Received: 3 April 2023 | Revised: 20 October 2023 | Accepted: 26 October 2023

DOI: 10.1111/mec.17198

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Association between the skin microbiome and MHC class II diversity in an amphibian

M. Cortazar-Chinarro[1,2,3] | A. Richter-Boix[4] | P. Rödin-Mörch[1] | P. Halvarsson[5] | J. B. Logue[6,7] | A. Laurila[1] | J. Höglund[1]

[1]Animal Ecology/Department of Ecology and Genetics, Uppsala University, Uppsala, Sweden

[2]MEMEG/Department of Biology, Lund University, Lund, Sweden

[3]Department of Earth Ocean and Atmospheric Sciences, Faculty of Science 2020–2207, University of British Columbia, Vancouver, British Columbia, Canada

[4]Department of Political and Social Science, Pompeu Fabra University, Barcelona, Spain

[5]Parasitology/Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden

[6]Aquatic Ecology/Department of Biology, Lund University, Lund, Sweden

[7]SLU University Library, Swedish University of Agricultural Sciences, Uppsala, Sweden

Correspondence
M. Cortazar-Chinarro, Animal Ecology/Department of Ecology and Genetics, Uppsala University, Norbyvägen 18D, SE-75236 Uppsala, Sweden.
Email: maria.cortazar@ebc.uu.se

Funding information
Kungliga Vetenskapsakademin, Grant/Award Number: BS2018-0110; Stiftelsen Lars Hiertas Minne, Grant/Award Number: FO2018-0540; Stiftelsen Oscar och Lili Lamms Minne, Grant/Award Number: DO2013-0013; Stilftensen for zoologiska forskning; Svenska Forskningsrådet Formas, Grant/Award Number: 146400178; Swedish research Council, Grant/Award Number: 621-2013-4503

Handling Editor: Nick Fountain-Jones

Abstract

Microbiomes play an important role in determining the ecology and behaviour of their hosts. However, questions remain pertaining to how host genetics shape microbiomes, and how microbiome composition influences host fitness. We explored the effects of geography, evolutionary history and host genetics on the skin microbiome diversity and structure in a widespread amphibian. More specifically, we examined the association between bacterial diversity and composition and the major histocompatibility complex class II exon 2 diversity in 12 moor frog (*Rana arvalis*) populations belonging to two geographical clusters that show signatures of past and ongoing differential selection. We found that while bacterial alpha diversity did not differ between the two clusters, MHC alleles/supertypes and genetic diversity varied considerably depending on geography and evolutionary history. Bacterial alpha diversity was positively correlated with expected MHC heterozygosity and negatively with MHC nucleotide diversity. Furthermore, bacterial community composition showed significant variation between the two geographical clusters and between specific MHC alleles/supertypes. Our findings emphasize the importance of historical demographic events on hologenomic variation and provide new insights into how immunogenetic host variability and microbial diversity may jointly influence host fitness with consequences for disease susceptibility and population persistence.

KEYWORDS
bacterial diversity, host-associated microbiome, MHC class II beta chain, MHC IIB, *Rana arvalis*

1 | INTRODUCTION

All multicellular organisms host microbes. These form diverse communities that associate with a host's organs, such as the skin, lungs and gut (Antwis et al., 2020; Müller et al., 2016; Zilber-Rosenberg & Rosenberg, 2008). These microbial communities contribute to the functioning of the associated organs because of tight interconnections shaped by long co-evolutionary history between a host and its microbiome have forged pervasive interconnections between both parties. Furthermore, the microbiome plays a fundamental role in the development and function of the host immune system. At the same time, the host immune system acts as a resistant environment that imposes ecological filters on the microbial organisms, and thereby has the potential to shape host microbial communities (Hooper et al., 2012; Lee & Mazmanian, 2010; Thaiss et al., 2016). While the potential importance of the interactions between the microbiome and immune system in determining host health has been studied for a number of species, within-species microbiome diversity in non-human hosts deserves further investigation (Becker et al., 2017; Bolnick et al., 2014; Garud & Pollard, 2020; Montero et al., 2021).

The major histocompatibility complex (MHC) plays a vital role in the adaptive immune system of vertebrates (Flajnik & Kasahara, 2001; Ohta et al., 2000; Potts & Wakeland, 1990). MHC genes encode for cell surface proteins that are essential for cell-mediated immunity. They appeared early in the evolution of the adaptive immune system 500 million years ago (Flajnik & Kasahara, 2001; Rock et al., 2016). The extensive population-level allelic diversity in these genes, alongside their central role in the vertebrate immune system, makes them ideal candidates for studying the interaction between the immune system and the microbiome in wild host populations. Examining this relationship in wild populations will also help in understanding the reciprocal interplay between the microbiome and the immune system shaping beneficial host–microbial combinations, pathogen elimination and disease resistance.

The influence of MHC genotype on microbiome diversity and composition has been studied for all major vertebrate groups, including fish (Bolnick et al., 2014), amphibians (Belasen et al., 2021; Hernández-Gómez et al., 2018), birds (Darolová et al., 2021; Leclaire et al., 2019) and mammals (Khan, Stephens, et al., 2019; Kubinak et al., 2015; Lin et al., 2014). In humans, MHC (known as human leukocyte antigen, HLA) variants have been found to be influenced by the composition of the microbiome (Bolnick et al., 2014; Bonder et al., 2016). The results from these studies offer three conflicting predictions for MHC-microbiome interactions: (1) A negative correlation between MHC diversity (heterozygosity) and microbiome diversity (Bolnick et al., 2014; Leclaire et al., 2019), where a higher MHC allele diversity leads to a higher antimicrobial peptide diversity eliminating a higher number of microbial species. (2) A positive correlation between MHC diversity and microbiome diversity (Hernández-Gómez et al., 2018; Khan, Yurkovetskiy, et al., 2019), in which a higher diversity of MHC alleles allows tolerance of a higher number of microbial species. (3) No correlation because MHC diversity is not correlated with the diversity of a microbiome but with its composition, so that certain MHC alleles are linked to the presence of specific microbial species. This results in covariation between an MHC genotype and microbiome composition (Bonder et al., 2016; Olivares et al., 2015). Note that predictions 2 and 3 are not mutually exclusive.

Studies on chimpanzees have shown a clear relationship between a healthy and diverse immune system and internal body regulation of gut microbiome composition (Barbian et al., 2018; Björk et al., 2019). Consequently, individuals suffering from immunodeficiency due to a pathogenic infection experience substantial alterations in their gut microbiome communities (Dillon et al., 2014; Moeller et al., 2015), confirming that immune responses shape microbes (Salas & Chang, 2014). In humans, patients with a lack of immunogenetic diversity show higher gut bacterial diversity and higher frequency of low abundance genes likely encoding nonessential functions than patients with a regular immune system in terms of diversity (Bosák et al., 2021). Most studies have focused on the gut microbiome and its direct association with the mammalian immune system, and very little research has been done on other bacterial communities 'including skin microbiota' and on other host vertebrate groups. Therefore, studies investigating the role of skin microbiome diversity in shaping immune response are urgently needed in order to gain a better understanding of the factors causing the bacterial community compensation effect on the host.

Despite recent calls for the integration of microbiome research in evolutionary and conservation biology (Cullen et al., 2020; Henry et al., 2021; West et al., 2019), little progress has been made on the fundamental association between host population history and genetic variation and the diversity and composition of host microbiomes in wild populations. In this paper, we studied the variation in MHC class II exon 2 in 12 moor frog (*Rana arvalis*) populations from Scandinavia originating from different environments and having different evolutionary histories (see Section 2 for a more detailed description). Previous studies have demonstrated that postglacial colonization processes after the Last Glacial Maximum had a profound impact on the geographical distribution of *R. arvalis* and its genetic diversity (Cortázar-Chinarro et al., 2017; Knopp & Merilä, 2009; Meyer-Lucht et al., 2019; Rödin-Mörch et al., 2019). These findings indicate that current patterns of neutral single nucleotide polymorphism (SNP), as well as MHC and other adaptive variations, across Scandinavia reflect two different postglacial colonization routes and show signatures of past and ongoing differential selection patterns, drift and historical demographic events. Southern populations have higher allele richness than those in the north (Cortazar-Chinarro et al., 2018; Cortázar-Chinarro et al., 2017; Meyer-Lucht et al., 2019; Rödin-Mörch et al., 2019). Investigating how these different genetic backgrounds, and especially the MHC variation, are correlated with microbiome diversity could provide promising avenues for understanding the distribution of host–microbiome biodiversity, its evolutionary association history and the forces that have shaped and continue to shape it (Groussin et al., 2020).

Understanding the causal connections between the host's MHC and its microbiome are especially relevant in groups in which virulent wildlife diseases are contributing to population declines (Fisher et al., 2012). Among these diseases, chytridiomycosis, caused by the chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*), stands out as an emerging disease inflicting amphibian mass die-offs worldwide (Kilpatrick et al., 2010; Martel et al., 2014; Scheele et al., 2019). Recent studies demonstrate the importance of the skin microbiome in the innate immune defence of amphibians against *Bd* (Bates et al., 2018; Rebollar et al., 2016; Torres-Sánchez & Longo, 2022). In the present study, the 12 *R. arvalis* populations included differed regarding *Bd* infection. *Bd* has not been detected in Northern Sweden, whereas the fungus is present in many of the southern populations (Meurling et al., 2020). An earlier study found that individuals at northern latitudes show lower adaptive and neutral genetic variation than southern individuals (Cortázar-Chinarro et al., 2017). This might increase the risk of infection by and vulnerability to *Bd* if it reaches northern latitudes in the future. Hence, investigating how host MHC genetics, the environment and evolutionary history determine the skin microbial diversity and composition of amphibian populations is a priority in amphibian conservation (Jiménez & Sommer, 2017; Trevelline et al., 2019).

We have asked the following four questions: (i) Does geography and/or host evolutionary history affect the diversity and composition of the skin microbiome in *R. arvalis*? (ii) What is the nature of the correlation between MHC heterozygosity and microbiome diversity? (iii) How does MHC diversity affect the skin microbiome? (iv) Does MHC allele similarity correlate with skin microbiome diversity and/or composition?

2 | METHODS

2.1 | Study sites and sampling

Rana arvalis has a broad longitudinal and latitudinal distribution in Eurasia and is relatively common in most of Fennoscandia (Wielstra et al., 2014). Previous studies show a bidirectional postglacial colonization route of the species to Scandinavia, with a western lineage coming from the south via Denmark to Southern Sweden and another lineage arriving from the east via Finland to Northern Sweden (Cortázar-Chinarro et al., 2017; Knopp & Merilä, 2009; Rödin-Mörch et al., 2019). Eight sites close to Uppsala (Uppland region, henceforth termed 'South') and four sites in Luleå (Norrbotten region, henceforth called 'North') were selected as sampling locations for this study (Figure 1). 'South' corresponds to the western lineage, and 'North' to the eastern lineage. Study sites within each region were at least 8 km apart and differed in habitat, ranging from open farm ponds to forest ponds. Sampling was conducted during the breeding season in March–April (South) and May (North) 2016 (Figure 1 and Table S1).

A total of 207 adult frogs were captured using hand nets (South: $n = 146$; $n_{average} = 18$, North: $n = 61$, $n_{average} = 15$; Table S3_A). Each individual was handled with a new pair of sterile nitrile gloves to avoid cross-contamination. All individuals were sexed and weighed prior to sample collection. A piece of tissue was removed from the toe webbing and stored in 90% alcohol for DNA extraction. To sample the skin microbiome, each frog was transferred to an individual 250 mL container containing sterile distilled water (Millipore Milli-Q™; Fisher Scientific) to remove transient microbes from the environment. After 5 min, each animal was moved to a new container with sterile distilled water and kept there for another 2 min.

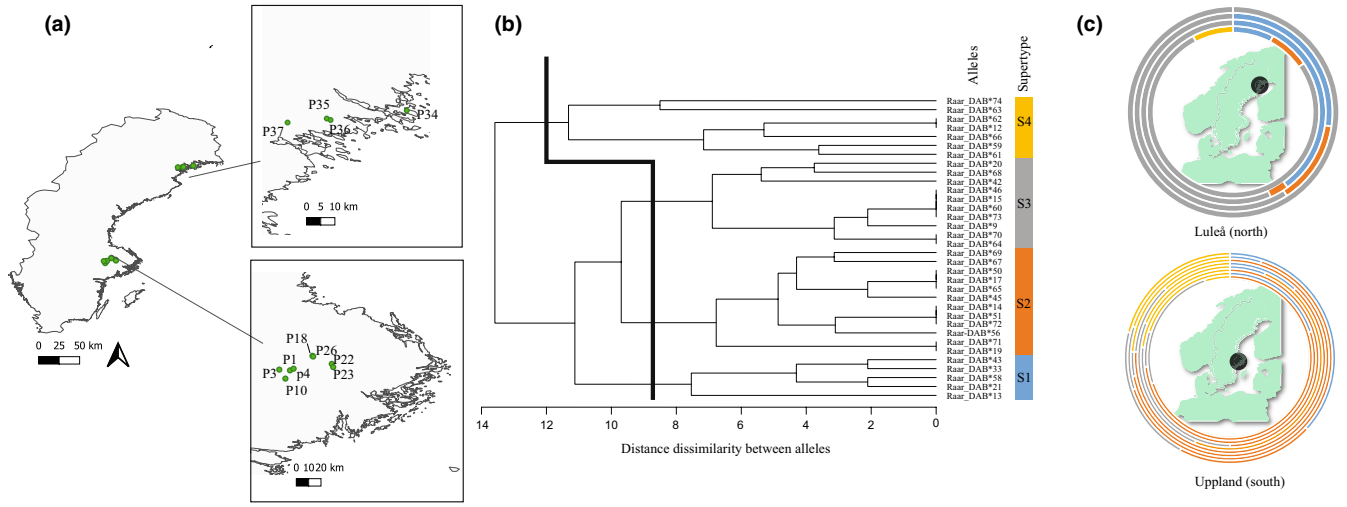

FIGURE 1 (a) Map of sampling locations ($n = 4$ in the north, $n = 8$ in the south). The average distance between collection sites in the same region was 20 km (range 8–50 km). The exact coordinates can be found in the supplementary information (Table S1). (b) The hierarchical clustering tree illustrates the grouping of a total of 34 alleles into four distinct supertypes. Determination of the number of clusters (supertypes) was based on the divergence observed among the branches in the phylogenetic tree. Within each cluster, alleles were collapsed into a single supertype using z-descriptors. The optimal number of clusters, represented by supertypes (S1, S2, S3 and S4), is indicated by the black line, determined using the *hclust* function in R. (c) The pie chart illustrates the frequencies of supertypes in two regions: North and South.

Finally, each frog was cleansed manually (again with sterile-distilled water; ddH_2O). The frogs were then carefully swabbed with a sterile rayon-tipped MW100 (mwe; Medical Wire & Equipment Co) six times on both dorsal and ventral surfaces, covering as much skin as possible. After sampling, the frogs were released back into the pond. Swabs were transported on ice in cooler boxes prior to storage at −80°C in the laboratory.

To control for environmental microbes that might be found on the frogs' skin, a 2 L water sample was taken from every study site. Water samples were taken in close proximity to where the frogs were captured using a sterilized Durham glass bottle and kept cold and dark until processed in the laboratory. Water samples were filtered under a sterilized hood in the laboratory on the night of collection. As a pre-filtration step, two blank filtered samples (FNC1 and FNC2) were obtained from every water sample after filtering 200 mL DNA/RNA-free Milli-Q water. Bacterioplankton cells were collected onto 0.2 μm membrane filters (Super-200 Membrane Disc Filters; Pall Corporation), filtering 0.2 L of pre-filtered (0.7 μm; membrane filter) water. Pre-filtration was carried out to avoid capturing larger particles. Four water samples were taken at each site. The filters were kept at −80°C until DNA extraction.

The temperature of every pond was recorded on the day of sampling using a portable multiparameter meter. Monthly temperature and precipitation (Worldclim database: http://www.worldclim.org average of 30 years) at each sampling location were extracted to estimate the average values of these bioclimatic variables from the beginning of the breeding season in March to the end of the growing season in October.

2.2 | DNA extraction and Illumina MiSeq library preparation and sequencing

2.2.1 | MHC class II exon 2

DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. The complete second exon (272 bp) of the single MHC II gene (corresponding to the β −2 domain) in *R. arvalis* was amplified using the primers ELF_1 (3′-GAGGTGATCCCTCCAGTCAGT-5′) and ELR_2 (3′-GCATAGCAGAC GGAGGAGTC-5′) (Cortázar-Chinarro et al., 2017). Both forward and reverse primers were modified for Illumina MiSeq sequencing with an individual 8 bp barcode and an NNN sequence (to facilitate cluster identification). PCR reactions and library preparation are described in detail in Cortázar-Chinarro et al. (2017). A total of six libraries were generated using the ThruPLEX DNA-seq 6S (12) Kit (Takara Bio Europe). The concentration of each sample pool was measured with Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen Life Technologies) on a fluorescence microplate reader (Ultra 384; Tecan Group Ltd.). The six libraries were combined in equimolecular amounts of each sample into a MiSeq run prior to sequencing. Sequencing of two MiSeq 2x250 (rxn) runs was carried out at the NGI/SciLifeLab Uppsala (Sweden).

2.2.2 | Bacterial DNA extraction and library construction

The whole community DNA was extracted from both the swabs and filters using the DNeasy PowerSoil Kit (Qiagen) following the manufacturer's protocol. The extracted DNA was sized and quantified using agarose (1.5%) gel electrophoresis, GreenGel staining (Biotium Inc.) and safe blue light transillumination prior to PCR amplification.

The bacterial swab and lake samples were subjected to 16S rRNA gene amplicon sequencing on an Illumina MiSeq platform (Illumina Inc.). The sequencing library was prepared according to a two-step PCR. The first PCR step (30 cycles) amplified the bacterial hypervariable region V4 of the 16S rDNA gene, using bacterial forward primer 515F (5′-GTGCCAGCMGCCGCGGTAA -3′) and reverse primer 806R (5′- GGACTACHVGGGTWTCTAAT -3′) (Varg et al., 2022). The second PCR step (20 cycles) attached indices to both ends of the 16S amplicons in order to create a unique dual barcode for each individual sample (see Table S2 and additional information A1). The 16S primers used in the first PCR step were thus modified by extending their 5′-ends with Illumina adapter sequences. These barcoding primers also comprised the Illumina sequencing handle sequence, which attaches the amplicons onto the Illumina flow cell to initiate sequencing. In both PCR steps, Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific) was used. PCR mixtures were prepared each time according to the manufacturer's instructions with the addition of 20 mg/mL of BSA (Bovine Serum Albumine, Thermo Fisher). Amplicons were purified after each PCR step using the Agencourt AMPure XP purification kit (Beckman Coulter Inc.). Amplicon fragment size and quantification were checked using a Bioanalyzer (Agilent) and a fluorescence microplate reader (Ultra 384; Tecan Group Ltd.), employing the Quant-iT PicoGreen dsDNA quantification kit (Invitrogen). Finally, equimolar amounts of samples were mixed and the final amplicon sequenced using the Illumina MiSeq platform (Illumina Inc.) at NGI/SciLifeLab Uppsala (Sweden).

2.2.3 | MHC class II exon 2 sequence processing

The raw amplicon sequencing was combined into single forward reads using FLASH v1.2 (Magoč & Salzberg, 2011). Each of the six amplicon pools was analysed independently. A total of six fastq files were generated and transformed to fasta by using the AVALANCHE NEXTGEN package (DNA Baser Sequence Assembler v4 (2013), Heracle BioSoft, www.DnaBaser.com). AMPLICHECK (Sebastian et al., 2016) was used to remove primer sequences and for de-multiplexing, chimera removal and counting variants for each amplicon, while AMPLISAS (Sebastian et al., 2016) was used for final allele verification. We used the DOC method (Lighten et al., 2014), where variants are sorted top-down by coverage, followed by the calculation of the coverage break point (DOC statistic) around each variant. We retained a total of 177 samples that revealed an identical genotype for at least two out of three replicates. All retained allele sequences were imported and aligned in MEGA X (Kumar et al., 2018). All sequences were extensively compared

to other sequences from the same locus (*R. arvalis*: GenBank: isolates from h1 to h57 [MT002608.1–MT002664.1]). We used the MHC nomenclature by Klein (1975) for the valid retained alleles. This nomenclature consists of a four-digit abbreviation of the species name followed by gene*numeration, for example, Raar_ DAB*01.

2.2.4 | Bioinformatic processing of bacterial data

Raw sequences were processed using software DADA2 (Callahan et al., 2016). Forward and reverse reads were trimmed to 240 and 200 bp, respectively, using default parameters. Default parameters were also employed to correct for amplicon errors and to identify chimeras and merge-end reads. Taxonomic assignment following amplicon sequence variant approach (ASV) was performed using the bacterial 16S rRNA SILVA reference database (version v132) training set (Yilmaz et al., 2014). All unassigned ASVs were removed from the samples (Costa et al., 2022; Couch et al., 2021). To further minimize erroneous ASVs, all singletons were removed using the default settings of DADA2. The data were filtered by sample or taxa, using the functions *subet_sample*, *prune_taxa* () implemented in the 'PHYLOSEQ' R package (McMurdie & Holmes, 2013). Data were transformed into proportions (compositional data) in order to minimize erroneous ASVs and for direct count comparisons to produce correct results for statistical analyses (Cameron et al., 2020; McMurdie & Holmes, 2014; Willis, 2019). We used the function *transform_sample_counts (ps, function (out) out/sum(out))* implemented in the 'PHYLOSEQ' package (McMurdie & Holmes, 2013) or the 'MICROBIOME' R package (Shetty & Lahti, 2019). We used the *compositional* method and *clr* (i.e. relative abundance methods). The analyses were verified using all two methods.

2.2.5 | MHC class II exon 2 data analyses

We assessed genetic diversity in the MHC class II exon 2 using standard diversity indices (expected heterozygosity [H_E], observed heterozygosity [H_O], allelic richness and nucleotide diversity, among others, Table S3A). These were calculated for each locality in ARLEQUIN v 3.5 (Excoffier & Lischer, 2010). Global pairwise F_{st} between all populations and an AMOVA test were computed in ARLEQUIN v 3.5 (Table S3_B and Table S4). Allelic richness was calculated in FSTAT 2.9.3.2 (Goudet, 1995; see Table S3). Allele frequency plots were created in R using the GGPLOT2 package (Wickham, 2016).

To collapse MHC alleles into functional supertypes, we extracted the 12 codon positions for the peptide binding region (PBR) in accordance with Cortazar-Chinarro et al. (2018). We then characterized each codon based on five physiochemical descriptor variables: z1 (hydrophobicity), z2 (steric bulk), z3 (polarity), z4 and z5 (electronic effects; Sandberg et al., 1998). A hierarchical clustering tree for the MHC class II exon 2 in *R. arvalis* was constructed with the z-descriptors in R (version 4.0.5) by using the function *hclust* in R. The optimal number of clusters was chosen based on divergence between the branches in the phylogenetic tree, following Meyer-Lucht et al. (2010). Alleles within each cluster were collapsed into a single supertype (Figure 1). Supertype allele frequency plots were created in Excel (Figure 1). We consider supertyping a useful method for investigating broad associations between MHC, infection and microbiome in field studies (e.g. While the use of MHC supertypes comes with important caveats, Kaufman, 2020; Tregaskes & Kaufman, 2021), they have a strong track record as a tool in ecological applications of MHC (other refs), including in controlled infection experiments (Phillips et al., 2018, 2021; Smallbone et al., 2021).

2.2.6 | Bacterial diversity data analyses

Differences in composition between the environmental pond and amphibian skin communities were examined using permutational multivariate analysis of variance (PERMANOVA, analysis of differences in group means based on distances, 999 mutations) and permutational multivariate analysis of dispersion (PERMDISP, analysis of differences in group homogeneities based on distances).

Bacterial alpha diversity was estimated using observed richness, Shannon diversity and phylogenetic diversity indices. Comparisons between regions and sexes were carried out using Wilcoxon and Kruskal–Wallis tests due to the unresolvable distribution issues of our data. All comparisons were implemented in 'PHYLOSEQ' (McMurdie & Holmes, 2013) and 'PICANTE' (Kembel & Kembel, 2014) R packages. Pearson correlation coefficients between ASVs, observed richness and total number of reads were used to assess whether the asymptote had been reached, and thus whether there was a risk of us missing diversity (Figure S1). Generalized linear models (GLMs) and generalized linear mixed models (GLMMs) with Gaussian error structure were used to assess whether alpha diversity (Shannon) could be explained by the following environmental factors: (1) temperature at time of sample collection, 'TemCollection', (2) average temperature, 'TemMean' and (3) average precipitation, 'PreMean' on ASV bacterial diversity. Population was included as a random factor. GLMMs models were run in R.

The analysis of differences in bacterial composition among communities between the two regions (South and North) and sexes involved using a PERMANOVA test. The *Adonis2*() function in the 'VEGAN' package was used for this analysis, run with 999 permutations. Additionally, the homogeneity of group dispersion (PERMDISP) was tested on weighted and unweighted UniFrac distances. Relationships between the bacterial assemblages from the south and north were explored by employing hierarchal cluster analyses (Bray–Curtis distance and UniFrac distances) using 'VEGAN' (Oksanen et al., 2019) and circlize (Gu et al., 2014) packages implemented in R.

2.2.7 | Associations between bacterial diversity and host MHC class II exon 2

Relationships between MHC genetic and bacterial diversity (Shannon, Simpson and Chaos1) were analysed using several tests.

The Shannon diversity index was the only diversity index suitable for the data interpretation and visualization that followed. We assessed the effect of MHC heterozygosity and MHC nucleotide diversity and bacterial Shannon diversity at both the population and individual level. For both, multiple regression on distance matrices (MRM) and linear regression models (lm) were implemented with the packages 'Ecodist' (Goslee & Urban, 2007) and 'NLME' in R.

Relationships between heterozygosity at the allelic, supertype level and the Shannon bacterial diversity index between regions were explored by running a GLMM model in R. For this purpose, individuals were divided into two categories: homozygous (0; individuals carrying two identical alleles) and heterozygous (1; individuals carrying two distinct alleles). Additionally, individuals were categorized as *SameS* or *DistinctS* based on their functionality, as determined by the supertype information. *SameS* individuals were defined as individuals in which the two alleles belonged to the same supertype group (e.g. Supertype2_2), while *DistinctS* individuals carried two alleles that belong to different supertype groups (e.g. Supertype1_3). The individual Shannon diversity index was used as the response variable, and the region as a fixed factor with population as a random effect. Additional GLMM analyses were carried out to test for differences between bacterial diversity and specific supertype_genotype groups within the regions. Redundancy analyses (RDA) using the 'VEGAN' package (Oksanen et al., 2019) were carried out to find potential indications of a relationship between bacterial community composition and supertype_genotype structure (e.g. Supertype 1_1, see Figure S3). Likewise, we used RDA to summarize linear relationships between the composition of the bacterial community and MHC-specific supertypes.

DESeq2 and ANCOMBC2 analyses were performed to explore whether specific bacterial taxa differed in abundance between homozygous and heterozygous individuals, as well as between *SameS* and *DistinctS* groups of individuals between specific supertypes (Lin Peddada & Lin, 2021; Lin et al., 2014; Love et al., 2014). In addition, ASV abundance and supertype data were cross-correlated using Spearman rank correlation to explore whether specific taxa were correlated with specific supertypes. First, we transformed abundance data into compositional data by using the 'MICROBIOME' package (Shetty & Lahti, 2019). A neighbour-joining tree, showing the phylogenetic relationships among ASVs negatively and positively correlated with MHC supertypes, was constructed using MEGA X (Kumar et al., 2018).

3 | RESULTS

3.1 | MHC II exon 2 and skin microbiome characterization

We obtained a total of 4.2 million reads with intact primers and attached barcode information that could be assigned to 207 individuals. We amplified and sequenced 81.8% of the samples in duplicates or triplicates. One sample out of 421 failed due to PCR amplification problems. The average number of reads per amplicon was 13,085.37 ranging from 420 to 106,172 reads. After filtering and quality control analysis, 2.7 million reads remained. We then excluded individuals that did not provide identical genotypes in at least two replicates, as well as those for whom we lacked microbiome information due to PCR failing during 16S region amplification. We ultimately included a total of 177 individuals in all subsequent analyses. We assigned 34 valid MHC class II exon 2 alleles with a length of 272 bp and 27 polymorphic nucleotide positions. All the 34 valid MHC II exon 2 allele sequences were translated into unique amino acid alleles. A previous study by Cortázar-Chinarro et al. (2017) had identified 17 out of the 34 alleles, and another 17 were new alleles were discovered in the present study (Raar_58 to Raar_74). Using the DOC method (Lighten et al., 2014), we detected a single locus in 193 out of 207 individuals. Three individuals showed evidence of a second MHC class II locus with a lower number of reads in two of the three replicates, pointing to the possible existence of a very rare MHC class II duplication. We concluded that we were working with a single MHC class II locus in our data set. However, we cannot rule out the possibility that our primers amplified an additional MHC class II locus in a few cases (two individuals).

For the skin microbiome characterization, a total of 37,148 reads were obtained from both amphibian ($n = 177$) and water samples ($n = 12$), with amphibian swabs contributing 84.84% to the total number of reads. The most abundant phyla were Proteobacteria (45% of the total number of sequences), Bacteroidetes (16%), Actinobacteria (9.9%), Acidobacteria (6.77%), Verrucomicrobia (5.39%) and Firmicutes (3.71%). The rest of the phyla represents less than 2.5% of the total number of reads: Planctomycetes (2.1%), Chloroflexi (1.77%), Armatimonadetes (1.4%), Candidatus Saccharibacteria (0.81) and Gemmatimonadetes (0.75%) (Figure S2). After the removal of uncharacterized taxa ($n = 1280$ ASVs; 7.8% of the total abundance), 15,017 taxa remained.

3.2 | Genetic diversity

3.2.1 | MHC class II exon 2

The number of alleles per population varied between the northern and southern region (overall AR; South = 9.87, North = 3.75, see Figure 2 and Figure S3, and Table S3). Levels of expected heterozygosity for the MHC locus between populations ranged from 0.23 to 0.84 (overall HE = 0.79, Table S3) and allelic richness ranged from 3 to 11 (overall AR = 7.83, Table S3). The northern region showed lower diversity than the southern region in terms of HE and AR. The overall Fst among the groups (North vs. South) was 0.52 (95% C.I. = −0.56 to 0.39), while the overall Fst between populations within the two geographical clusters was 0.07 (95% C.I. = 0.1 to 0.09) and 0.004 for the northern and southern cluster respectively (95% C.I. = −0.01 to 0.016, see Table S4). Two alleles occurred only in a single population in the southern region (Raar_69 and Raar_65; Figure 2 and Figure S2). Three alleles were

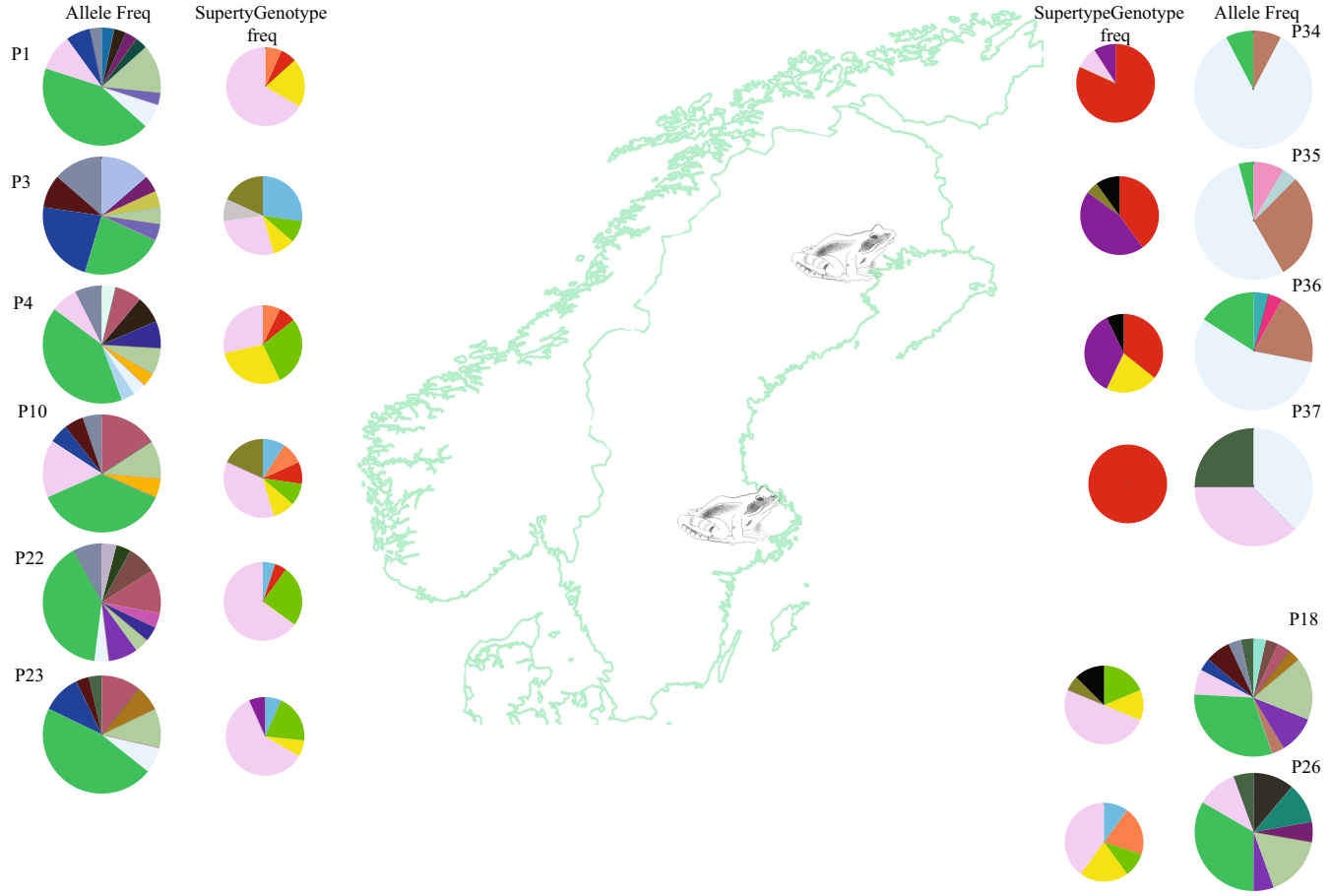

FIGURE 2 Allelic frequency distribution of MHC class II alleles and supertype$_{Genotypes}$ in 12 *R. arvalis* populations (P1: Ekeborg, P3: Eneby, P4: Valsbrunna, P10: Kroklösa, P22: Högbyhatt, P23: Dalkarlskärret, P18: Mosta, P26: Ströbykärret, P34: Lillträsket, P35: Vittjärnen, P36: Djurhustjärnen, P37: Dalbacka). Colour coding scheme for MHC alleles is given in Figure S2, and the exact coordinate locations are described in Table S1.

only present as private alleles to a single population in the northern region (Raar_42, Raar_43, Raar _68). The alleles spanning from Raar_DAB*1 to 57 were previously identified, while the alleles from Raar_DAB*58 to Raar_DAB*74 were newly discovered in the current study. Consequently, all the alleles ranging from Raar_DAB*1 to 57 and absent in Figure 1 are unique to the earlier study (Cortázar-Chinarro et al., 2017).

The 34 alleles were converted into four different MHC class II exon 2 supertypes based on physiochemical binding properties (Figure 1). Supertype_2 was the most common supertype in the southern region, while Supertype_3 was the most abundant in the northern region (Figure 1). Supertypes were also grouped by genotypes. Supertype$_{Genotype}$ diversity was defined as the diversity within each genotype. Supertype$_{Genotype}$ was higher in the southern region (Supertype$_{Genotype_south}$ = 9; Supertypes$_{Genotype_north}$ = 6). We observed that 49% of the southern individuals carried the Supertype$_{Genotype}$ 2_2, while only 1.18% carried the Supertype$_{Genotype}$ 2_2 in the North. By contrast, Supertype$_{Genotype}$ 3_3 and Supertype$_{Genotype}$ 1_3 occurred with a higher frequency in the north than in the south (3_3: 58.1% and 27.2% and 1_3: 3.53% and 0.88% respectively) (Figure 2 and Figure S3).

3.2.2 | Skin bacterial diversity patterns in relation to environmental variables, regions and sex

We found significant differences between the composition of the bacterial community in the water filters and on the skin of the amphibians (PERMANOVA; $p < .05$, PERMDISP; $p < .05$) (Figure S4, Table S5). We did not find significant differences in alpha diversity between sexes (Wilcoxon Observed; W = 3486, $p = .33$; Wilcoxon Shannon; W = 3453, $p = .39$; Wilcoxon PD; W = 3469, $p = .36$) or regions (Wilcoxon Observed; W = 2901.5, $p = .88$; Wilcoxon Shannon; W = 2954, $p = .96$; Wilcoxon PD; W = 2959, $p = .95$; Figure S5). A GLM controlling for average precipitation (PreMean) and temperature at data collection (TemCollection) found a significant effect between North and South in alpha diversity (Table S6). Average precipitation had a significant negative effect on alpha diversity. However, we did not observe a significant relationship between the temperature at the collection time and the skin microbiota diversity in the GLMMs models when we controlled by population (Table S6). Additionally, we found support for a geographical effect on beta diversity, showing significant differences in bacterial community composition between the two regions (PERMANOVA; $p < .05$ weighted and unweighted;

UniFrac distances, Figure 3 and Figure S6, Table S7) but no differences in group dispersions (PERMDISP; $p > .05$, Figure S7, Table S8).

3.3 | Effects of MHC class II heterozygosity and skin microbiota composition

Multiple regression analyses showed that populations with higher MHC heterozygosity exhibited more diverse microbiota at the population level (Figure S8, Table S9). However, individuals with more divergent MHC sequences based on nucleotide diversity had less diverse microbiota at both individual and population levels (Figure S9, Table S9).

We next quantified the relationship between individuals that carried two identical alleles (homozygous) versus individuals carrying two distinct alleles (heterozygous). Using GLMs, we did not find significant differences in alpha diversity between homozygous and heterozygous individuals. However, we found that *DistinctS* individuals from the north showed significantly higher microbial diversity in comparison to southern *DistinctS* individuals in terms of the functional clustering (Figure S10, Table S10). We did not observe such an effect on the *SameS* individuals, suggesting that bacterial diversity potentially compensates for the deficiency of MHC diversity in the northern region. We did not find significant differences in bacterial diversity between specific MHC genotypes present in both geographical regions (Supertype 2_3 and Supertype 3_3). Furthermore, RDA and PERMANOVA analyses did not show any differences in the beta bacterial diversity patterns between *DistinctS* and *SameS* individuals. However, we found significant differences in community structure among specific $\text{Supertype}_{\text{Genotype}}$ (Table S11). Additionally, we found that individuals carrying supertypes 1 or 2 had specific bacterial compositions (Figure 3b,c; Figure S10, Table S12). A more detailed analysis of individual genotypes showed that $\text{Supertype}_{\text{Genotype}}$1_3, 2_2, 2_4 and 3_3 had specific bacterial compositions (Table S13). Both these results might be explained by strong direct regional effects between North and South, as well as

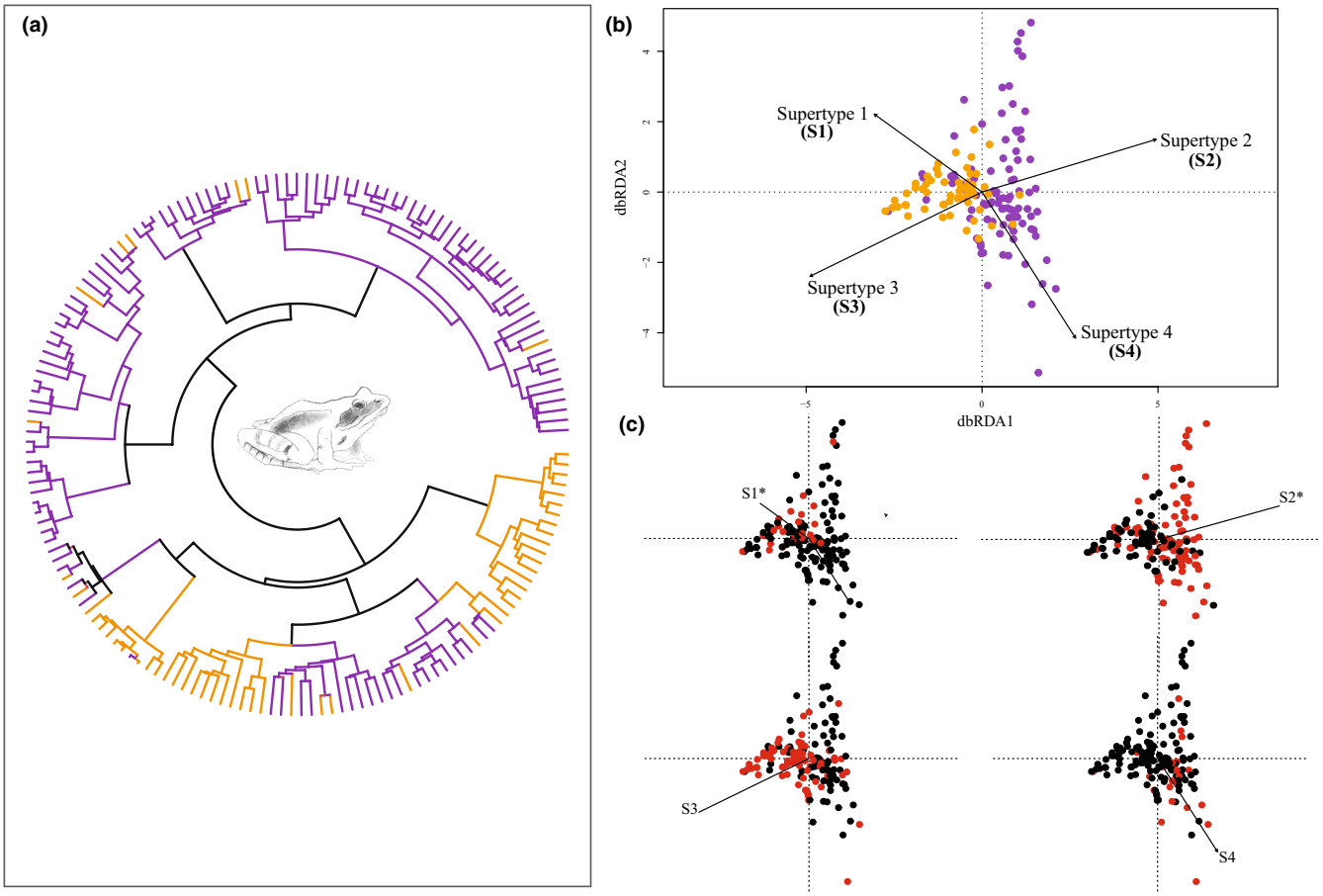

FIGURE 3 (a) Differences in bacterial community composition of 16S DNA skin microbiota between regions represented by hierarchal clustering of samples (Ward's clustering; Bray–Curtis distance). Clusters representing the 16S DNA skin microbiota composition from the south are coloured in purple and from the north in orange respectively. (b) RDA performed with the bacteria identified in skin microbiome clustered in two main groups according to amphibian's origin. Each point represents the skin microbial community of an individual *R. arvalis*. 16S skin samples of an individuals from the south are shown in purple and samples of individuals from the north are shown in orange. The supertypes (S1, S2, S3 and S4) are indicated as variables represented by arrows. (c) The RDA plots show the separation pattern for each supertype (S1, S2, S3 and S4). Individuals carrying the supertypes S1, S2, S3 and S4 are highlighted in red and labelled in bold following the nomenclature in panel B. Significant supertypes are marked with (*) with a $p < .05$ according to PERMANOVA (*Adonis* test).

an effect of a specific combination of MHC class II exon 2 on the microbial structure (Figure S11).

3.4 | Associations between MHC supertypes and microbial taxa

We did not find bacterial ASVs that were significantly different in abundance between Homozygous/Heterozygous and *SameS/DistincS* individuals according to the MHC supertype clustering. In contrast, we found bacterial ASVs that were significantly different in abundance depending on the supertype that an individual carried (Figure S12; Tables S14 and S15). The *Oxalobacteraceae* family was the most common taxon found by using both DESEq2 and ANCOMBC2 (Lin, Peddada & Lin, 2021). Likewise, the heat map (Figure 4b) illustrated positive and negative correlations (Spearman rank correlation $p < .04$) between supertypes and specific ASVs. Families exhibiting significant MHC correlations (*Comamonadaceae*, *Oxalobacteraceae and Pseudomonadaceae*) are taxonomically clustered (Figure 4a). Considering all the results obtained, we suggest that supertype 4 may influence the abundance of a specific *Bacteroidetes* family, while supertypes 1, 2 and 3 may affect the abundance of at least two families within *Proteobacteria*. Surprisingly, supertypes 2 and 3, which are the most dominant supertypes in the southern and northern regions, respectively, showed an opposite association with specific bacterial taxa, especially *Oxalobacteraceae*. The negative and positive associations with MHC may thus simply be a by-product of regional differences in MHC composition and microbiome composition.

4 | DISCUSSION

We characterized the skin microbiota composition and MHC class II exon 2 diversity in 12 *R. arvalis* populations from two separate geographical regions representing different evolutionary histories due to different postglacial colonization patterns (Cortázar-Chinarro et al., 2017; Luquet et al., 2019; Rödin-Mörch et al., 2019). We assessed the relationships between MHC genotype and microbial community diversity to investigate potential associations between the host MHC genes and skin microbiome, and to elucidate differences between regions and evolutionary histories. Our results indicate that the skin microbial community of frog populations varies substantially across populations and regions. Climatic and pond environmental factors appeared to influence the diversity and structure of microbial communities, but most of the differences identified could not be explained by the environmental factors included in our study. Therefore, although our data cannot prove the relationship, our results suggest that skin microbial community structure could be influenced by host genetic variation. While further investigations

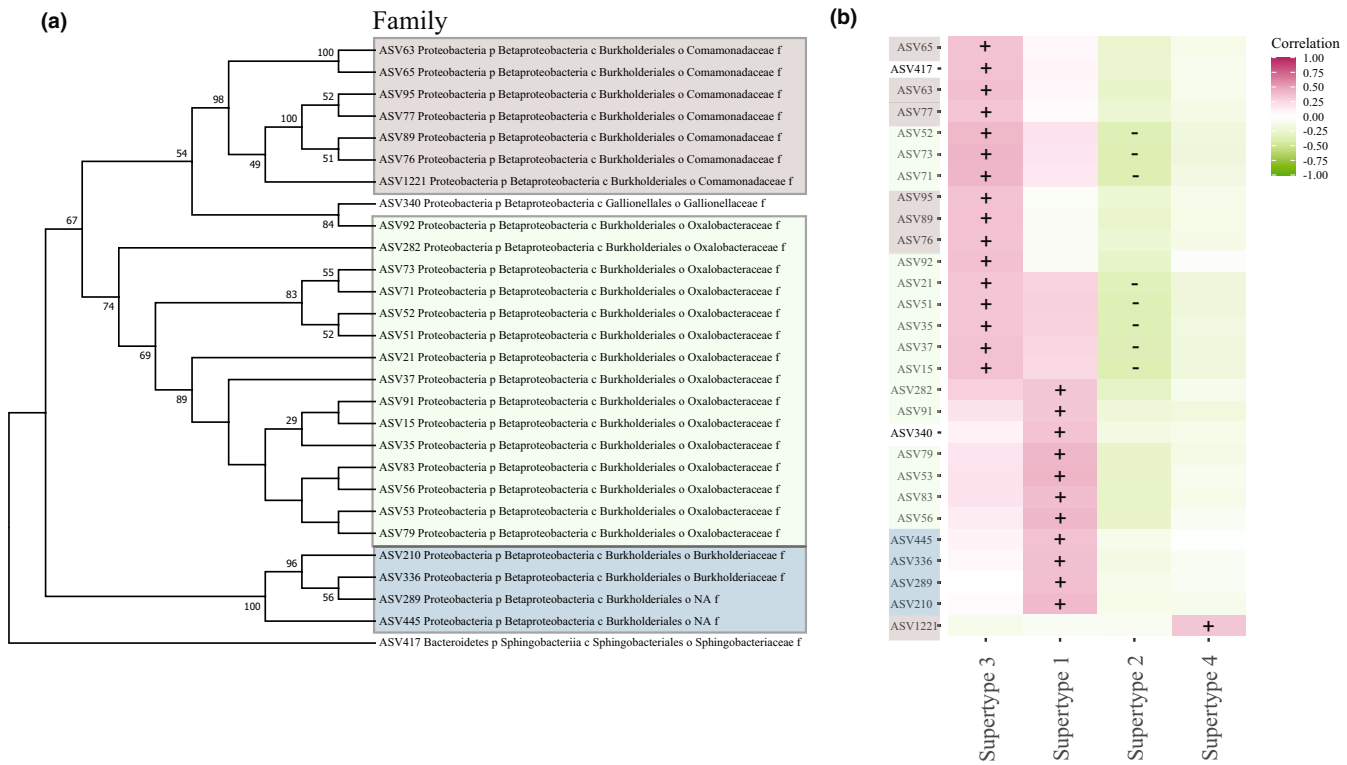

FIGURE 4 (a) The neighbour-joining tree of the phylogenetic relationships among ASVs correlated with MHC supertypes at family level. *Commamonadaceaeae* family are shown in dark pink, *Oxalobacteraceae* in light green and bacteria belonging to the *Burkholderiaceae* family in light blue. (b) Heatmap showing the correlations between supertypes and specific ASVs. Significant Spearman cross-correlations ($p < .05$) are labelled with (+) or (−). Positive correlations are shown in pink (+) and negative correlations (−) in green.

should be carried out in this regard, four main results can be derived from our analyses. First, there was a significant difference in alpha diversity between regions after controlling for both precipitation and temperature. Additionally, beta diversity, which is associated with microbial composition, exhibited a significant difference between the regions. Second, within populations, MHC heterozygosity was positively correlated with microbial alpha diversity. Third, heterozygous individuals from the north showed higher alpha diversity than heterozygous individuals from the south, where MHC class II diversity was higher. Fourth, there were indications of opposing associations between MHC class II alleles and specific bacterial taxa at the regional level. We will discuss each of these results in detail below.

4.1 | Microbiome variation between regions and populations

Previous studies of *R. arvalis* showed that the genetic composition of MHC class II alleles was strongly influenced by evolutionary processes. These studies included neutral markers such as SNPs and microsatellites, as well as adaptive markers like MHC (Cortázar-Chinarro et al., 2017; Meyer-Lucht et al., 2019; Rödin-Mörch et al., 2019). We also observed distinct historical selection patterns in the MHC between populations in the north and south. In the northern cluster, uniform directional selection emerged as the primary driving force behind the modulation of genetic variation, whereas divergent selection was more prevalent in the south (for detailed information, see Cortazar-Chinarro et al., 2018). Recent studies have revealed the significance of both demography and evolutionary processes in shaping genetic variation (Cortázar-Chinarro et al., 2017). However, very little is known about how evolutionary processes influence skin microbiota diversity in amphibians (Belasen et al., 2021; Torres-Sánchez & Longo, 2022).

In the present study, we found similar patterns of alpha diversity in the skin microbiome when comparing regions and populations, but also found that the relative abundance of shared ASVs, that is, beta diversity, and thereby, the composition of the bacterial community structure, varied between regions and populations. Different regions and populations exhibited distinct skin microbial communities, likely influenced by differential environmental conditions and host-specific filtering. We suggest that the historical genetic background of different colonizing lineages, along with different selective pressures, may play a significant role in shaping the distribution of host–microbiome biodiversity in *R. arvalis* populations. The effect of the population-level genetic background of the host has been proposed as a strong predictor of skin microbiome structure in other systems (Amato et al., 2016; Dimitriu et al., 2019; Muletz Wolz et al., 2018; Weinstein et al., 2021). In line with this, co-occurring Panamanian frog species host unique skin bacterial communities (Belden et al., 2015). However, it is not known whether host-associated traits, such as the immune genes, select for specific host bacterial communities in amphibians as they do in other organismal groups such as humans (Shafquat et al., 2014; Wein & Sorek, 2022). Furthermore, we cannot rule out the possibility that host–pathogen associations might also be driven by linkage with other genes.

4.2 | Deterministic factors contributing to microbiome variation

Adaptive immune genes such as MHC have been extensively linked to susceptibility to infections in vertebrates (Savage et al., 2019; Savage & Zamudio, 2011). Parasite-specific immune responses driven by MHC polymorphism have been extensively studied (Eizaguirre & Lenz, 2010; Elbers & Taylor, 2016; Minias et al., 2017). However, how the complex relationship between MHC and a multitude of host-associated microbes influences the host immune response is still poorly understood. While mammalian studies have highlighted that the host's genetic background can influence microbial communities via the immune system (Blekhman et al., 2015; Tabrett & Horton, 2020; Woodhams et al., 2020), less is known for other taxa. However, recent investigations have shed light on important associations between host immunity and microbiomes (Bolnick et al., 2014; Fleischer et al., 2020; Hernández-Gómez et al., 2018), not only for MHC genes but also for other immune and cell signalling genes linked to MHC class I and II (Flajnik, 2018; Grogan et al., 2018; Richmond et al., 2009). In sticklebacks, high MHC variation has been associated with diverse microbiota (Bolnick et al., 2014), while in amphibians high MHC variability may influence host health indirectly by shaping bacterial communities (Belasen et al., 2021). In accordance with previous findings for *R. arvalis* (Cortázar-Chinarro et al., 2017), we found lower MHC class II diversity in northern latitudes, conferring a possible increased susceptibility to infection. However, we did not find regional differences in bacterial alpha diversity but in the composition of the microbial community.

We found a positive link between expected MHC heterozygosity and bacterial alpha diversity. These results are in line with the heterozygote advantage (overdominance) theory which posits that heterozygous individuals might successfully carry a highly diverse bacterial community on the skin and consequently heighten their resistance to infection (Khan, Yurkovetskiy, et al., 2019). Additionally, we found that more divergent MHC alleles are negatively associated with alpha diversity; heterozygous individuals from northern populations carried a more diverse bacterial community than individuals from the southern populations. Given these results, we infer that nucleotide sequence MHC dissimilarity within a host reduces the diversity of skin microbial communities. Nucleotide diversity was directly correlated to the *Theta k* value, which is a proxy for MHC sequence dissimilarity (Cortazar-Chinarro et al., 2018). Furthermore, *Theta k* values clearly differed between populations and regions, being lower in the northern region where MHC sequences were more similar. This suggests that the lower genetic variation commonly observed at northern latitudes could be compensated for by higher bacterial richness, lending support to the idea that more diverse bacterial communities compensate for the lower individual MHC diversity in northern latitudes.

We hypothesize that host MHC alleles selectively target specific bacterial communities, co-evolving in a manner that increases host survival in the face of pathogenic infections. No infection data exist to fully test this hypothesis and further investigations in this regard are needed. However, one of the main results of this study indicates that individuals carrying $Supertype_{Genotype}$ 1_3, 2_2, 2_4 and 3_3 have specific bacterial compositions. Moreover, supertypes 2 and 3, the most abundant supertypes in the south and north, respectively, are oppositely linked to specific bacterial taxa. For instance, taxonomic units ASV52, ASV73 and ASV71, which are included within the Proteobacteria group from the *Oxalobacteraceae* family, are positively correlated with Supertype 3 but negatively correlated with Supertype 2. Bacteria from family *Oxalobacteraceae* have been recently detected in amphibian skin among individuals with different *Bd* infection intensities (Ellison et al., 2019). This finding might indicate differences in the strategy to combat infectious diseases between regions. We suggest that specific bacteria from the *Oxalobacteraceae* family could act differently on infected individuals depending on their MHC class II supertype configuration and bacterial abundance, but this hypothesis requires further investigation.

Several studies support the idea that local environmental conditions might directly predict the amphibian skin microbiome structure by influencing the pool of potential symbionts in the habitat (Amato et al., 2016; Kueneman et al., 2014; Rebollar et al., 2016). However, none of these studies have investigated the associations between the skin microbiome composition and adaptive markers such as MHC. Our study demonstrates the interplay of (1) evolutionary and biogeographical processes (as inferred from previous studies, e.g. Cortázar-Chinarro et al., 2017), (2) local environmental conditions and (3) host adaptive gene characteristics shaping the diversity and heterogeneity of the skin microbiota. Investigating these factors is crucial for comprehending the interactions between the host, microbiome and immunity. Expanding the survey of wild populations across environmental gradients could provide valuable insights into the environmental characteristics and evolutionary processes that shape host-associated microbial communities. This comprehensive approach would involve incorporating both putatively neutral markers (SNPs from low coverage genome sequencing) and adaptive genes. Our findings suggest that historical demographic events impact hologenomic variation and provide new insights into how immunogenetic host variability and microbial diversity may jointly influence host fitness with consequences for disease susceptibility and population persistence.

AUTHOR CONTRIBUTIONS

MC-C carried out conceptualization, field work, laboratory work, data curation, formal analyses, funding and writing—original draft and editing. AR-B carried outfield work, conceptualization, formal analyses, writing—original draft, review and editing. PR-M carried outfield work, statistical-bioinformatic support, writing—review and editing. P-H was involved in statistical-bioinformatic support, writing—review and editing. JBL carried out field work, writing—review and editing. AL carried out conceptualization, funding and writing—review and editing. JH was involved in conceptualization, funding and writing: review and editing.

ACKNOWLEDGEMENTS

We thank David Åhlen for invaluable help in the field. Many thanks to Gunilla Engström for all the help and support in the laboratory. We also want to thank Javier Edo Varg for the help with microbiome pipeline analysis and all the guidance in the laboratory, Yvonne Meyer-Lucht for invaluable help during the study design, Alba Cortazar Chinarro for the artistic frog painting and Karl Phillips and Anna Savage for constructive comments on the earlier version of the manuscript.

FUNDING INFORMATION

Funding for the field work was provided by the Swedish Research Council Formas (grant 146400178 to JH), Stiftelsen Oscar och Lili Lamms Minne (DO2013-0013 to JH) and the Swedish Research Council (621-4503 to AL). Funding for the laboratory work was provided by Stiftelsen för Zoologisk Forskning to MC, Hiertas Minne Foundation (FO2018-0540 to MC) and the Kungliga Vetenskapsakademin to MC (BS2018-0110).

CONFLICT OF INTEREST STATEMENT

We declare we have no competing interests.

DATA AVAILABILITY STATEMENT

Electronic supplementary material is available online: https://figshare.com/s/fa4e49bd4aa8e9f8819b. Raw data available from Figshare: https://figshare.com/s/104cd48dd9b1e64d644a.

ORCID

M. Cortazar-Chinarro https://orcid.org/0000-0003-4604-1441
P. Rödin-Mörch https://orcid.org/0000-0001-6737-1488
A. Laurila https://orcid.org/0000-0001-8090-3776
J. Höglund https://orcid.org/0000-0002-5840-779X

REFERENCES

Amato, K. R., Martinez-Mota, R., Righini, N., Raguet-Schofield, M., Corcione, F. P., Marini, E., Humphrey, G., Gogul, G., Gaffney, J., Lovelace, E., Williams, L., Luong, A., Dominguez-Bello, M. G., Stumpf, R. M., White, B., Nelson, K. E., Knight, R., & Leigh, S. R. (2016). Phylogenetic and ecological factors impact the gut microbiota of two neotropical primate species. *Oecologia*, *180*(3), 717–733.

Antwis, R. E., Fry, E., James, C. E., & Ferry, N. (2020). Microbial biotechnology. In R. E. Antwis, X. A. Harrison, & M. J. Cox (Eds.), *Microbiomes of soils, plants and animals: An integrated approach* (pp. 182–221). Cambridge University Press.

Barbian, H. J., Li, Y., Ramirez, M., Klase, Z., Lipende, I., Mjungu, D., & Lonsdorf, E. V. (2018). Destabilization of the gut microbiome marks the end-stage of simian immunodeficiency virus infection in wild chimpanzees. *American Journal of Primatology*, *80*(1), e22515.

Bates, K. A., Clare, F. C., O'Hanlon, S., Bosch, J., Brookes, L., Hopkins, K., & Fisher, M. C. (2018). Amphibian chytridiomycosis outbreak dynamics are linked with host skin bacterial community structure. *Nature Communications*, *9*(1), 693.

Becker, C., Longo, A., Haddad, C., & Zamudio, K. (2017). Land cover and forest connectivity alter the interactions among host, pathogen and skin microbiome. *Proceedings of the Royal Society B*, *284*(1861), 20170582.

Belasen, A. M., Riolo, M. A., Bletz, M. C., Lyra, M. L., Toledo, L. F., & James, T. Y. (2021). Geography, host genetics, and cross-domain microbial networks structure the skin microbiota of fragmented Brazilian Atlantic forest frog populations. *Ecology and Evolution*, *11*(14), 9293–9307.

Belden, L. K., Hughey, M. C., Rebollar, E. A., Umile, T. P., Loftus, S. C., Burzynski, E. A., & Becker, M. H. (2015). Panamanian frog species host unique skin bacterial communities. *Frontiers in Microbiology*, *6*, 1171.

Björk, J. R., Dasari, M., Grieneisen, L., & Archie, E. A. (2019). Primate microbiomes over time: Longitudinal answers to standing questions in microbiome research. *American Journal of Primatology*, *81*(10–11), e22970.

Blekhman, R., Goodrich, J. K., Huang, K., Sun, Q., Bukowski, R., Bell, J. T., & Gevers, D. (2015). Host genetic variation impacts microbiome composition across human body sites. *Genome Biology*, *16*(1), 191.

Bolnick, D. I., Snowberg, L. K., Caporaso, J. G., Lauber, C., Knight, R., & Stutz, W. E. (2014). Major H istocompatibility C omplex class II b polymorphism influences gut microbiota composition and diversity. *Molecular Ecology*, *23*(19), 4831–4845.

Bonder, M. J., Kurilshikov, A., Tigchelaar, E. F., Mujagic, Z., Imhann, F., Vila, A. V., Deelen, P., Vatanen, T., Schirmer, M., Smeekens, S. P., Zhernakova, D. V., Jankipersadsing, S. A., Jaeger, M., Oosting, M., Cenit, M. C., Masclee, A. A., Swertz, M. A., Li, Y., Kumar, V., … Zhernakova, A. (2016). The effect of host genetics on the gut microbiome. *Nature Genetics*, *48*(11), 1407–1412.

Bosák, J., Lexa, M., Fiedorová, K., Gadara, D. C., Micenková, L., Spacil, Z., & Šmajs, D. (2021). Patients with common variable immunodeficiency (CVID) show higher gut bacterial diversity and levels of low-abundance genes than the healthy housemates. *Frontiers in Immunology*, *12*, 671239.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583.

Cameron, E. S., Schmidt, P. J., Tremblay, B. J.-M., Emelko, M. B., & Müller, K. M. (2020). To rarefy or not to rarefy: Enhancing diversity analysis of microbial communities through next-generation sequencing and rarefying repeatedly. *bioRxiv*, https://doi.org/10.1101/2020.09.09.290049

Cortázar-Chinarro, M., Lattenkamp, E. Z., Meyer-Lucht, Y., Luquet, E., Laurila, A., & Höglund, J. (2017). Drift, selection, or migration? Processes affecting genetic differentiation and variation along a latitudinal gradient in an amphibian. *BMC Evolutionary Biology*, *17*(1), 189.

Cortazar-Chinarro, M., Meyer-Lucht, Y., Laurila, A., & Höglund, J. (2018). Signatures of historical selection on MHC reveal different selection patterns in the moor frog (*Rana arvalis*). *Immunogenetics*, *70*(7), 477–484.

Costa, D., Tavares, R. M., Baptista, P., & Lino-Neto, T. (2022). The influence of bioclimate on soil microbial communities of cork oak. *BMC Microbiology*, *22*(1), 163.

Couch, C. E., Stagaman, K., Spaan, R. S., Combrink, H. J., Sharpton, T. J., Beechler, B. R., & Jolles, A. E. (2021). Diet and gut microbiome enterotype are associated at the population level in African buffalo. *Nature Communications*, *12*(1), 2267.

Cullen, C. M., Aneja, K. K., Beyhan, S., Cho, C. E., Woloszynek, S., Convertino, M., & Alvarez-Ponce, D. (2020). Emerging priorities for microbiome research. *Frontiers in Microbiology*, *11*, 136.

Darolová, A., Poláček, M., Krištofík, J., Lukasch, B., & Hoi, H. (2021). First evidence of a relationship between female major histocompatibility complex diversity and eggshell bacteria in house sparrows (*Passer domesticus*). *Frontiers in Ecology and Evolution*, *9*, 615667.

Dillon, S., Lee, E., Kotter, C., Austin, G., Dong, Z., Hecht, D., & Landay, A. (2014). An altered intestinal mucosal microbiome in HIV-1 infection is associated with mucosal and systemic immune activation and endotoxemia. *Mucosal Immunology*, *7*(4), 983–994.

Dimitriu, P. A., Iker, B., Malik, K., Leung, H., Mohn, W., & Hillebrand, G. G. (2019). New insights into the intrinsic and extrinsic factors that shape the human skin microbiome. *MBio*, *10*(4), e00839-19.

Eizaguirre, C., & Lenz, T. (2010). Major histocompatibility complex polymorphism: Dynamics and consequences of parasite-mediated local adaptation in fishes. *Journal of Fish Biology*, *77*(9), 2023–2047.

Elbers, J. P., & Taylor, S. S. (2016). Major histocompatibility complex polymorphism in reptile conservation. *Herpetological Conservation and Biology*, *11*, 1–12.

Ellison, S., Knapp, R. A., Sparagon, W., Swei, A., & Vredenburg, V. T. (2019). Reduced skin bacterial diversity correlates with increased pathogen infection intensity in an endangered amphibian host. *Molecular Ecology*, *28*(1), 127–140.

Excoffier, L., & Lischer, H. E. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, *10*(3), 564–567.

Fisher, M. C., Henk, D., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L., & Gurr, S. J. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature*, *484*(7393), 186–194.

Flajnik, M. F. (2018). A cold-blooded view of adaptive immunity. *Nature Reviews Immunology*, *18*(7), 438–453.

Flajnik, M. F., & Kasahara, M. (2001). Comparative genomics of the MHC: Glimpses into the evolution of the adaptive immune system. *Immunity*, *15*(3), 351–362.

Fleischer, R., Risely, A., Hoeck, P. E., Keller, L. F., & Sommer, S. (2020). Mechanisms governing avian phylosymbiosis: Genetic dissimilarity based on neutral and MHC regions exhibits little relationship with gut microbiome distributions of Galápagos mockingbirds. *Ecology and Evolution*, *10*(23), 13345–13354.

Garud, N. R., & Pollard, K. S. (2020). Population genetics in the human microbiome. *Trends in Genetics*, *36*(1), 53–67.

Goslee, S. C., & Urban, D. L. (2007). The ecodist package for dissimilarity-based analysis of ecological data. *Journal of Statistical Software*, *22*, 1–19.

Goudet, J. (1995). FSTAT (version 1.2): A computer program to calculate F-statistics. *Journal of Heredity*, *86*(6), 485–486.

Grogan, L. F., Robert, J., Berger, L., Skerratt, L. F., Scheele, B. C., Castley, J. G., Newell, D. A., & McCallum, H. I. (2018). Review of the amphibian immune response to chytridiomycosis, and future directions. *Frontiers in Immunology*, *9*, 2536.

Groussin, M., Mazel, F., & Alm, E. J. (2020). Co-evolution and co-speciation of host-gut bacteria systems. *Cell Host & Microbe*, *28*(1), 12–22.

Gu, Z., Gu, L., Eils, R., Schlesner, M., & Brors, B. (2014). Circlize implements and enhances circular visualization in R. *Bioinformatics*, *30*(19), 2811–2812.

Henry, L. P., Bruijning, M., Forsberg, S. K., & Ayroles, J. F. (2021). The microbiome extends host evolutionary potential. *Nature Communications*, *12*(1), 1–13.

Hernández-Gómez, O., Briggler, J. T., & Williams, R. N. (2018). Influence of immunogenetics, sex and body condition on the cutaneous microbial communities of two giant salamanders. *Molecular Ecology*, *27*(8), 1915–1929.

Hooper, L. V., Littman, D. R., & Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. *Science*, *336*(6086), 1268–1273.

Jiménez, R. R., & Sommer, S. (2017). The amphibian microbiome: Natural range of variation, pathogenic dysbiosis, and role in conservation. *Biodiversity and Conservation*, *26*(4), 763–786.

Kaufman, J. (2020). From chickens to humans: The importance of peptide repertoires for MHC class I alleles. *Frontiers in Immunology*, *11*, 601089.

Kembel, S. W., & Kembel, M. S. W. (2014). *Package "picante"*. R Foundation for Statistical Computing, Vienna, Austria: https://cran.r-project.org/web/packages/picante/picante.pdf

Khan, A. A., Yurkovetskiy, L., O'Grady, K., Pickard, J. M., de Pooter, R., Antonopoulos, D. A., & Chervonsky, A. (2019). Polymorphic immune

mechanisms regulate commensal repertoire. *Cell Reports*, *29*(3), 541–550.e544.
Khan, M. W., Stephens, W. Z., Mohammed, A. D., Round, J. L., & Kubinak, J. L. (2019). Does MHC heterozygosity influence microbiota form and function? *PLoS One*, *14*(5), e0215946.
Kilpatrick, A. M., Briggs, C. J., & Daszak, P. (2010). The ecology and impact of chytridiomycosis: An emerging disease of amphibians. *Trends in Ecology & Evolution*, *25*(2), 109–118.
Klein, J. (1975). Biology of the Mouse Histocom-patibility-2 Complex. Springer Verlag, New York.
Knopp, T., & Merilä, J. (2009). The postglacial recolonization of northern Europe by *Rana arvalis* as revealed by microsatellite and mitochondrial DNA analyses. *Heredity*, *102*(2), 174–181.
Kubinak, J. L., Stephens, W. Z., Soto, R., Petersen, C., Chiaro, T., Gogokhia, L., & Morrison, L. (2015). MHC variation sculpts individualized microbial communities that control susceptibility to enteric infection. *Nature Communications*, *6*(1), 8642.
Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., & McKenzie, V. J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Molecular Ecology*, *23*(6), 1238–1250.
Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549.
Leclaire, S., Strandh, M., Dell'Ariccia, G., Gabirot, M., Westerdahl, H., & Bonadonna, F. (2019). Plumage microbiota covaries with the major histocompatibility complex in blue petrels. *Molecular Ecology*, *28*(4), 833–846.
Lee, Y. K., & Mazmanian, S. K. (2010). Has the microbiota played a critical role in the evolution of the adaptive immune system? *Science*, *330*(6012), 1768–1773.
Lighten, J., Van Oosterhout, C., Paterson, I. G., McMullan, M., & Bentzen, P. (2014). Ultra-deep Illumina sequencing accurately identifies MHC class II b alleles and provides evidence for copy number variation in the guppy (*Poecilia reticulata*). *Molecular Ecology Resources*, *14*(4), 753–767.
Lin, H., Peddada, S. D., & Lin, M. H. (2021). *Package "ANCOMBC"*.
Lin, P., Bach, M., Asquith, M., Lee, A. Y., Akileswaran, L., Stauffer, P., & Dorris, M. (2014). HLA-B27 and human β2-microglobulin affect the gut microbiota of transgenic rats. *PLoS One*, *9*(8), e105684.
Love, M., Anders, S., & Huber, W. (2014). Differential analysis of count data–the DESeq2 package. *Genome Biology*, *15*(550).
Luquet, E., Rödin Mörch, P., Cortázar-Chinarro, M., Meyer-Lucht, Y., Höglund, J., & Laurila, A. (2019). Post-glacial colonization routes coincide with a life-history breakpoint along a latitudinal gradient. *Journal of Evolutionary Biology*, *32*(4), 356–368.
Magoč, T., & Salzberg, S. L. (2011). FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, *27*(21), 2957–2963.
Martel, A., Blooi, M., Adriaensen, C., van Rooij, P., Beukema, W., Fisher, M. C., Goka, K., Lips, K. R., Muletz, C., Zamudio, K. R., Bosch, J., Lötters, S., Wombwell, E., Garner, T. W., Cunningham, A. A., Spitzen-van der Sluijs, A., Salvidio, S., Ducatelle, R., Nishikawa, K., … Pasmans, F. (2014). Recent introduction of a chytrid fungus endangers Western palearctic salamanders. *Science*, *346*(6209), 630–631.
McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*, *8*(4), e61217.
McMurdie, P. J., & Holmes, S. (2014). Waste not, want not: Why rarefying microbiome data is inadmissible. *PLoS Computational Biology*, *10*(4), e1003531.
Meurling, S., Kärvemo, S., Chondrelli, N., Chinarro, M. C., Åhlen, D., Brookes, L., & Höglund, J. (2020). Occurrence of *Batrachochytrium dendrobatidis* in Sweden: Higher infection prevalence in southern species. *Diseases of Aquatic Organisms*, *140*, 209–218.
Meyer-Lucht, Y., Luquet, E., Jóhannesdóttir, F., Rödin-Mörch, P., Quintela, M., Richter-Boix, A., & Laurila, A. (2019). Genetic basis of amphibian larval development along a latitudinal gradient: Gene diversity, selection and links with phenotypic variation in transcription factor C/EBP-1. *Molecular Ecology*, *28*(11), 2786–2801.
Meyer-Lucht, Y., Otten, C., Püttker, T., Pardini, R., Metzger, J. P., & Sommer, S. (2010). Variety matters: Adaptive genetic diversity and parasite load in two mouse opossums from the Brazilian Atlantic forest. *Conservation Genetics*, *11*, 2001–2013.
Minias, P., Whittingham, L. A., & Dunn, P. O. (2017). Coloniality and migration are related to selection on MHC genes in birds. *Evolution*, *71*(2), 432–441.
Moeller, A. H., Peeters, M., Ayouba, A., Ngole, E. M., Esteban, A., Hahn, B. H., & Ochman, H. (2015). Stability of the gorilla microbiome despite simian immunodeficiency virus infection. *Molecular Ecology*, *24*(3), 690–697.
Montero, B. K., Schwensow, N., Gillingham, M. A., Ratovonamana, Y. R., Rakotondranary, S. J., Corman, V., & Sommer, S. (2021). Evidence of MHC class I and II influencing viral and helminth infection via the microbiome in a non-human primate. *PLoS Pathogens*, *17*(11), e1009675.
Muletz Wolz, C. R., Yarwood, S. A., Campbell Grant, E. H., Fleischer, R. C., & Lips, K. R. (2018). Effects of host species and environment on the skin microbiome of plethodontid salamanders. *Journal of Animal Ecology*, *87*(2), 341–353.
Müller, D. B., Vogel, C., Bai, Y., & Vorholt, J. A. (2016). The plant microbiota: Systems-level insights and perspectives. *Annual Review of Genetics*, *50*(1), 211–234.
Ohta, Y., Okamura, K., McKinney, E. C., Bartl, S., Hashimoto, K., & Flajnik, M. F. (2000). Primitive synteny of vertebrate major histocompatibility complex class I and class II genes. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(9), 4712–4717.
Oksanen, J., Blanchet, F., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., & Solymos, P. (2019). *Vegan: Community ecology package*. Ordination Methods, Diversity Analysis and Other Functions for Community and Vegetation Ecologists https://cran.r-project.org/web/packages/vegan/index.html
Olivares, M., Neef, A., Castillejo, G., De Palma, G., Varea, V., Capilla, A., & Polanco, I. (2015). The HLA-DQ2 genotype selects for early intestinal microbiota composition in infants at high risk of developing coeliac disease. *Gut*, *64*(3), 406–417.
Phillips, K. P., Cable, J., Mohammed, R. S., Chmielewski, S., Przesmycka, K. J., Van Oosterhout, C., & Radwan, J. (2021). Functional immunogenetic variation, rather than local adaptation, predicts ectoparasite infection intensity in a model fish species. *Molecular Ecology*, *30*(21), 5588–5604.
Phillips, K. P., Cable, J., Mohammed, R. S., Herdegen-Radwan, M., Raubic, J., Przesmycka, K. J., van Oosterhout, C., & Radwan, J. (2018). Immunogenetic novelty confers a selective advantage in host–pathogen coevolution. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(7), 1552–1557.
Potts, W. K., & Wakeland, E. K. (1990). Evolution of diversity at the major histocompatibility complex. *Trends in Ecology & Evolution*, *5*(6), 181–187.
Rebollar, E. A., Hughey, M. C., Medina, D., Harris, R. N., Ibáñez, R., & Belden, L. K. (2016). Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *The ISME Journal*, *10*(7), 1682–1695.
Richmond, J. Q., Savage, A. E., Zamudio, K. R., & Rosenblum, E. B. (2009). Toward immunogenetic studies of amphibian chytridiomycosis: Linking innate and acquired immunity. *Bioscience*, *59*(4), 311–320.
Rock, K. L., Reits, E., & Neefjes, J. (2016). Present yourself! By MHC class I and MHC class II molecules. *Trends in Immunology*, *37*(11), 724–737.
Rödin-Mörch, P., Luquet, E., Meyer-Lucht, Y., Richter-Boix, A., Höglund, J., & Laurila, A. (2019). Latitudinal divergence in a widespread amphibian: Contrasting patterns of neutral and adaptive genomic variation. *Molecular Ecology*, *28*(12), 2996–3011.
Salas, J. T., & Chang, T. L. (2014). Microbiome in human immunodeficiency virus infection. *Clinics in Laboratory Medicine*, *34*(4), 733–745.

Sandberg, M., Eriksson, L., Jonsson, J., Sjöström, M., & Wold, S. (1998). New chemical descriptors relevant for the design of biologically active peptides. A multivariate characterization of 87 amino acids. *Journal of Medicinal Chemistry*, *41*(14), 2481–2491.

Savage, A. E., Muletz-Wolz, C. R., Campbell Grant, E. H., Fleischer, R. C., & Mulder, K. P. (2019). Functional variation at an expressed MHC class IIβ locus associates with Ranavirus infection intensity in larval anuran populations. *Immunogenetics*, *71*(4), 335–346.

Savage, A. E., & Zamudio, K. R. (2011). MHC genotypes associate with resistance to a frog-killing fungus. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(40), 16705–16710.

Scheele, B. C., Pasmans, F., Skerratt, L. F., Berger, L., Martel, A., Beukema, W., Acevedo, A. A., Burrowes, P. A., Carvalho, T., Catenazzi, A., De la Riva, I., Fisher, M. C., Flechas, S. V., Foster, C. N., Frías-Álvarez, P., Garner, T. W. J., Gratwicke, B., Guayasamin, J. M., Hirschfeld, M., … Canessa, S. (2019). Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science*, *363*(6434), 1459–1463.

Sebastian, A., Herdegen, M., Migalska, M., & Radwan, J. (2016). AMPLISAS: A web server for multilocus genotyping using next-generation amplicon sequencing data. *Molecular Ecology Resources*, *16*(2), 498–510.

Shafquat, A., Joice, R., Simmons, S. L., & Huttenhower, C. (2014). Functional and phylogenetic assembly of microbial communities in the human microbiome. *Trends in Microbiology*, *22*(5), 261–266.

Shetty, S. A., & Lahti, L. (2019). Microbiome data science. *Journal of Biosciences*, *44*(5), 1–6.

Smallbone, W., Ellison, A., Poulton, S., van Oosterhout, C., & Cable, J. (2021). Depletion of MHC supertype during domestication can compromise immunocompetence. *Molecular Ecology*, *30*(3), 736–746.

Tabrett, A., & Horton, M. W. (2020). The influence of host genetics on the microbiome. *F1000Research*, *9*, 84. https://doi.org/10.12688/f1000research.20835.1

Thaiss, C. A., Zmora, N., Levy, M., & Elinav, E. (2016). The microbiome and innate immunity. *Nature*, *535*(7610), 65–74.

Torres-Sánchez, M., & Longo, A. V. (2022). Linking pathogen–microbiome–host interactions to explain amphibian population dynamics. *Molecular Ecology*, *31*(22), 5784–5794.

Tregaskes, C. A., & Kaufman, J. (2021). Chickens as a simple system for scientific discovery: The example of the MHC. *Molecular Immunology*, *135*, 12–20.

Trevelline, B. K., Fontaine, S. S., Hartup, B. K., & Kohl, K. D. (2019). Conservation biology needs a microbial renaissance: A call for the consideration of host-associated microbiota in wildlife management practices. *Proceedings of the Royal Society B*, *286*(1895), 20182448.

Varg, J. E., Outomuro, D., Kunce, W., Kuehrer, L., Svanbäck, R., & Johansson, F. (2022). Microplastic exposure across trophic levels: Effects on the host-microbiota of freshwater organisms. *Environmental Microbiomes*, *17*(1), 1–15.

Wein, T., & Sorek, R. (2022). Bacterial origins of human cell-autonomous innate immune mechanisms. *Nature Reviews Immunology*, *22*, 629–638.

Weinstein, S. B., Martínez-Mota, R., Stapleton, T. E., Klure, D. M., Greenhalgh, R., Orr, T. J., & Dearing, M. D. (2021). Microbiome stability and structure is governed by host phylogeny over diet and geography in woodrats (*Neotoma* spp.). *Proceedings of the National Academy of Sciences of the United States of America*, *118*(47), e2108787118.

West, A. G., Waite, D. W., Deines, P., Bourne, D. G., Digby, A., McKenzie, V. J., & Taylor, M. W. (2019). The microbiome in threatened species conservation. *Biological Conservation*, *229*, 85–98.

Wickham, H. (2016). *Data analysis. ggplot2* (pp. 189–201). Springer.

Wielstra, B., Sillero, N., Vörös, J., & Arntzen, J. W. (2014). The distribution of the crested and marbled newt species (*amphibia: Salamandridae: Triturus*): An addition to the new atlas of amphibians and reptiles of Europe. *Amphibia-Reptilia*, *35*(3), 376–381.

Willis, A. D. (2019). Rarefaction, alpha diversity, and statistics. *Frontiers in Microbiology*, *10*, 2407.

Woodhams, D. C., Bletz, M. C., Becker, C. G., Bender, H. A., Buitrago-Rosas, D., Diebboll, H., Huynh, R., Kearns, P. J., Kueneman, J., Kurosawa, E., LaBumbard, B. C., Lyons, C., McNally, K., Schliep, K., Shankar, N., Tokash-Peters, A. G., Vences, M., & Whetstone, R. (2020). Host-associated microbiomes are predicted by immune system complexity and climate. *Genome Biology*, *21*(1), 1–20.

Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., & Glöckner, F. O. (2014). The SILVA and "all-species living tree project (LTP)" taxonomic frameworks. *Nucleic Acids Research*, *42*(D1), D643–D648.

Zilber-Rosenberg, I., & Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: The hologenome theory of evolution. *FEMS Microbiology Reviews*, *32*(5), 723–735.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Cortazar-Chinarro, M., Richter-Boix, A., Rödin-Mörch, P., Halvarsson, P., Logue, J. B., Laurila, A., & Höglund, J. (2024). Association between the skin microbiome and MHC class II diversity in an amphibian. *Molecular Ecology*, *33*, e17198. https://doi.org/10.1111/mec.17198