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Blurred Lines Between Determinism and Stochasticity in an Amphibian Phylosymbiosis Under Pathogen Infection

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ABSTRACT

Selection, dispersal and drift jointly contribute to generating variation in microbial composition within and between hosts, habitats and ecosystems. However, we have limited examples of how these processes interact as hosts and their microbes turn over across latitudinal gradients of biodiversity and climate. To bridge this gap, we assembled an extensive dataset of 580 skin bacteriomes from 22 amphibian species distributed across a 10° latitudinal range in Chile. Amphibians are susceptible to the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), which infects their skin, potentially leading to changes in the normal skin microbiome (i.e., dysbiosis). Using comparative methods, accounting for pathogen infection and implementing resampling schemes, we found evidence of phylosymbiosis, characterised by more similar bacterial communities in closely related amphibian species. We also compared how neutral processes affected the assembly of skin bacteria by focusing on two widespread species from our dataset: the Chilean four-eyed frog (*Pleurodema thaul*) and Darwin's frog (*Rhinoderma darwinii*). Neutral models revealed that dispersal and chance largely facilitated the occurrence of ~90% of skin bacteria in both species. Deterministic processes (e.g., phylosymbiosis, active recruitment of microbes, microbe–microbe interactions) explained the remaining fraction of the bacteriomes. Amphibian species accounted for 21%–32% of the variance found in non-neutral bacterial taxa, whereas the interaction with *Bd* carried a weaker but still significant effect. Our findings provide evidence from ectotherms that most of their skin bacteria are subject to dispersal and chance, yet contemporary and historical contingencies leave strong signatures in their microbiomes even at large geographical scales.

1 | Introduction

Emerging infectious pathogens are widely recognised for their ability to disrupt host populations (Russell et al. 2020), yet many species exhibit mechanisms that allow population persistence in the face of disease. Pathogens not only affect survival (Lips et al. 2006; Stegen et al. 2017), but also have sublethal effects on host reproduction and other fitness-related traits (Chatfield et al. 2013; Wu et al. 2018). Host-associated microbial communities form part of these individual traits and represent one of the

most significant knowledge gaps in the ecology and evolution of infectious diseases (Bernardo-Cravo et al. 2020; Hird 2019; Lively et al. 2014), particularly for threatened species (Jiménez and Sommer 2017; West et al. 2019). Host-associated microbes can provide critical functions that determine disease outcomes (Allender et al. 2018; Jani et al. 2017; Keady et al. 2023; Muletz et al. 2012). For example, microbes shape host immunity (Hanson et al. 2023), mediate reproductive signalling and mate choice (Brunetti et al. 2022, 2019; Comizzoli et al. 2021) and produce chemical defence against predators (Vaelli et al. 2020). Furthermore, host-associated microbes vary across climatic gradients and hosts' immune complexity (Kueneman et al. 2019; Woodhams et al. 2020). Although it is widely accepted that both selection and stochasticity modulate host-microbe interactions in the presence of emerging infectious diseases (Longo and Zamudio 2017; Wilber et al. 2020), there is still a lack of integrative studies investigating microbe assembly processes at latitudinal scales where multiple host species coexist with emerging pathogens.

Amphibians provide an ideal study system to investigate large-scale shifts in microbiome composition and structure in relation to pathogen infection. The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) has been detected in more than 1375 species of amphibians globally (Olson et al. 2021). *Bd*-mediated population declines have occurred in one-third of these species (Scheele et al. 2019). Host-associated microbes have been proposed as mediators of susceptibility to infection and disease (Bletz et al. 2013; Buttimer et al. 2024; Rebollar et al. 2020). However, it has not been possible to establish generalities for the observed patterns of microbial diversity between infected and uninfected individuals across amphibian species and geographical regions. For example, in hylid frogs, *Bd* infection was not associated with alpha and beta diversity metrics of skin microbiomes (Ellison et al. 2019). In contrast, in ranids, uninfected frogs carried higher bacterial richness than *Bd*-infected frogs (Schmeller et al. 2022). Although hylids and ranids are comparable in clade age and species diversity (Wiens et al. 2009), other traits such as their habitat use, physiology and behaviour can confound cross-species comparisons. Ideally, investigating species-level microbiome composition requires robust empirical approaches to reduce variation introduced by many variables at the host and environmental levels (Couch and Epps 2022; Degregori et al. 2024; Trevelline et al. 2020).

Disentangling the roles of determinism and stochastic processes in generating differences in host-associated microbial diversity can be achieved using a combination of phylogenetic comparative methods and neutral models. Determinism describes predictable events such as environmental filtering, biotic interactions and phenological patterns (Clements 1916), whereas stochasticity refers to random variation introduced by chance and dispersal (Gleason 1917). Phylogenetic comparative methods can demonstrate whether host evolutionary history influences microbial diversity, which is typically analysed as a continuous trait or by comparing distance matrices (Lim and Bordenstein 2020). Host evolutionary relationships are deterministic and can explain clustering patterns of microbiome diversity and community structure through the convergence of diets, habitats, behaviours, physiology and genetics (Belasen et al. 2021; Buttimer et al. 2021; Ellison et al. 2019; Harrison et al. 2021). The term 'phylosymbiosis' has been applied to describe this pattern (Lim and Bordenstein 2020), in which closely related species tend to harbour more similar microbial communities than distantly related species (Blomberg et al. 2003; Kohl 2020; Lim and Bordenstein 2020; Losos 2008). Host specificity allows for increased instances of vertical transmission of symbionts (Kouete et al. 2023), which in turn influences speciation and hybridization (Miller et al. 2021). Thus, overlapping host traits can functionally constrain microbiomes if there are limitations related to microbial dispersal.

On the other hand, neutral models assume that microbial community assembly is uncoupled from host phylogenetic history, as microbes should have equivalent fitness responses in their local environments. Neutral community models can identify specific taxa facilitated by dispersal, chance and drift, as well as those deviating from the expectations that are responding to selection (Sloan et al. 2006). However, because each host species represents an environment with a unique evolutionary history, contemporary microbial diversity may reflect a combination of both deterministic and stochastic processes operating at different timescales (Zhou and Ning 2017). For example, abiotic and biotic factors are considered deterministic processes, but when selection is heterogeneous across space and time, different taxa can be selected at distinct times, leading to uncertainty. This uncertainty has been further defined into three distinct classifications: (1) demographic stochasticity, (2) environmental stochasticity and (3) measurement error (Shoemaker et al. 2020), but it is also affected by metabolic processes (Saito et al. 2021). Demographic stochasticity is related to intrinsic processes such as birth, death, or migration, whereas environmental stochasticity reflects extrinsic conditions such as temperature, precipitation and disturbance events (Shoemaker et al. 2020). The ambiguity present in these parameters blurs the line between determinism and stochasticity, as it can be argued that birth, death and migration rates are bound to some extent by evolutionary history. In addition, hosts can die of infection, and their associated microbes will also be limited if they lack the ability to disperse. Therefore, examining the relative roles of determinism and stochasticity in shaping bacterial diversity requires developing novel approaches to resolve these challenging dynamics.

Here, we examine amphibian skin microbial communities across a large latitudinal gradient in South America. We specifically focused on 22 amphibian species from Chile to investigate the influence of host evolutionary history, the presence of *Bd* and geographic variation in bacterial composition. In the study area, amphibian species diversity shows considerable latitudinal variation (Vidal et al. 2009), providing a spatial gradient to study compositional changes of hosts, associated microbes and the enzootic pathogen *Bd*. *Bd* infections in Chilean amphibians have been detected since the 1970s, and today the pathogen is found across most of the country (Soto-Azat et al. 2013a, 2013b; Valenzuela-Sánchez et al. 2018). Evidence from wild hosts indicates that the prevalence of *Bd* infections in this country decreases from north to south (Bacigalupe et al. 2017). Furthermore, a recent study on the widely distributed Chilean four-eyed frog (*Pleurodema thaul*) revealed that *Bd* infection, but not climate nor human footprint, influenced the richness of bacterial families in their skin (Bacigalupe et al. 2024). However,

it is unclear whether or not this pattern is shared across host species in Chile.

Based on the latitudinal variation operating at this geographical scale (Ladau and Eloe-Fadrosh 2019), we hypothesised that Chilean amphibians and their associated microbes would similarly respond to assembly processes affected by dispersal, connectivity and environmental filtering (Härer and Rennison 2023). Deviations from this expectation would indicate that deterministic processes (e.g., phylosymbiosis, active recruitment of microbes, microbe-microbe interactions) are involved in microbial community assembly. We first tested for phylogenetic signal in skin bacterial diversity between host species and then fitted linear models to test the effect of *Bd* presence/absence and geography on the diversity of bacteria. In addition, we carried out a sensitivity analysis to estimate the effect of removing particular host species on the robustness of the significant factors, including phylosymbiosis. To further investigate the processes driving the community assembly of host microbiomes, we selected two of the most widespread amphibian species from our dataset as sentinels of bacterial occupancy, fitted Sloan's neutral models and identified bacterial taxa deviating from neutral expectations in the microbiome of these two species. Finally, we compared the sentinels' neutral and deviant bacterial taxa across all 22 amphibian species to determine the contribution of host effects and infection to the clustering of host-associated microbiomes.

2 | Material and Methods

2.1 | Amphibian Skin Microbiomes and *Bd* Infection

Our microbiome dataset consists of 751 individual samples from 22 amphibian species collected from 2008 to 2017 across a latitudinal gradient of 1,900 km in Chile and sample controls (PRJNA1003691; Table S1). Only one species of this dataset is invasive in Chile (*Xenopus laevis*). All samples from free-living amphibians were obtained using rayon-tipped skin swabs (MW100; Medical Wire & Equipment, UK). The same sample was used for both detecting *Bd* and for microbiome characterisation using the PrepMan Ultra reagent (Thermo Fisher Inc) for DNA extraction following the standard protocol detailed in Boyle et al. (2004). Full details on swabbing and *Bd* quantification via qPCR can be found in Soto-Azat et al. (2013a) and Bacigalupe et al. (2024). We classified *Bd* positives as any swab sample in which more than 0.1 genomic equivalents were detected in both of two replicate PCR assays. To profile bacterial diversity, DNA from each sample was PCR-amplified using the 16S rRNA V3–V4 region with primers 341F (5′CCTACGGGNGGCWGCAG3′) and 805R (5′CTACHVGGGTATCTAATCC3′) described by Klindworth et al. (2013). PCR products were sequenced in five runs of 300 bp paired-end libraries on an Illumina MiSeq at the AUSTRAL-omics bioscience core facility at the Universidad Austral de Chile. Each run had between 39 and 270 samples, including negative controls. We analysed all sequencing runs in parallel with the same parameters and the DADA2 pipeline as done in Bacigalupe et al. (2024), which consisted of removing short reads below 100 bp, reads with an average quality below 20 and reads with mismatches at the barcode sequences. We assigned bacterial taxonomy to Amplicon Sequence Variants (ASVs) using SILVA 138 bacterial 16S rRNA database (Quast et al. 2012), and constructed a phylogenetic tree using *iqtree* (Nguyen et al. 2014). We obtained a range of 14–386,661 reads per swab sample; thus, all samples were rarefied at 5,000 reads (Hong et al. 2022). This filtering step retained 77% of the ASV richness across 90% of the rarefied samples (total frog samples = 580).

2.2 | Testing the Role of Individual, Geographic and Temporal Factors on Bacterial Diversity

We collapsed ASVs at the family level and calculated the number of unique bacterial families per individual. We used this level because families can capture broader geographic patterns, whereas ASVs at their highest resolution often reflect differentiation among sites (Marshall et al. 2019). We employed an information-theoretic approach (Burnham and Anderson 2002) to evaluate the level of support for models explaining the role of factors potentially shaping the skin microbiome structure (i.e., developmental stage, year of sampling, geographic location, *Bd* infection and host evolutionary history). Bacterial family richness was log-transformed prior to all analyses carried out using the amphibian phylogeny by Jetz and Pyron (2018). We conducted analyses in two steps. First, we assessed the potential impact of individual developmental stage (i.e., tadpole, juvenile, adult), year of sampling (samples were collected in different years and the year was included to account for potential temporal effects of the sampling) and geographic structure (latitude × longitude) on bacterial richness at the family level. To estimate Pagel's λ, we ran a phylogenetic generalised least squared (*pgls*) model using Restricted Maximum Likelihood (REML) with individual developmental stage, year of sampling and latitude × longitude as predictors (model: bacterial family richness ~ predictors). Afterwards, we fit four univariate *pgls* models using ML and λ set to 0.68 with those same predictors in addition to the null model. The model with latitude and longitude had an Akaike weight of 0.971 (model with most explanatory power (Wagenmakers and Farrell 2004), Table S2), and therefore, it was the one used in step two. Secondly, we evaluated the effect of *Bd* infection status on bacterial family richness by fitting the following two *pgls* models using ML and λ set to 0.56: (i) latitude × longitude, (ii) latitude × longitude + *Bd*. As the model with *Bd* had the highest support (see Results), we implemented a cross-validation scheme to assess the influence of amphibian sample sizes and host species identity on the estimated effect of *Bd*. This involved removing one species at a time, recalculating Pagel's λ as well as the *pgls* model including the contrast for *Bd* infection (i.e., the weighted sum of group means between *Bd*-positive and *Bd*-negative individuals). Analyses were carried out using *nlme* (Pinheiro et al. 2023) and *MuMIn* packages (Barton 2023) in R 4.2.2 (R Core Team 2022).

2.3 | Comparing the Effect of *Bd* Infection on the Abundance of Shared Bacterial Families

We filtered ASVs to select shared bacterial families across all amphibian species. We applied differential abundance analyses using *Bd* status (*Bd*+ versus *Bd*−) implemented with *DESeq2* in R with multiple test correction (Love et al. 2014). These analyses are suitable for comparisons that rely on small sample

sizes and large dynamic ranges (not normally distributed and high variation) using shrinkage estimators for dispersion (Love et al. 2014).

2.4 | Quantifying the Strength of Phylosymbiosis Using Randomizations

We modelled the congruence of host-bacterial associations by randomising samples because each species had multiple individuals. This approach provided an accurate estimate of phylosymbiosis considering the background of high latitudinal variation and stochasticity. To do this, we randomly selected one sample from each species, saved its ASVs into a matrix and calculated weighted Unifrac distances for the simulated data. To obtain the spatial distances, we used the centroid of the geographical distribution of each species within the sampled populations. We repeated this process 9999 times (see R script deposited in Zenodo). To quantify phylosymbiosis, we used the function *MRM* in the R package *ecodist* to perform multiple regressions testing host evolutionary history (amphibian phylogeny) and the geographical distances between the samples as the predictors in the model (Goslee and Urban 2007). We saved the R^2 and p values of all models and visualised the results at different taxonomic levels. These findings represented the limits of phylosymbiosis across taxonomic levels and determined whether host-microbe relationships are consistent or context dependent. We applied a Mantel test to the rarefied ASV table to determine the correlation between host evolutionary distances and unweighted Unifrac distances.

2.5 | Fitting Sloan Neutral Community Models for Widespread Amphibian Species

For this analysis, we focused on two widespread species with the highest sample sizes: *P. thaul* and *R. darwinii*. We examined if neutral processes accounted for large-scale latitudinal variation in microbiome membership for each of these species. We implemented Sloan Neutral Community Models to quantify deviations in abundance using the frequencies of all bacterial ASVs across samples against a binomial and Poisson model (Sloan et al. 2006). This analysis predicts that abundant bacterial taxa will be widespread and subject to dispersal and chance, whereas rare taxa are lost from hosts via drift. We used the terms neutral ASV, below prediction ASV (or negatively selected) and above prediction ASV (or positively selected) to refer to the results of the Sloan community models. We fitted independent Sloan neutral community models for each one of the two amphibian species to determine if ASVs exhibited similar processes despite stark differences in host evolutionary history, ecology and *Bd* infection prevalence. We expected that *P. thaul* would host more neutral ASVs as its latitudinal distribution is larger than that of *R. darwinii* (Barria et al. 2020; Soto-Azat et al. 2013b).

We pooled bacterial ASVs across *P. thaul* and *R. darwinii* into three partitions depending on their deviation from the 95% CI of the predictions of the neutral models (i.e., more frequently = positive, less frequently = negative and within predictions = neutral). We conducted permutational multivariate analyses (functions *betadisper* and *adonis*) for each partition using *Bd* infection status and host species as covariates of interest using unweighted Unifrac distances.

To further understand any potential contribution of these ASVs to microbe-microbe and host–microbe interactions, we queried the sequences against the anti-*Bd* bacteria database generated by Woodhams et al. (2015). We used a strict threshold of 100% sequence similarity and constructed Venn diagrams to compare ASV distribution in *P. thaul* and *R. darwinii*. The ASVs were classified based on their function in the database as facilitators or inhibitors of *Bd* infections.

3 | Results

3.1 | Patterns of Bacterial Diversity Across Host Species

Amphibian species exhibited high variability in bacterial family diversity and composition (Figure 1 and Figure S1). We found that Amplicon Sequence Variants (ASVs) predominantly belonged to several bacterial families including *Flavobacteriaceae*, *Chitinophagaceae*, *Microbacteriaceae*, *Dermabacteraceae*, *Sphingomonadaceae*, *Cellulomonadaceae*, *Beijerinckiaceae*, *Sanguibacteriaceae* and *Sphingobacteriaceae*, among others (Figure 1b). The richness of bacterial families significantly varied across host species (Figure 1c), with values ranging from 5 to 150 bacterial families after rarefaction. *Pleurodema thaul*, The species with the largest geographical distribution and highest *Bd* infection prevalence (45.5%, Table S1), carried 89.1% of the families found, whereas *R. darwinii* exhibited lower diversity of bacterial families with only 62.3% of families detected (*Bd* prevalence = 6.8%; Figure 1c and Table S1).

3.2 | *Bd* Infection and Geography Were the Main Factors Influencing Skin Bacterial Diversity at the Family Level Across Host Species

As bacterial family richness was influenced by host evolutionary relationships (Pagel's $\lambda = 0.56$, $p < 0.001$), we included phylogenetic relatedness in all models (Table S2). Richness of bacterial families was affected by *Bd* infection and geographic location (i.e., latitude × longitude). In particular, *Bd*-infected individuals carried on average 1.86 more bacterial families than uninfected frogs (overall average values 71.5 versus 69.6 families, respectively; Figure S1). Our sensitivity analysis allowed us to quantify the contribution of each host species, including their phylogenetic and sample size weight, to the contrast estimate of *Bd* in the *pgls* model (Figure 2a and Figure S1; *contrast* is the weighted sum per category). Overall, the removal of host species did not substantially affect the estimated value of the *Bd* contrast (min: 0.043, max: 0.0442), as many species harbour only a few *Bd* infections (Table S1 and Figure S1). Removing species within the same clade such as four-eyed frogs (*P. thaul*, *P. bufoninum)* and Concepción Toad (*Rhinella arunco*) slightly decreased the value of the *Bd* contrast in the model (Figure 2a), whereas the removal of aquatic and evolutionary divergent hosts such as the helmeted water toad (*Calyptocephalella gayi*) and the African clawed frog

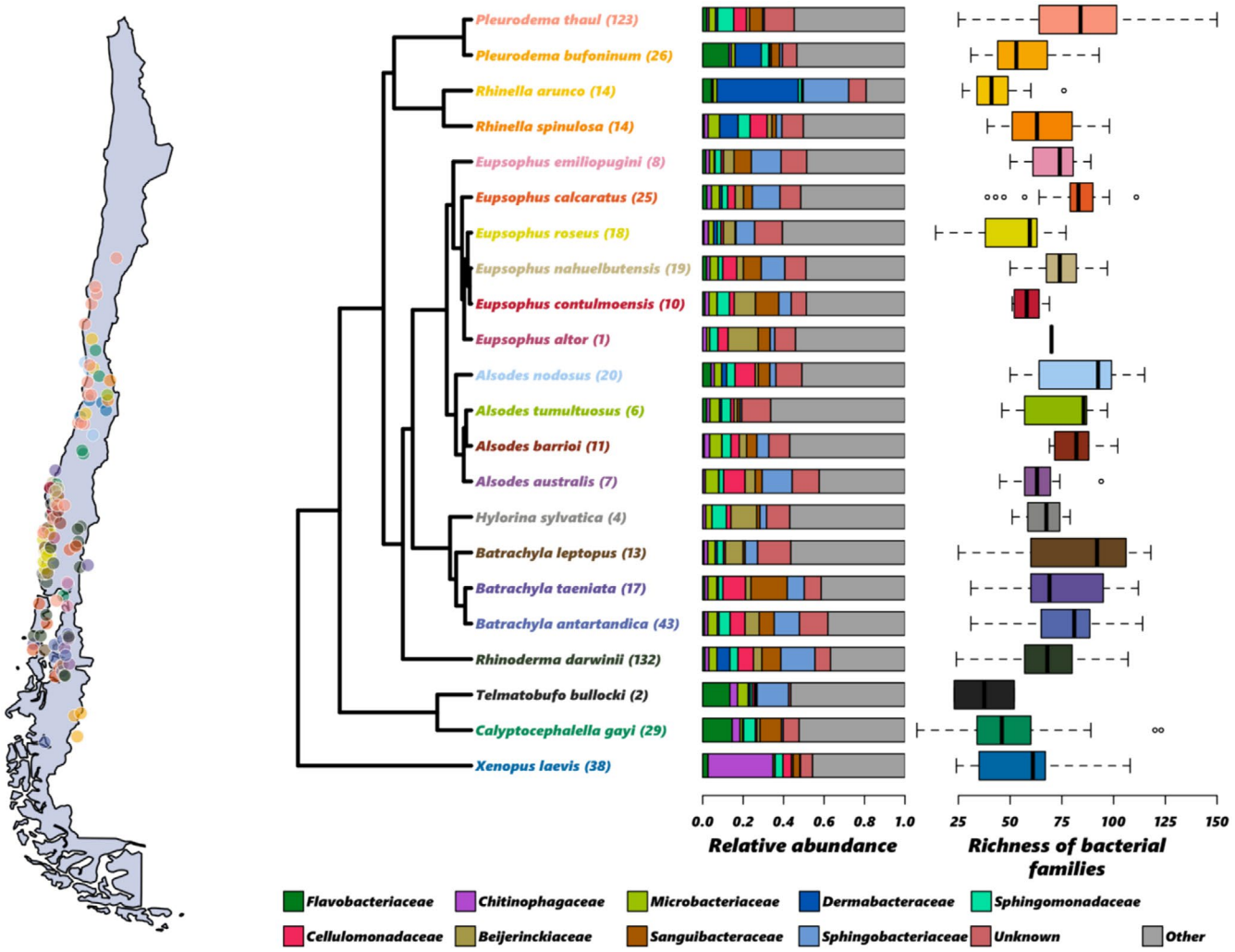

FIGURE 1 | Distribution of amphibian hosts and their major skin bacterial families across a latitudinal gradient of biodiversity in South America. (Left) Map of Chile indicating the approximate locations and distribution of each host species sampled as part of this study. Points have added jitter to prevent the overlap of locations. (Center) Phylogeny of the 22 species of amphibians (Jetz and Pyron 2018) and the relative abundances of the top 10 bacterial families. The number in parenthesis after the species names indicates the sample size. All species, except for *Rhinoderma darwinii*, inhabit aquatic environments, where their larvae hatch from aquatic eggs. (Right) Boxplots of the richness of bacterial families for each host species sampled and rarefied at 5000 reads.

(*Xenopus laevis*) positively increased the contrast. This reduction is directly related to the number of *Bd* positives in the dataset that belong to each species. In addition, our sensitivity analysis showed that removing these two divergent host species (i.e., *C. gayi* and *X. laevis*) led to bacterial diversity becoming more similar than expected after accounting for host evolutionary relationships (Pagel's λ increased towards 1; Figure 2a). For example, *P. thaul Bd*-positive individuals were characterised by decreased abundances across many phyla such as Actinobacteriota, Bacteroidota, Chloroflexi, Spirochaetota and Synergistota (Figure 2b; significant $\log_2$ fold changes). However, *P. bufoninum*, a closely related species with contrasting habitat preferences, primarily showed significant changes in the abundances of Bacteroidota and Firmicutes (Figure 2b). Other host species such as the La Parva spiny-chest frog (*Alsodes tumultuosus*) and *C. gayi* showed contrasting patterns in families belonging to phylum Bacteroidota (higher $\log_2$ fold changes; Figure 2b).

3.3 | Strength of Phylosymbiosis Was Context Dependent

We found significant correlations between host evolutionary distances and the beta diversity of skin bacterial communities (Unweighted UniFrac distances) in 12.8% of the randomizations (Figure 3 and Table S3; 9999 Randomizations), indicating phylosymbiosis. In contrast, we detected fewer significant correlations with geographic distances (7.6%; Figure 3 and TableS3). However, the chances of finding phylosymbiosis depended on individual samples and site combinations. After merging ASVs from the 9999 randomizations and performing a single rarefaction at 5000 reads, host phylogeny had greater explanatory power than geographical distances (Table S4 and Figures S2–S6). Both approaches showed a predictable loss of the phylosymbiosis signal when grouping ASVs by their taxonomic rank from genus to family (Figure 2, Tables S3, S4 and Figure S2). We observed consistent clustering among amphibian species when

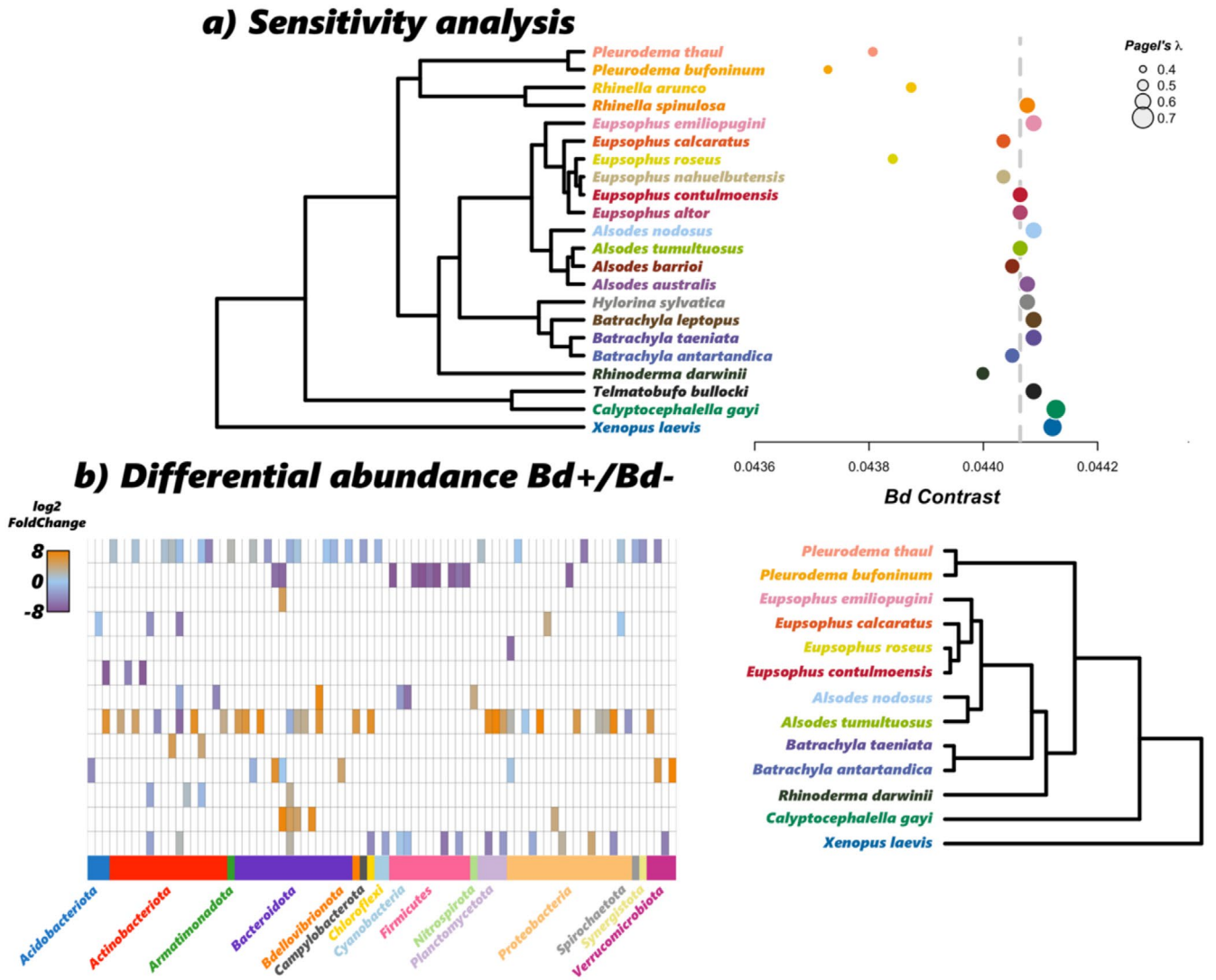

FIGURE 2 | Effect of *Batrachochytrium dendrobatidis* (*Bd*) infection on skin bacterial diversity in Chilean amphibians. (a) Model results of re-sampling scheme removing each host species. Each point represents the estimate of the *Bd* contrast from the phylogenetic generalised least square model without the host species indicated on the amphibian phylogeny (left side). Grey dashed line represents the value of the model including all species. Higher values indicate more bacterial families per infected individual for each host species. (b) Heatmap of logarithmic fold changes in the abundance of bacterial families between *Bd*+ and *Bd*− samples. Bacterial phyla are indicated in the bottom of the figure. Only amphibian species with positive *Bd* detections are included on the heatmap and phylogeny (right side). Positive $\log_2$ fold changes (>0) indicate increased number of reads in *Bd* + frogs.

comparing the tree topologies (Figure S6). *Xenopus laevis* stood out as an outlier in both trees.

3.4 | Neutral Processes Operate Differently Across Host Species

We found that the number of ASVs departing from the neutral expectations (i.e., subject to stochastic dispersal and drift) was relatively small for both *P. thaul* and *R. darwinii* compared to the total number of ASVs present in their skin microbiomes (Figure 4 and Figure S3, $R^2 = 22\%–52\%$). These two species were selected for this analysis due to their comparatively large sample sizes and broad geographical distributions (Figure 1). Skin bacterial microbiomes of *R. darwinii* harboured a small proportion of non-neutral ASVs (6.64% above and 3.47% below predictions, respectively; Figure 4a). *P. thaul* showed a similar proportion of above-prediction ASVs (6.88%), but a much smaller fraction of ASVs was below neutral expectations (0.08%; Figure 4b). Overall, these results indicate that between 7% and 10% of the ASVs are selected for or against (above/below prediction) in both species, while ~90% of ASVs were subject to stochastic dispersal and drift (Figure 4). While most ASVs above and below neutral predictions were unique to each species, a large proportion of neutral ASVs were shared (Figure 4 and Figure S3). As expected, the differences in the number of bacterial taxa decreased at higher taxonomic levels (Figures S3, S4). Furthermore, when comparing beta diversity among all host species and partitioning their bacterial ASVs into the four categories (see Figure 5), the centroids of each host species visually overlap, yet both species and *Bd* infection status were significant factors associated with bacterial composition (Table S5; all comparisons $p = 0.001$). This microbial

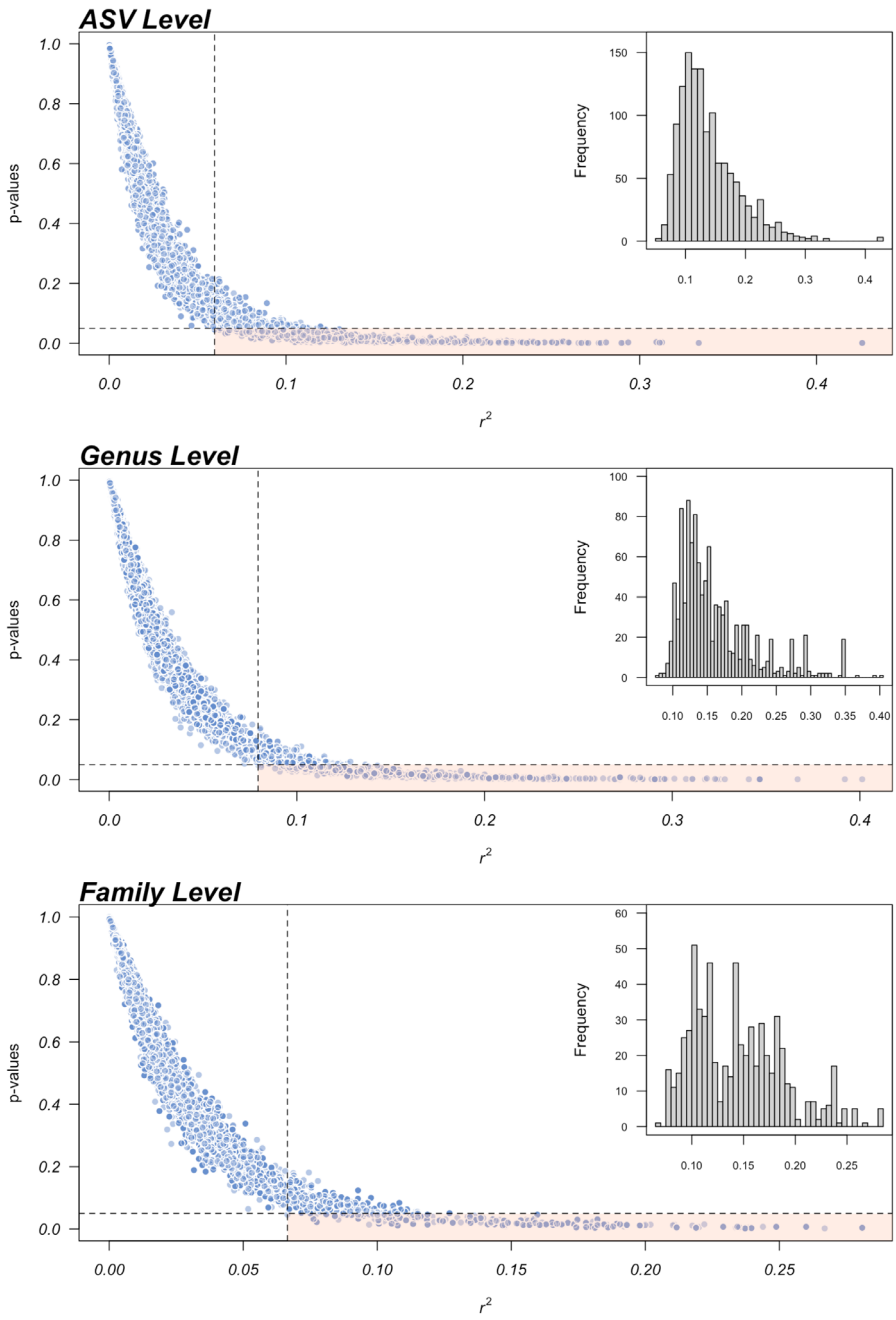

FIGURE 3 | Results of randomizations testing phylosymbiosis in Chilean amphibians. Amphibian-bacterial phylosymbiosis was detected in 6.7%–12.8% of all multiple regression models with randomised data and depended on the taxonomic level of the amplicon sequence variants (ASVs). Each point represents one of 9999 randomizations. The shaded area highlights significant models at $p < 0.05$. The multiple regressions on distance matrices analyses showed a greater proportion of models with significant effects of host phylogeny over geography using bacterial UniFrac distances (refer to Tables S3, S4 for the relative proportions). The histograms indicate the distribution of models with significant R^2 values, consistently demonstrating that phylosymbiosis explains 10%–20% of the randomised data, as the average frequencies fall between 0.10–0.20.

association with *Bd* infection remained significant in *P. thaul* across all ASVs classifications (Table S6). In contrast, for *R. darwinii*, we did not find differences in the centroid position related to *Bd* infection (Table S6), but the dispersion was significantly different for all ASVs, neutral ASVs and those below prediction; Table S7. ASVs that can putatively facilitate *Bd* infection only represented 6% of the bacterial taxa selected against by both amphibian species (below neutral expectations, Figure S5), whereas 13.4% of anti-*Bd* bacteria were above neutral predictions.

4 | Discussion

Identifying the factors that determine the diversity, composition and stability of host-associated microbiomes has generated considerable interest in ecology and evolution (Härer and Rennison 2023; Perreau and Moran 2022); yet many questions remain regarding the mechanisms that operate under infected and healthy states (Lively et al. 2014). Here, using a large-scale latitudinal dataset, we show that variability in the skin

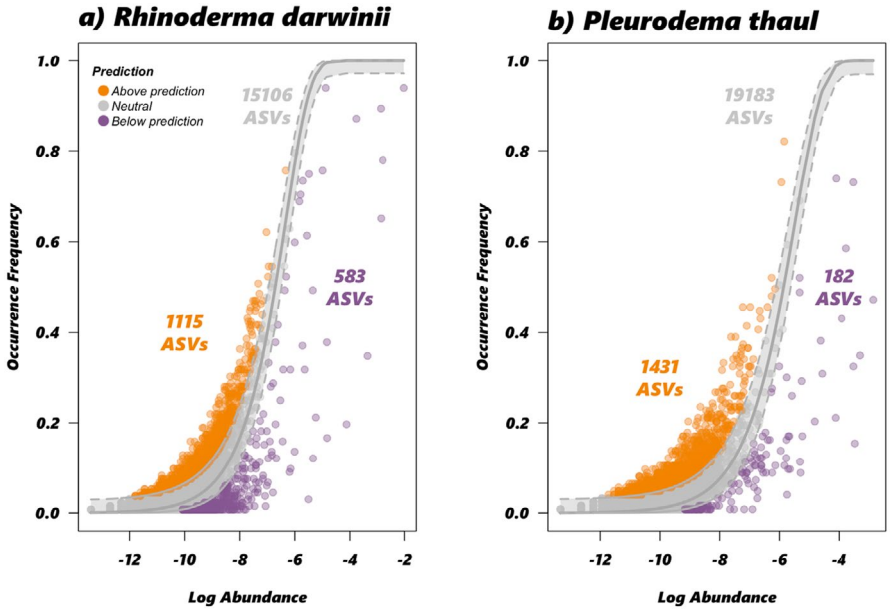

FIGURE 4 | Bacterial amplicon sequence variant (ASV) abundance distributions under Sloan neutral community models for: (a) *Rhinoderma darwinii* [Poisson fit: $M = 0.04$, $R^2 = 0.52$, *ASV richness* = 16,804, $N = 132$] and (b) *Pleurodema thaul* [Poisson fit: $M = 0.01$, $R^2 = 0.22$, *ASV richness* = 21,155, $N = 123$]. Bacterial ASVs that deviated from the model are identified by colours (orange: More frequent than predicted or positively selected; purple: Less frequent than predicted or negatively selected). Points coloured in grey represent neutrally distributed ASVs, which are those subject to dispersal and chance. Grey lines represent 95% confidence intervals of the predictions. ASV overlap between species is shown in a Venn Diagram (Figure S3).

bacteriome of Chilean amphibians reflects interactions between random and predictable processes. Closely related amphibian hosts typically share traits in terms of reproductive mode, home ranges, diets, habitats, behaviour and susceptibility to pathogens (Youngblut et al. 2019). Although we did not test each trait directly, these attributes influence the occurrence of host-associated microbes and can further affect microbe –microbe interactions. We detected a significant phylogenetic signal in the number of bacterial families and evidence of phylosymbiosis, two measures that point to predictable patterns of community assembly driven by host evolutionary relationships. However, our findings also showed that almost 90% of bacterial taxa in the skin of the two most widespread amphibian species from our dataset were determined by dispersal and chance, as identified by the neutral community models. These models indicated that most bacterial taxa were rare (low occurrence frequency and abundance) and confirmed differences in dispersal capacity among the two species. Pathogen infection and geographic distance had statistically significant but limited contributions, highlighting the complex processes involved in determining bacterial composition in the amphibian skin.

4.1 | Blurred Lines Between Determinism and Stochasticity

Our findings underscore strong interspecific associations driven by evolutionary and ecological processes with a joint role for determinism and stochasticity in community assembly. Researchers have recently expanded the concept of stochasticity in ecological communities to include the confluence of environmental, demographic factors, measurement error (Shoemaker et al. 2020) and metabolic processes (Saito et al. 2021). Stochasticity may arise from dispersal limitation across host species and populations, disturbance from pathogen infection (Wilber et al. 2020), environmental variation through the individual's lifetime and the emergence of new genetic variants. However, stochastic processes may show some degrees of determinism or vice versa, which can be driven by aspects of the host's natural history and evolution (Kohl 2020; Stegen et al. 2012; Zhou and Ning 2017).

Host phylogeny can play a deterministic role in the diversity of skin microbes even at fine spatial scales (e.g., sites that located within one latitudinal/longitudinal degree). In lungless salamanders (genera *Pseudoeurycea* and *Bolitoglossa*) in Mexico and Guatemala, the influence of evolutionary history was only detected at shallower scales (genera and species). This fact is primarily attributed to distinct preferences for terrestrial versus arboreal habitats and variation in skin characteristics, including chemical composition (Ellison et al. 2019). However, in the most extreme case where host species are part of an adaptive radiation at an island scale, such as coqui frogs (*Eleutherodactylus* spp.) and *Anolis* lizards, phylogeny did not or only weakly explained microbiome composition (Garcia-Recinos et al. 2019; Ren et al. 2016). Rapid radiation events may reduce the time for co-diversification processes occurring between hosts and microbes, as detected in mammals (Nishida and Ochman 2018). This lack of concordance between studies can also be due to habitat specialisation among species, high dispersal capacity of microbial communities (Bird et al. 2018), or pathogen effects. For instance, amphibian species within a taxonomic family can occupy contrasting habitats (e.g., *Rhinoderma darwinii* and *Insuetophrynus acarpicus*, found in fully terrestrial habitats versus streams, respectively), leading to differences in exposure to microbes and pathogens.

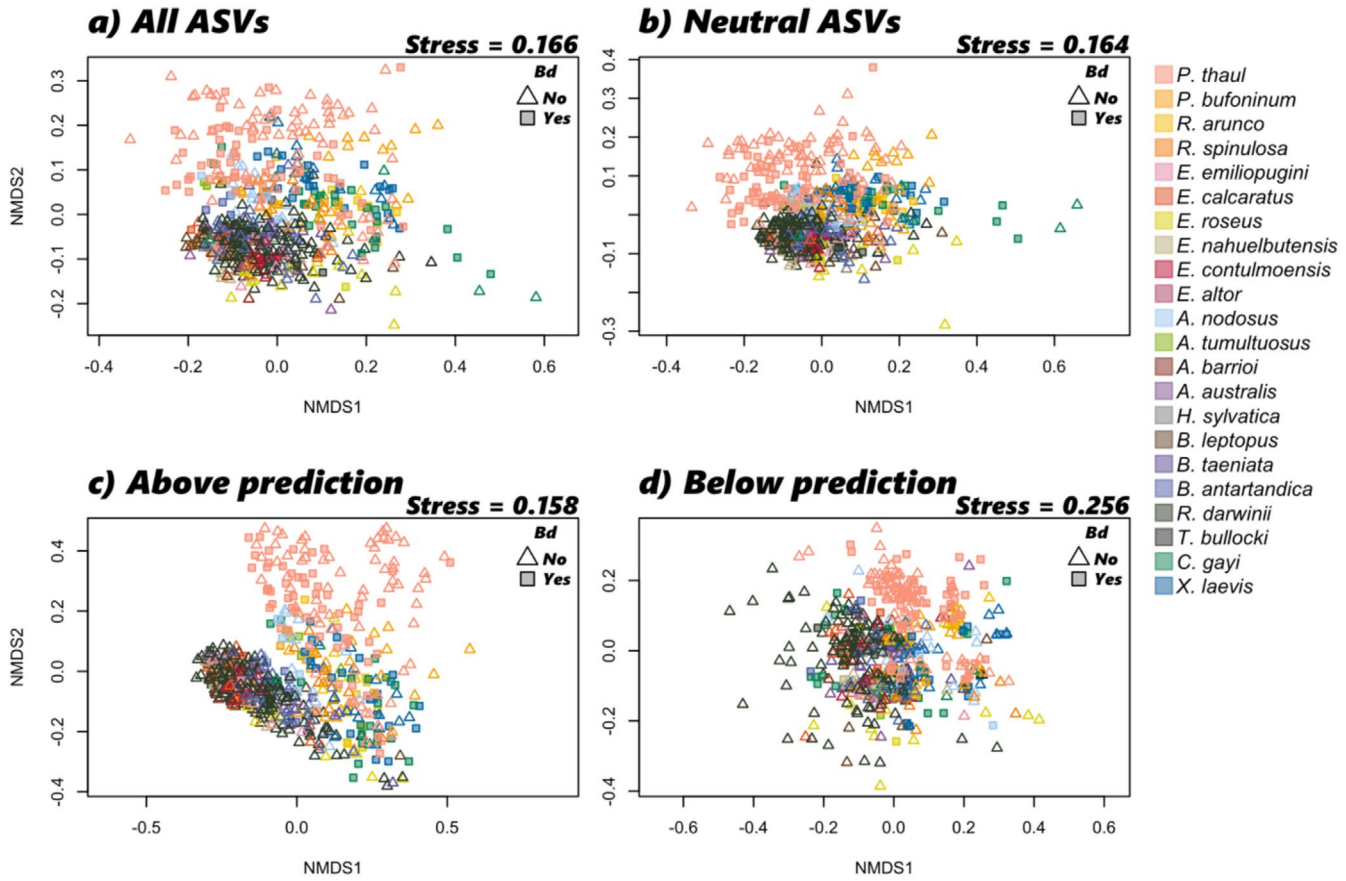

FIGURE 5 | Ordination plots comparing skin bacterial composition (unweighted UniFrac distances) between amphibian species. Each colour represents a different species and follows the same colour scheme in Figure 1. Each panel shows a subset of: (a) all ASVs detected in the study, (b) ASVs identified as *neutral*, which are those subject to chance and dispersal, (c) ASVs above prediction, or (d) below prediction in *Rhinoderma darwinii* and *Pleurodema thaul* but detected across the 22 species. Point shape indicates if the sample showed positive detection of the pathogen *Bd* (squares) or negative detection (triangles). Stress values indicate how well the ordinations summarise the distance between the samples. Significance tests for the centroid and dispersion of the bacterial communities are reported in Tables S4–S7.

The question of whether amphibian species can be solely predicted by their skin bacteriomes seems possible based on our findings, but remains unanswered as it was not part of the main objectives of this work. Future research can address it by implementing experimental approaches and supervised clustering methods, such as partial least squares discriminant analyses and random forest classifiers (Bates et al. 2022; Lam and Fong 2024). In addition, invasive species make good candidate hosts to test the sensitivity of species as a factor relative to environmental variables. Recent studies suggest that environmental filters shape microbial community assembly in invasive species (Abarca et al. 2018; Kueneman et al. 2019; Leonhardt et al. 2023); however, these studies lacked measures of host evolutionary relationships. Our dataset only included one invasive species (*Xenopus laevis*), which also served as an outgroup given its phylogenetic placement. Removing *Xenopus laevis* resulted in the highest phylogenetic signal in the richness of bacterial families (Pagel's $\lambda \sim 0.7$, Figure 2), indicating that invasive species can dampen the effects of phylosymbiosis (Figure S6).

Pathogen infection has shown ambiguous correlations with microbial diversity and composition across susceptible and tolerant amphibians (Jani et al. 2021; Kaganer et al. 2023; Kruger 2020; Wilber et al. 2020). Our findings agree with previous studies indicating that pathogen infection partly generates and explains variation in alpha and beta diversity of bacteria in our dataset (Bacigalupe et al. 2024; Jani et al. 2021; Muletz-Wolz et al. 2019), but to a much lesser extent than other deterministic processes such as host phylogenetic relationships (Ramírez-Barahona et al. 2023). Establishing correlations between skin microbiomes and the presence of a pathogen, as well as classifying infection status as a deterministic process, can be difficult when individuals are constantly losing and gaining infection (Ellison et al. 2021), or have different susceptibilities to become infected. Our 22 species persist with varying levels of *Bd* infection prevalence (Alvarado-Rybak et al. 2021; Bacigalupe et al. 2017), which we hypothesised would lead to dysbiosis (Jani and Briggs 2014). The overall prevalence of *Bd* infection across our dataset was low (7%, Table S1), and our sentinel species, *R. darwinii* and *P. thaul*, also differed in infection prevalence (6.8% and 45.5%, Table S2) (Alvarado-Rybak et al. 2021; Valenzuela-Sánchez et al. 2017). *Bd* infection causes systemic effects in susceptible hosts as part of disease progression; thus, we expect that this disturbance will likely affect microbial communities not only in the skin, but also in other tissues such as the gut (Knutie et al. 2018). In some species, infected amphibians cannot

recover their original microbial diversity even after clearing the infection (Jani et al. 2021). Again, these findings emphasise that pathogen status can be both a deterministic and stochastic process affecting skin bacterial assembly. Infections do not always produce the same outcome as susceptibility varies across individuals, populations and species. Researchers have begun to describe this type of interaction in microbial pathogenesis as a chaotic system (i.e., a system that is highly sensitive to initial conditions; Sella et al. 2024). We also have little information about the nature of bacterial associations within the host. For instance, what is the relative composition of skin bacteria that are endosymbionts, ectosymbionts and commensals, and how these ratios vary across host species? Quantifying these relationships will allow us to determine potential interactions with *Bd*, differences in the strength of selection, dispersal and drift, and the predictability of the system.

One of the recurring problems that can also introduce randomness in large datasets is uneven sampling and/or incomplete sampling of host lineages. We overcame this issue by testing the robustness of our inferences, removing each host species at a time and recalculating the phylogenetic signal and the status of *Bd* infection. Species removal operated as incomplete sampling, which resulted in an accurate method to assess the strength of the phylogenetic signal, accounting for both stochastic and deterministic variation. Furthermore, the randomizations showed that the pattern of phylosymbiosis and geographic distance weakened by changing the taxonomic level of the microbes and individuals sampled in the analysis of beta diversity (Figure 3). This result points to the importance of evaluating different levels in the taxonomical hierarchy of microbes to understand the evolutionary limits of phylosymbiosis. In comparative analyses, high stochasticity paired with limited host forces (e.g., immune filtering) is expected to produce microbiome alternative states characterised by weak self-organising feedbacks and low resilience (Degregori et al. 2024), thereby dampening phylosymbiosis effects.

4.2 | Valuable Contributions of Neutral Models for Widespread Species

We aimed to compare how neutral processes affected the assembly of skin bacteria by focusing on the two most widespread species, *R. darwinii* and *P. thaul* (Figure 4). *R. darwinii* is listed as an Endangered species by the IUCN Red List of Threatened Species and is highly susceptible to chytridiomycosis (Azat et al. 2021; Soto-Azat et al. 2013a, 2013b; Valenzuela-Sánchez et al. 2017), whereas *P. thaul* is a non-threatened species. Extinction risk has been associated with lower bacteriome diversity in threatened amphibians (Greenspan et al. 2022). Our results support this association (Figure 4), and the neutral community models point to the dependence on dispersal and connectivity to maintain microbial diversity. Any barriers limiting the dispersal of essential microbes could significantly impact physiological processes in these endangered species, including pathogen defence. Our findings are consistent with previous bacteriome work on *P. thaul* (Bacigalupe et al. 2024), in which pathogen infection plays a more significant role than climate and human impact variables. However, our comparisons expand the availability of bacteriome data spanning across multiple latitudinal degrees and focused on species with widespread distributions, which so far have been limited to studies with invasive amphibian species (Kueneman et al. 2019; Weitzman et al. 2023). By focusing on native amphibian species, we can study community assembly process patterns without confounding factors such as novel interactions occurring during invasion and rapid population spread.

Neutral models assume that species in the assemblage are functionally equivalent, thus insensitive to the environment (Sloan et al. 2006). In other words, microbial taxa must die or move out from the skin, then be replaced by microorganisms from the same community or by immigrants. Some authors have criticised these models, arguing they could be oversimplistic (Gotelli and McGill 2006). However, the environment here is embedded within the host; thus, unfavourable conditions, such as pathogen infection or abiotic factors, can simultaneously determine the fate of both the host and its microbes.

Interestingly, the most abundant skin bacterial taxa tended to be positively or negatively selected in both host species (i.e., non-neutral; Figure 4 and Figure S4). Using these models by themselves might not be of great utility, but when combined with other analyses incorporating deterministic processes, we gained a better understanding of the mechanisms affecting the assembly of microbial communities, such as *Bd* infection (Tables S5–S7). Neutral models predict that abundant taxa will be more widespread among local microbial communities. Although both amphibian species overlap in parts of their distribution, *R. darwinii* occupies a smaller fraction of the range (Figure 1). Neutral community models in *R. darwinii* accurately reflected less dispersal limitation of bacterial taxa with slightly higher migration rate values than *P. thaul* ($m = 0.04$; Figure 4). Our data also indicated that larger distributional ranges in *P. thaul* resulted in a ~4 K difference in ASV richness when compared to *R. darwinii* (Figure 4; 16,804 versus 20,796 ASVs), indicating a higher ability for recruiting novel microbial taxa or evidence of diversification. In addition, differences in habitat preferences between these two species (*P. thaul* = pond-breeder; *R. darwinii* = fully terrestrial) can contribute to microbial assembly, as co-occurrence with other amphibian species may facilitate the movement of bacteria from species to species. Although some pond-breeding amphibian species can disperse long distances (Cayuela et al. 2020), anurans typically exhibit high site fidelity and have physiological limitations to movement related to water balance and thermoregulation (Sinsch 1990). Future studies should quantify parameters related to dispersal, colonisation probability and survival of microbes and contrast these variables with evolutionary measures of differentiation and dispersal distance of the host. Bacteria consistently isolated from amphibian skin belonging to genera *Acinetobacter* and *Pseudomonas* could serve as candidate groups to explore the extent to which co-evolution occurs across the amphibian phylogeny (Brunetti et al. 2022; Cevallos et al. 2022). Genomic and functional characterisation of these taxa can reveal traits associated with pathogenicity or as mediators of host defences as found in several isolates of *Acinetobacter* spp. (Cevallos et al. 2022).

Our results revealed that, when concurrently analysing *P. thaul* and *R. darwinii*, a remarkably low number of bacterial ASVs shared the same direction in the prediction under the neutral community models (84 ASVs above and 13 ASVs below predictions, respectively, Figure S3). These findings are likely driven by the high number of unique ASVs hosted by each species (Figure S4), which is expected given the influence of deterministic processes such as host phylogeny (selection) and local adaptation of hosts/microbes (Figures 1, 2). However, within each species, the prediction is that the majority of ASVs will be neutral; thus, drift and dispersal will generate a significant proportion of microbial diversity (Burns et al. 2016). Diversification introduces new genetic variants to the communities through stochastic (mutation) and deterministic (selection) processes, as highlighted by Hayashi et al. (2024). Sampling multiple populations with variable effort across years can also contribute to the reduction in the number of non-neutral ASVs. We further investigated these ASVs relative to the rest of the dataset, with the goal of understanding species effects in beta diversity. Our analyses not only showed that the species factor carried higher explanatory power than *Bd* infection (Figure 5 and Table S5) but also that its predictability was higher for the ASVs below prediction ($R^2 = 0.32$, Figure 5d and Table S5). Negatively selected ASVs are often dispersal-limited (Shade and Stopnisek 2019), may reflect antagonistic interactions (host-microbe and microbe-microbe) or represent rare bacterial taxa. Experimental infections with *Bd* focusing on the two widespread species (*P. thaul* and *R. darwinii*) could shed some light on the functional properties of these non-neutral bacterial taxa (Figure S4). Because the fit of the neutral models was better in *R. darwinii* than *P. thaul* (Figure 4), deviation from neutrality can be a consequence of *Bd* infection and disease severity (Venkataraman et al. 2015). If these ASVs are opportunistic taxa, we expect to see an increase in abundance after *Bd* infection under controlled conditions. Therefore, our analyses provide a unique perspective on community assembly reflecting contemporary effects and historical contingencies in the amphibian skin.

5 | Conclusions

Quantifying bacterial diversity and assembly processes using a single gene marker is far from perfect (Abellan-Schneyder et al. 2021); therefore, addressing the well-known Baas Becking's statement "Everything is everywhere, but, the environment selects" for host-associated microbes still presents challenges in its interpretation. Here, we provide evidence from ectotherm species that most of their bacteria are subject to dispersal and chance, yet contemporary (pathogen infection) and historical (evolution) contingencies leave strong signatures in their microbiomes. Microorganisms have always been challenging to study because the nutrients and metabolic interactions required to culture and isolate taxa remain uncharacterised. However, sequencing technologies, combined with community modelling and phylogenetic comparative methods, have helped us understand how different bacterial taxa are distributed across space and hosts (Walkup et al. 2023). Advancing research on amphibian microbiomes can significantly benefit conservation because microbes mediate essential processes such as disease resistance, mate choice (Brunetti et al. 2022, 2019; Comizzoli et al. 2021) and chemical defences (Vaelli et al. 2020). Shifts in microbial abundance or composition can make amphibians more vulnerable but are not easily quantified without robust data. Thus, our study provides a comprehensive collection of host-associated bacteria that can serve as a baseline for future comparisons in the face of climate change and emerging infectious diseases.

Author Contributions

A.V.L. and L.D.B. conceptualised the study; J.J.S.-I. analysed the data and built plots. A.V.-S., M.A.-R. and C.A. collected the samples and performed laboratory work. A.V.L. wrote the first draft of the manuscript. All coauthors substantially contributed to revising and editing the manuscript.

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Disclosure

Benefit-Sharing Statement: Our research collaboration has played a crucial role in fostering and enhancing international scientific partnerships. We have addressed a key priority: the conservation of amphibians threatened by emerging infectious diseases. The data and scripts generated from our work have been shared with the broader scientific community.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The dataset and R code to generate all the analyses of this manuscript are available at Zenodo https://doi.org/10.5281/zenodo.14256951. 16S rRNA sequences were deposited at NCBI PRJNA1003691 (https://trace.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA1003691&o=acc_s%3Aa).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.