samples with highest numbers of unique ASVs (20,031 and 9,902 respectively), while metamorphic and non metamorphic samples had 8,916, and 6,650 unique ASVs respectively. Interestingly only 677 ASVs were shared between metamorphic and non metamorphic salamanders.

Taxonomic diversity showed that, Burkholderiaceae was the most abundant bacterial family in all four sample types accounting for 32.6% and 51.1% of the relative abundance in metamorphic and non metamorphic samples respectively, and 14.6% and 40.8% of sediment and water respectively (Additional file 1, Supplementary Fig. 1). For the axolotl samples we found that Chitinophagaceae and Pseudomonadaceae varied in relative abundance according to host metamorphic status, being Chitinophagaceae (metamorphic 2.7% / non metamorphic 27%) and Pseudomonadaceae more abundant in metamorphic samples (metamorphic 18.1% / non metamorphic 6.4%).

Bacterial alpha diversity was significantly different between sample types (metamorphic, non metamorphic, sediment and water) with Observed ASVs (Kruskal-Wallis (KW), $\chi 2$ = 278.46, p-value < 0.001), Shannon index (KW, $\chi 2$ = 276.28, p-value < 0.001) and Faith´s phylogenetic diversity (PD) (KW, $\chi 2$ = 286.91, p-value < 0.001) (Fig. 1B). *Post hoc* pairwise comparisons for each alpha diversity index showed significant differences among all sample types (Additional file 2, Supplementary Table 1) except for metamorphic salamanders and water in observed ASVs (Wilcoxon, p-value = 0.48) and Shannon diversity index (Wilcoxon, p-value = 0.66). Sediment samples showed the highest alpha diversity values while non metamorphic salamanders always had the lowest values.

Bacterial beta diversity based on the weighted UniFrac distance matrix varied significantly among sample types (PERMANOVA, pseudo-F = 64.76, p-value < 0.001) (Fig. 1C, Additional file 2, Supplementary Table 2). Sample

dispersion also significantly differed among sample types (PERMUTEST, F = 34.5, p-value = 0.001) (Fig. 1D, Additional file 2, Supplementary Table 3) with non metamorphic samples showing the greatest dispersion.

The skin bacterial composition of A. altamirani is mainly influenced by metamorphosis

Clear differences in skin bacterial alpha and beta diversity were found between metamorphic and non metamorphic salamanders (Fig. 1B, C, D). To look deeper into the bacterial taxa driving these differences we used an analysis of composition of microbiomes (ANCOM) which identified 45 bacterial families (out of 392 families in the axolotl skin samples) that were differentially abundant between metamorphic and non metamorphic samples (Fig. 2). Most of these bacterial families (40 out of 45) were enriched in metamorphic samples, being Verrucomicrobiaceae, Caulobacteraceae and Sphingomonadaceae the families with higher W values. In contrast, five bacterial families were enriched in non metamorphic samples with Burkholderiaceae, Chitinophagaceae being the families with higher W values.

To identify the core microbiota of each *A. altamirani* metamorphic stage we calculated the bacterial ASVs that were highly prevalent in either metamorphic or non metamorphic individuals but absent from environmental samples. We found that seven bacterial ASVs represent the bacterial core of metamorphic axolotls accounting for a cumulative relative abundance of 17.4%. Meanwhile, four bacterial ASVs represent the bacterial core of non metamorphic axolotls accounting for 52.26% of the relative abundance (Table 2). Interestingly, two of the four core ASVs present in non metamorphic samples accounted for 25.9% and 20.75% of the relative abundance in these samples and belonged to the bacterial families Chitinophagaceae and Burkholderiaceae respectively. In addition, we identified that two ASVs from the Pseudomonadaceae family were part of the core of both metamorphic and non metamorphic samples.

Table 2
Amplicon sequence variants (ASVs) defining the core skin microbiota of metamorphic and non metamorphic *A. altamirani*.

ASV ID	Sample Type	Family	Relative abundance	Persistence
9936daae333af6e517a9deb4b9e18ffa	Metamorphic	Pseudomonadaceae	13.83	95.12%
6d0c9d0395e6a2a7667eb0b07c17a275	Metamorphic	Burkholderiaceae	1.4	97.56%
17d60505100c3cf44d4f9fad620d1636	Metamorphic	Pseudomonadaceae	0.74	93.90%
be8eb25874b4202cf98050dbadeeb7ce	Metamorphic	Burkholderiaceae	0.33	93.90%
8fd9eab61a0f63db6cbb201ce66b484b	Metamorphic	Burkholderiaceae	0.36	82.93%
6ddbdbae5830daa9d7d08270857b02b8	Metamorphic	Rhizobiaceae	0.27	82.93%
d21b6c5e4a5c9d5f40bda6b543e4208f	Metamorphic	Xanthomonadaceae	0.21	82.93%
3c28f0caf9183357de05d1882a943f8e	Non metamorphic	Chitinophagaceae	25.09	96.84%
ed5a79897d0f82525c3854759d384c26	Non metamorphic	Burkholderiaceae	20.75	98.42%
9936daae333af6e517a9deb4b9e18ffa	Non metamorphic	Pseudomonadaceae	6.03	83.16%
17d60505100c3cf44d4f9fad620d1636	Non metamorphic	Pseudomonadaceae	0.39	87.37%

ASVs were considered part of the skin bacterial core if they were present in ≥ 80% of the skin samples for metamorphic or non metamorphic axolotls.

Seasonality And Location Differentially Influence Skin Bacterial Diversity In Metamorphic And Non Metamorphic Axolotls

Physicochemical variables measured at each sampling location (pH, conductivity, dissolved oxygen, maximin, minimum mean and delta seasonal temperatures) varied significantly across seasons (MANOVA, Wilks = 0.002, p-value < 0.001) and sampling locations (MANOVA, Wilks = 0.0009, p-value < 0.001). While all physicochemical variables varied across seasons, dissolved oxygen was the only variable that did not vary between sampling locations (Additional file 2, Supplementary Table 4).

Alpha PD of metamorphic axolotls varied significantly across seasons (KW, $\chi 2$ = 13.69, p-value = 0.003) (Fig. 3A) and post-hoc pairwise comparisons showed that only the transition between winter-spring was significant (Wilcoxon, p-value = 0.005) (Additional file 2, Supplementary Table 5). In contrast, PD of non metamorphic *A. altamirani* (Fig. 3B) did not differ across consecutive seasons (KW, $\chi 2$ = 0.21, p-value = 0.97) (Additional file 2, Supplementary Table 5).

Additionally, we found that seasonality significantly influenced skin bacterial beta diversity of metamorphic (PERMANOVA, pseudo-F = 12.37, p-value < 0.001) (Fig. 3D, Additional file 2, Supplementary Table 6) and non metamorphic (PERMANOVA, pseudo-F = 15.69, p-value < 0.001) (Fig. 3E, Additional file 2, Supplementary Table 7)

axolotls. Specifically, pairwise PERMANOVAs showed that metamorphic samples differed between winter-spring seasons (PERMANOVA, pseudo-F = 14.92, p-value = 0.001), while non metamorphic skin microbiota differed between autumn-winter (PERMANOVA, pseudo-F = 13.47, p-value < 0.001) and winter-spring seasons (PERMANOVA, pseudo-F = 12.61, p-value < 0.001).

Three bacterial families were identified by ANCOM as differentially abundant in metamorphic samples between winter-spring seasons (Fig. 4). In the case of non metamorphic individuals, ANCOM identified three bacterial families that were differentially abundant between autumn-winter and seven families as differentially abundant between winter-spring (Fig. 4). Psuedomonadaceae, Aquaspirillaceae and Shewanellaceae were shared between metamorphic and non metamorphic axolotls during winter and spring seasons. However, Pseudomonadaceae was differentially enriched according to host metamorphic status, being more abundant in metamorphic axolotls during spring and more abundant in non metamorphic axolotls during winter.

When analyzing the effect of location in the skin bacterial diversity, we found that PD of metamorphic samples differed significantly between sampling locations (KW, $\chi 2$ = 9.69, p-value = 0.02), however *post hoc* paired test showed that PD only differed significantly between sites 2 and 3 (Additional file 1, Supplementary Fig. 2A). Bacterial PD of non metamorphic samples also varied significantly between sampling locations (KW, $\chi 2$ = 40.9, p-value = 6.71e-9). *Post hoc* test showed that most pairwise comparisons were significant with the exception of sites 1 and 3 and sites 2 and 3 (Additional file 1, Supplementary Fig. 2C, Additional file 2, Supplementary Table 8). Beta diversity of the skin bacterial communities was also influenced by sampling location in metamorphic (PERMANOVA, pseudo-F = 2.71, p-value = 0.006) and in non metamorphic samples (PERMANOVA, pseudo-F = 31.34, p-value = 0.001) (Additional file 1, Supplementary Fig. 2B, D). Pairwise comparisons showed that bacterial beta diversity only differed between sites 2 and 3 in metamorphic axolotls (Additional file 2, Supplementary Table 9), while bacterial beta diversity differed between all sampling locations for non metamorphic samples (Additional file 2, Supplementary Table 10).

Biotic and abiotic factors influence the skin bacterial community structure of *A. altamirani*

Our results showed that beta bacterial diversity of *A. altamirani* skin is influenced by season and location. To assess the specific influence of all the biotic and abiotic factors measured in this study we performed a distance-based Redundancy Analysis (dbRDA) on the skin bacterial beta diversity. After forward model selection, that incorporates all the variables measured, only the following biotic and abiotic factors that resulted informative were included in the dbRDA regression model: host metamorphic status, host weight, pH, dissolved oxygen, conductivity, mean temperature, season delta temperature (difference between the maximum and minimum seasonal temperature) and site elevation.

The dbRDA calculated eight canonical components for the PCA, however anova.cca (by = axis) showed that only four of these canonical components were statistically significant. These four statistically significant canonical axes explained 26.47% of the variation in the weighted UniFrac distance matrix (Table 3, Additional file 1, Supplementary Fig. 3). Permutational analyses (anova.cca, by = terms) over each variable in the model showed that the metamorphic status of the host (PERMANOVA, pseudo-F = 39.1, p-value = 0.001) had the greatest effect-size over the variation, followed by seasonal delta temperature (PERMANOVA, pseudo-F = 19.8, p-value = 0.001), pH (PERMANOVA, pseudo-F = 15.85, p-value = 0.001) and seasonal mean temperature (PERMANOVA, pseudo-F = 12.05, p-value = 0.001) (Fig. 3C,Table 4).

Table 3
Variance explained of each canonical axis calculated by dbRDA.

	F	p-value	Variance explained	Cumulative variance
CAP1	63.3875	**0.001**	**0.173**	**0.173**
CAP2	18.4679	**0.001**	**0.050**	**0.223**
CAP3	9.8065	**0.001**	**0.026**	**0.250**
CAP4	5.1977	**0.006**	**0.014**	**0.264**
CAP5	2.5146	0.123	0.006	0.271
CAP6	1.5675	0.387	0.004	0.275
CAP7	1.1402	0.563	0.003	0.279
CAP8	0.7233	0.764	0.001	0.281

Columns indicate: F statistic, p-values, variance explaines by each canonical axis, and the cumulative variance calculated by the Permutational like ANOVA. Numbers in bold indicate significant p-values and the cumulative variance for each statistically significant canonical axis.

Table 4
Permutational like ANOVA results of each variable introduced in the dbRDA regression model.

	F	p-value
Developmental Stage	39.121	**0.001**
Delta Temperature	19.889	**0.001**
pH	15.854	**0.001**
Mean Temperature	12.053	**0.001**
Elevation	5.334	**0.001**
Dissolved Oxygen	4.478	**0.002**
Conductivity	3.470	**0.005**
Weight	2.604	**0.018**

Columns indicate: F statistic, p-values calculated by Permutational like ANOVA for each variable. Numbers in bold indicate significant p-value.

Skin bacterial diversity of *A. altamirani* is not influenced by Bd infection status but specific bacterial taxa abundance correlate with infection intensity

Alpha PD did not differ between infected and non-infected samples in both metamorphic (KW, = 0.09, p-value = 0.76) (Figure 5A) and non metamorphic (KW, = 0.51, p-value = 0.47) *A. altamirani* samples (Figure 5C). Additionally, beta diversity based on the weighted UniFrac distance matrix did not vary between infected and non-infected samples for metamorphic (PERMANOVA, pseudo-F = 1.37, p-value = 0.19) (Figure 5B) and non metamorphic salamanders (PERMANOVA, pseudo-F = 2.45, p-value = 0.08) (Figure 5D).

Even though alpha and beta diversity did not vary according to Bd infection status. Kendall's correlation test showed that the relative abundance of 139 and 129 ASV present in infected metamorphic and non metamorphic samples respectively, correlated with pathogen infection loads (Additional file 1, Supplementary Fig. 4). Specifically, 116 (out of 139) and 52 (out of 128) bacterial ASVs had positive correlations with pathogen infection loads in metamorphic and non metamorphic samples respectively.

All the ASVs that correlated with pathogen load in metamorphic samples had low relative abundances (0.001–0.67%) (Additional file 1, Supplementary Fig. 4A), while in non metamorphic samples the correlated ASVs ranged from 0.001–28.5% (Additional file 1, Supplementary Fig. 4B). Twelve ASVs with significant correlations were shared between metamorphic and non metamorphic samples and six of these showed different types of correlations with Bd loads according to axolotl metamorphic status. Interestingly, six of the twelve shared ASVs, had different correlations (negative vs positive) according to host metamorphic status (Additional file 2, Supplementary Table 11).

Discussion

The aim of this study was to evaluate the influence of metamorphosis, seasonality and pathogen presence over the skin microbiota of the axolotl *A. altamirani*. Since this is the first study exploring the skin microbiota of *A. altamirani*, we also evaluated if skin bacterial diversity differed from environmental bacterial communities of the streams where this species inhabits.

Consistent with previous studies showing differences between amphibian skin microbiota and their surrounding environmental bacterial communities [20, 46], we found that *A. altamirani* skin bacterial microbiota significantly differed from environmental samples, and that a great portion of the ASVs were unique to each sample type, supporting the idea that the amphibian skin hosts a distinctive bacterial repertoire [18, 47].

Several studies have shown that amphibian skin microbiota varies significantly across host development [26, 27, 48]. These studies focused on amphibian species that transition from an aquatic larval stage to a terrestrial adult stage [22, 34, 35, 38], making it difficult to tease apart the effects of host development stage and habitat conditions on skin microbial diversity [17, 18]. For species where adult and larval stages coexist in the same aquatic environment (i.e newts), host developmental stage had contrasting results in different species; for example adult and larvae of *Lissotriton boscai* showed clear differences in skin bacterial community composition, however this pattern was not observed in *Triturus marmoratus* [35].

In this study, we evaluated the influence of metamorphosis over skin bacterial diversity on a paedomorphic salamander species (axolotl) in which metamorphic and non metamorphic stages coexist in permanent streams along their life cycle [44, 49]. Our results showed that *A. altamirani* skin bacterial communities are strongly shaped by metamorphosis. Specifically, we found that non metamorphic individuals harbor less diverse and more disperse skin bacterial communities compared to metamorphic individuals. These differences could be explained by differences in skin mucus composition, immune response or gene expression before and after metamorphosis [30, 50, 51]. However, bacterial diversity patterns between metamorphic and non metamorphic *A. altamirani* axolotls contrast with a previous report on captive axolotl *A. mexicanum*, were non metamorphic individuals exhibited higher bacterial diversity over the skin compared to chemically induced metamorphic individuals [28].

Core microbiota analysis and ANCOM results highlighted differences in composition between metamorphic and non metamorphic axolotls. Specifically, non metamorphic skin microbiota is composed by less core members and have less differentially abundant bacterial ASVs when compared with metamorphic skin microbiota. It is interesting to highlight that both analyses identified that families Chitinophagaceae and Burkholderiaceae were enriched in non metamorphic samples, specially two ASVs from these families that accounted for 45.84% of the relative abundance. The high dominance of these bacterial taxa could explain in some extent the diversity patterns observed between metamorphic and non metamorphic samples, following previous observations in plant and animal microbiomes were bacterial diversity decreased in communities with highly dominant species, while more diverse communities lacked dominant species [52, 53].

Temporal and spatial dynamics of amphibian skin microbiota have been linked to variation in environmental factors such as temperature, precipitation or elevation [25, 26, 35–37]. Specifically, temperature fluctuations over short periods of time [22] and seasonal variation (dry – wet) [39] has been linked to differences in bacterial skin diversity on amphibians inhabiting aquatic environments. Our results showed that seasonal variation of temperature (delta temperature and mean temperature), pH, conductivity, and dissolved oxygen influence axolotl skin bacterial diversity.

Previous studies have shown clear location effects on alpha and beta skin bacterial diversity of terrestrial salamanders [14, 48, 54]; for example genetically and geographically different populations of *Ensatina eschscholtzii* vary in bacterial community composition [15]. In this study we found that sampling location significantly influences skin bacterial diversity, and this effect is stronger in non metamorphic axolotls. Due to differences in physicochemical conditions across sites we suggest that environmental differences might be driving the differences in skin microbial diversity. However, genetic differences across populations could also explain some of our results, since a previous study showed that *A. altamirani* populations of sites 2 and 3 are genetically different [55]. Additional work is needed to tease apart the effects of environment and host genetics on the skin microbial diversity of *A. altamirani*.

Differential responses to seasonal environmental variation and sampling location between metamorphic and non metamorphic skin bacterial diversity suggest that the metamorphic stage allows for a more stable skin microbiota possibly due to the development of a mature immune system [30] and skin gland maturation during metamorphosis [29, 50] which are involved in the synthesis of antimicrobial peptides.

An alternative hypothesis is that the higher bacterial diversity observed in metamorphic axolotl skin contributes to a higher resistance or resilience to seasonal environmental variation when compared to the less diverse skin bacterial communities of non metamorphic samples [56, 57]. However, recent results in *Rana sierrae* showed that individuals with low bacterial diversity are more stable across time [40]. We believe that future studies focusing on evaluating functional stability could be helpful to better understand how seasonality influences skin microbiota of *A. altamirani*.

Disruption of the skin microbiota following Bd infections has been previously documented in naive amphibian populations before and after Bd infection [20, 21], and in populations with different pathogen intensities where Bd seems to be present in an enzootic stage [23, 41]. Even when Bd was highly prevalent in the *A. altamirani* populations analyzed in this study [58], we did not find any significant influence of the presence of Bd when comparing the bacterial alpha and beta diversity of the skin microbiota between infected and non-infected *A.*

altamirani samples. This is particularly interestingly, since Bd prevalence and Bd infection intensity reached high levels [58].

Previous studies have shown significant correlations between the relative abundance of bacterial taxa and chytrid infection loads [19, 41, 42]. Our results showed significant correlations between several ASVs with Bd infection loads. However, most of these correlations were found with ASVs with very low relative abundances with the exception of two Proteobacteria and Bacteroidetes ASVs present in non metamorphic samples.

Inhibitory potential against Bd has been described for several bacterial isolates mainly form Burkholderiaceae, Yersiniceae, Pseudomonadaceae or Xanthamondaceae families [60–64]. We found that Burkholderiaceae and Chitinophagaceae were highly abundant over *A. altamirani* skin, and it has been shown that high abundance of Burkholderiaceae in *Anaxyrus boreas* skin microbiota correlates with reduced fungal presence over the skin during early life stages [27]. Additionally populations of *R. sierrae* with higher Bd loads showed lower abundances of Burkholderiaceae when compared to *R. sierrae* populations with lower Bd loads [21, 41]. In the case of Chitinophagaceae little is known about their inhibitory ability against Bd with only few isolates considered as putative Bd-inhibitory strains [27], and further work is needed to elucidate if members of this bacterial family present on *A. altamirani* skin display inhibitory functions against Bd.

Conclusion

Our results show that host metamorphic status is a major determinant of *A. altamirani*, influencing diversity and structure of the symbiotic skin bacterial communities. To our knowledge this study is the first to address how the effects of environmental variation over the skin microbiota are dependent on the amphibian developmental stage; we demonstrate that seasonal environmental variation significantly influences bacterial skin diversity of *A. altamirani*, and that metamorphic and non metamorphic axolotls response differently to environmental variation. Despite a growing body of literature suggesting that Bd influences skin bacterial diversity we did not find such effect. Nonetheless, we found that particular bacterial taxa are likely interacting with Bd. Further studies using metagenomics and cultivation techniques could elucidate if changes in skin microbiota across development and across seasons are reflecting functional differences regarding Bd inhibition or other host symbiotic traits [65, 66].

Methods

Sample collection

Skin samples were collected during four sampling periods at three-month intervals (July 2019, October 2019, January 2020, and April 2020) spanning all the seasons of a whole year at four localities at La Sierra de Cruces, Estado de México, México (Table 1). Individuals of *A. altamirani* were captured at each location using dip nets and held individually in sterile plastic containers filled with stream water until swabbing. Sampling effort consisted in the active search of salamanders for three consecutive hours across a 150m transect along each stream. Each captured salamander was manipulated with sterile nitrile gloves, rinsed with 25 ml of sterile deionized water to remove transient microorganisms from the skin and swabbed 30 times (five times in their ventral and dorsal surface each and five times in each limb joint) using sterile rayon swabs (MWE, Corsham UK). Swabs were stored in 1.5 ml microcentrifuge tubes containing 170µl of DNA/RNA Shield (Zymo Research, Irvine, USA) and kept at 4°C during field work. Once in the laboratory tubes were stored at -80°C until processing. Immediately after swabbing morphometric measurements of weight, tail and body length were registered for each individual. Once all axolotls

were swabbed and measured, they were released at the same site of capture. Sampling was approved by Subsecretaría de Gestión para la Protección Ambiental under the permit number: SGPA/DGVS/5673/19.

For the purposes of this work, we classified axolotl samples as metamorphic and non metamorphic according to the presence or absence of gills as reported previously [58]. Recognizing that gilled individuals of *A. altamirani* could be either juvenile or paedomorphic adults, we classified non-gilled axolotls as metamorphic and gilled axolotls as non metamorphic respectively in order to evaluate the effect of the metamorphic status of the host.

Additionally, five samples of sediment and water were collected at each location in all sampling periods. Water samples were obtained by submerging a sterile rayon swab at approximately 20 cm deep inside water for 10 seconds, and sediment samples were obtained by submerging swabs inside the bottom sediment of the stream for 10 seconds [46].

Environmental Characterization

Stream water temperature was recorded at 1h intervals during one year at each sampling location using Onset HOBO dataloggers (Onset Computer Corporation, Bourne, USA) from June 2019 to April 2020. Additionally, pH, dissolved oxygen and conductivity of the water was registered using a HANNA multiparameter HI98194 (HANNA Instruments, USA) during each sampling. Measurements were taken at each location in triplicate across 10 m transects.

To evaluate if these physicochemical variables vary between seasons and sampling location, we applied a two-way MANOVA test in R (v 4.0.2).

Dna Extraction And Sequencing

Amplicon libraries of the 16S rRNA gene spanning the V4 region were constructed using 515F/806R primers following the Earth Microbiome Project standard protocol (www.earthmicrobiome.org) and previously published studies [46, 67]. In brief, DNA was extracted from skin and environmental swabs using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Valencia, USA) following manufacturer instructions with an initial lysozyme incubation step at 37 ° for 1 h. Samples were PCR amplified in triplicate plus one negative control per sample, PCR products and negative controls were verified in 1% agarose gels, and PCR products were pooled in one tube per sample. Pools were quantified using a Qubit 4.0 fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, USA), samples were pooled in two amplicon libraries at a concentration of 240 ng per sample (221 and 217 samples each). Each pool was cleaned using the QIAquick PCR clean up kit (Qiagen, Valencia, USA). 16S amplicon libraries were sequenced in two sequencing runs (250 single end) using v2 Illumina chemistry at Dana-Farber Cancer Institute of Harvard University.

Bioinformatic Pipeline

Sequences were processed using Quantitative Insights Into Microbial Ecology (QIIME v2-2020.2) [68]. A total of 8,434,775 and 8,821,621 demultiplexed raw sequences were obtained for each sequencing run respectively. Sequences were quality filtered and denoised independently for each run using the DADA2 plugin to obtain two single feature table. Seven skin and eight environmental samples were discarded due to low quality reads. After

quality filtering feature tables were merged to generate a final Amplicon Sequence Variant (ASV) table containing 14,415,727 reads with a mean read depth of 32,900 reads per sample.

A phylogenetic tree was generated using the representative sequences of the ASV table using the q2-phylogeny plugin which first uses mafft to perform sequence alignment and then generate a phylogeny using FastTree. Samples were rarefied at 10,000 reads per sample according to observed ASV rarefaction curves in order to preserve the largest number of samples and sequences. Alpha and beta diversity metrics were calculated using the q2-diversity plugin using the rarefied table. Taxonomy was assigned using a naive Bayesian classifier pre-trained for the V4 region (515F / 806R 16s rRNA) on the SILVA 132 99% data base [69].

Microbial Diversity And Composition Analyses

Statistical analyses for alpha and beta diversity metrics were computed in R (v 4.0.2) unless otherwise stated. Kruskal-Wallis (KW) and *post hoc* Wilcoxon ranks sum test were used to determine differences in alpha diversity (Shannon, Faith's Phylogenetic Diversity (PD) and observed ASVs) across sample types (metamorphic, non metamorphic, sediment, and water), seasonality (summer, autumn, winter, and spring) and Bd infection status (infected and non-infected). Beta diversity was evaluated using weighted UniFrac distances to determine differences in bacterial community structure across sample types, seasons and Bd infection status, Statistical comparisons were conducted with permutational multivariate analyses (PERMANOVA) using the q2-diversity plugin in Qiime2 (v 2020.2). Beta diversity dispersion was calculated from the weighted UniFrac distance matrix using the function betadisper in the vegan package [70]. PERMUTEST based on 99 permutations was used to evaluate if dispersion differed between sample types.

ANCOM [71], was used to identify bacterial ASVs that were differentially abundant between metamorphic and non metamorphic salamanders and between samples from consecutive seasons (summer-autumn, autumn-winter, winter-spring). ANCOM applies a centered log ratio transformation on the relative abundance of each ASV and tests the null hypothesis that mean log absolute abundance of each ASV does not differ between sample types. An internal statistic (W) is calculated each time a taxa rejects this null hypothesis, then ANCOM generates an empirical distribution using W values in order to test which taxa are differentially abundant between sample types.

ANCOM between consecutive seasons was only applied if PERMANOVA results showed significant differences between consecutive seasons (winter-spring for metamorphic salamanders and autumn-winter and winter-spring for non metamorphic salamanders). Prior to performing the analyses low abundant ASVs (< 50 reads) were filtered out and ANCOM was performed using the q2-composition plugin in Qiime2.

Core microbiome of metamorphic and non metamorphic axolotls was calculated using feature-table plugin in Qiime2. In brief, two feature tables containing only ASVs present in metamorphic or non metamorphic axolotls were generated using the feature-table plugin. Then, using the core-features function ASVs present in ≥ 80% were selected as part of the core skin microbiota of metamorphic and non metamorphic axolotls.

Additionally, correlations between the relative abundance of each ASV of the infected samples and Bd infection intensities were calculated with Kendall rank correlation coefficient correcting for multiple comparisons (Benjamini-Hochberg) using cor.test function of the stats package in R [72]. To generate graphics for all the results Qiime2 artifacts were imported to R using the package qiime2R [73], then figures were generate using packages ggplot2 [74], Fantaxtic [75] and UpSetR [76].

Biotic and abiotic factors influencing the skin microbial structure.

In order to explore the specific influence of biotic (developmental stage, weight, tail length, snout vent length, Bd presence and Bd infection intensity) and abiotic factors (pH, conductivity, dissolved oxygen, mean season temperature, delta season temperature, and elevation) over the skin microbial structure, we applied a distance-based redundancy analysis (dbRDA) on the weighted UniFrac distance matrix using the capscale function of the vegan package [70]. dbRDA is a canonical ordination method that applies multiple linear regression to a distance matrix and then computes a principal component analysis (PCA) [77]. Prior to analyses non-categorical biotic and abiotic variables were z-scored to control for differences in magnitudes between factors. The ordistep function of the vegan package [70] was used for model selection in both directions with 999 permutations to select the best regression model. Once the dbRDa was obtained anova.cca function was used to perform an ANOVA like permutation test to evaluate the significance of each calculated canonical axis (anova.cca, by = axix) and the specific significance of each factor in the regression model (anova.cca, by = terms).

Abbreviations

ANCOM: Analysis of composition of microbiomes

ASV: Amplicon sequence variant

Bd: *Batrachochytrium dendrobatidis*

KW: Kruskal-Wallis

dbRDA: Distance based redundancy analysis

MANOVA: Multivariate analysis of variance

PD: Phylogenetic diversity

PERMANOVA: Permutational multivariate analysis of variance

PERMUTEST: Permutation-based test of multivariate homogeneity of group dispersions

Declarations

Ethics approval and consent to participate

Our research was approved by the ethical standards of Universidad Nacional Autónoma de México, additionally capture and sampling of *A. altamirani* was approved by Subsecretaría de Gestión para la Protección Ambiental under the permit number: SGPA/DGVS/5673/19.

Consent for publication

Not applicable.

Availability of data and material

All 16s rRNA gene raw data in this study are publicly available at the NCBI SRA under BioProject PRJNA819099. Sample metadata, output data of DADA2, R and Qiime2 scripts for analysis and figures included in this manuscript are available at https://github.com/EmanuelMartinez-Ugalde/A.-altamirani-16S-skin-microbiota.

Competing interests

The authors declare no competing interests.

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Authors' contributions

ER designed the study. All authors contributed to sample collection. EM-U performed the molecular work and bioinformatic analysis. EM-U and ER wrote the manuscript. All authors participated in the improvement of the manuscript.

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Figures

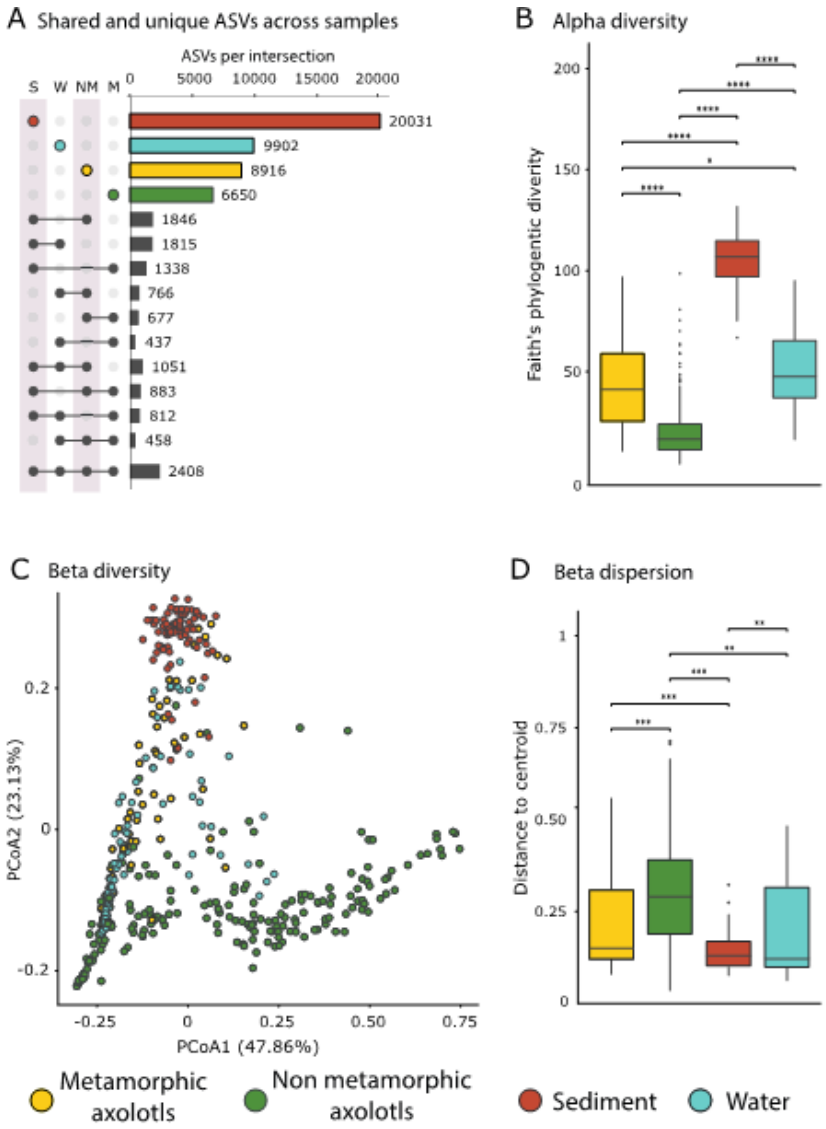

Figure 1

Bacterial diversity of *A. altamirani* skin and environmental samples. A) Upset plot illustrating the number of unique and shared ASVs. Numbers aside the color bars indicate how many ASVs were present on each sample type (color bars) and shared between sample types (gray bars). B) Alpha Faith's Phylogenetic diversity (PD) across sample types. C) Principal coordinate analysis (PCoA) based on weighted UniFrac distances across sample types. D) Beta dispersion using Analysis of multivariate homogeneity of groups dispersions. Brackets in B and D indicates statistically significant *post hoc* Wilcoxon pairwise comparisons (* = 0.01, ** = 0.001, *** = 0.0001, **** = 0.00001).

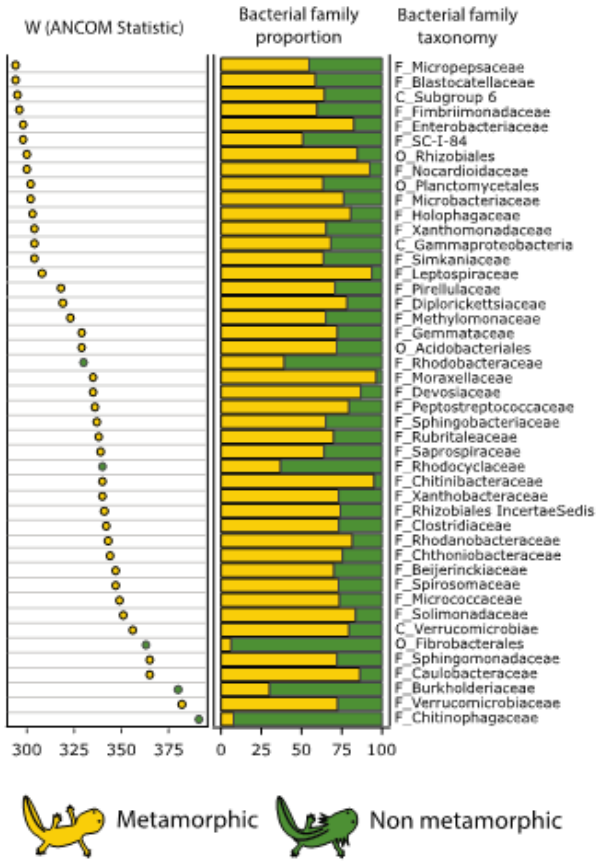

Figure 2

ANCOM results showing differentially abundant bacterial families between metamorphic and non metamorphic axolotls. Left panel shows ANCOM W values, middle panel shows the relative proportion for each bacterial family, and right panel shows the best taxonomic assignation according to SILVA database at order (O), class (C) or family (F) level. Circles and bars are color-coded according to the host metamorphic status.

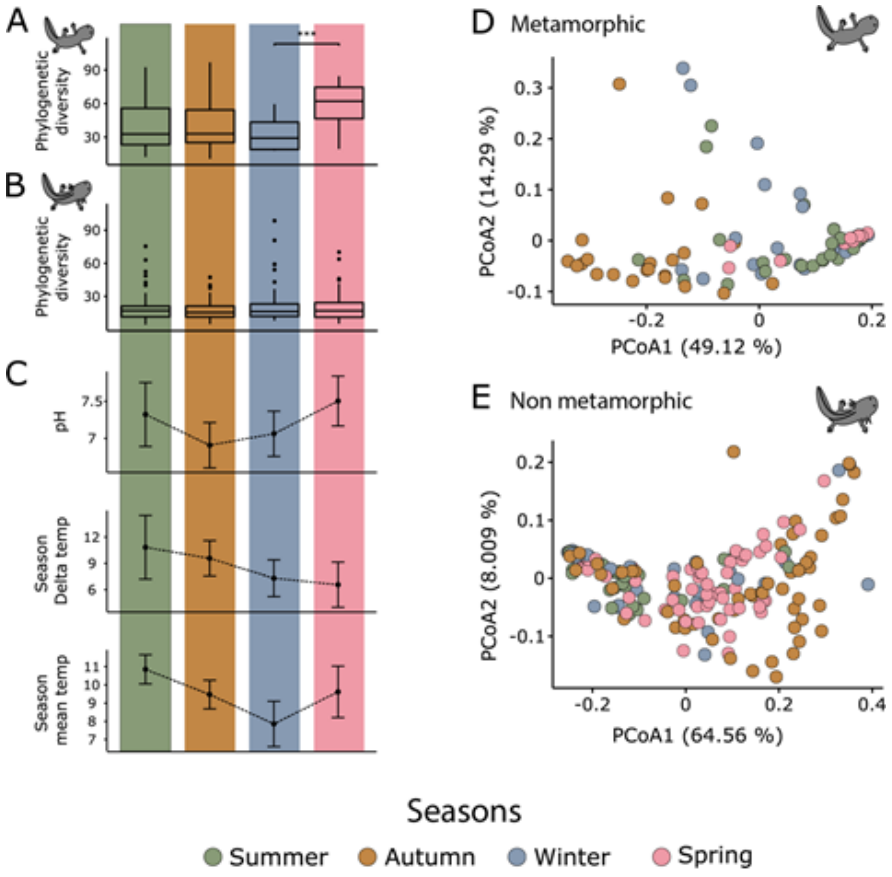

Figure 3

Seasonal influence over metamorphic and non metamorphic skin bacterial diversity. A) Phylogenetic diversity (PD) across seasons in metamorphic samples. Brackets indicate statistically significant *post hoc* Wilcoxon pairwise comparisons between seasons (* = 0.01, ** = 0.001, *** = 0.0001, **** = 0.00001). B) PD across seasons in non metamorphic samples. C) Seasonal variation of pH, delta temperature and mean temperature of the stream water. D) Principal coordinate analysis (PCoA) based on weighted UniFrac distances across seasons of metamorphic samples. E) PCoA based on weighted UniFrac distances across seasons in non metamorphic samples. Circles in D and E panels are color-coded by season.

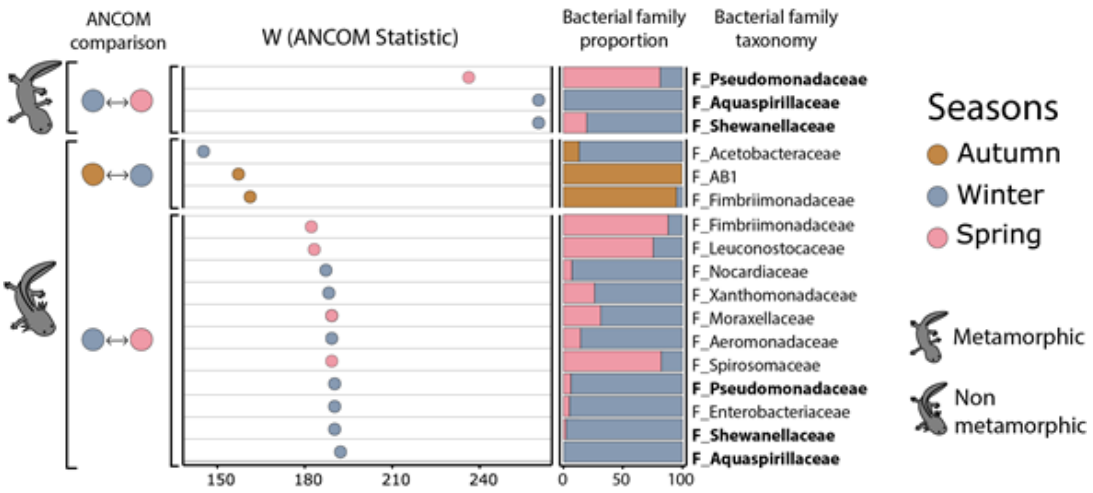

Figure 4

ANCOM results showing differentially abundant bacterial families in metamorphic and non metamorphic axolotls across consecutive seasons: autumn to winter seasons in non metamorphic axolotls, and winter to spring seasons for metamorphic and non metamorphic axolotls. From left to right: ANCOM comparisons color-coded by season, ANCOM W values, the relative bacterial family proportion and the best taxonomic assignation according to SILVA at order (O), class (C) or family (F) level. Circles and bars are color-coded by season. Shared bacterial families between metamorphic and non metamorphic axolotls between winter and spring seasons are shown in bold.

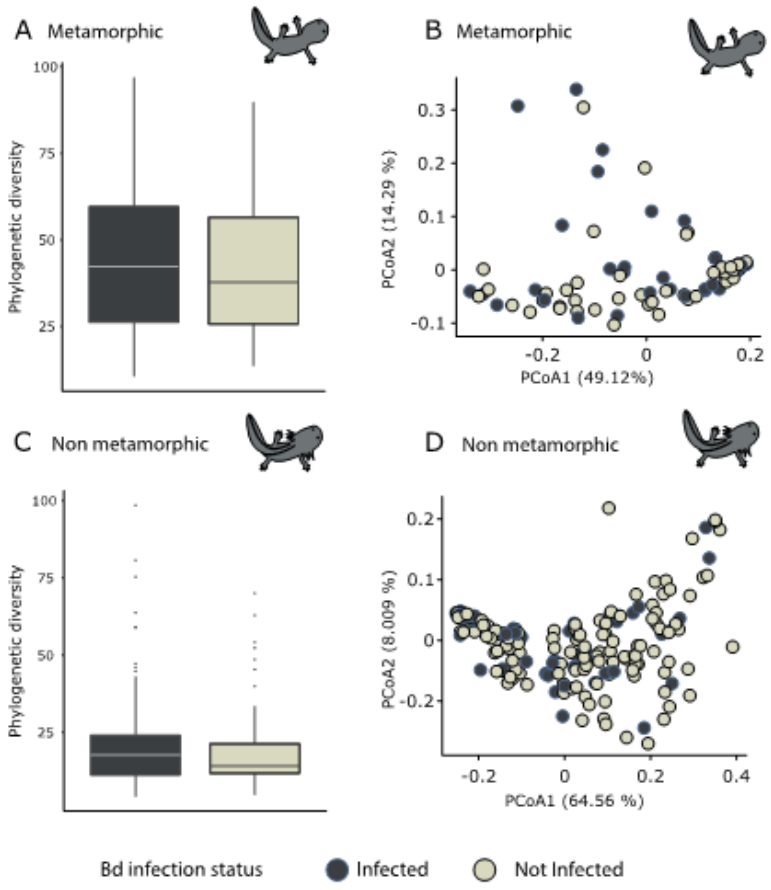

Figure 5

A. altamirani skin bacterial diversity with respect to Bd infection status. A) Alpha phylogenetic diversity (PD) between infected and non-infected in metamorphic axolotls. B) Principal coordinate analysis (PCoA) based on weighted UniFrac distances in infected vs non-infected of metamorphic samples. C) PD between infected and non-infected in non metamorphic salamanders. D) PCoA based on weighted UniFrac distances in infected vs non-infected in non metamorphic. Circles are color-coded by Bd infection status.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1SF14.pdf
- Additionalfile2ST111.pdf