





ORIGINAL ARTICLE



Environmental factors and host sex influence the skin microbiota structure of Hong Kong newt (*Paramesotriton hongkongensis*) in a coldspot of chytridiomycosis in subtropical East Asia

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Abstract

Chytridiomycosis, an infectious skin disease caused by the chytrid fungi, *Batrachochytrium dendrobatidis* and *B. salamandrivorans*, poses a significant threat to amphibian biodiversity worldwide. Antifungal bacteria found on the skin of chytrid-resistant amphibians could potentially provide defense against chytridiomycosis and lower mortality rates among resistant individuals. The Hong Kong newt (*Paramesotriton hongkongensis*) is native to East Asia, a region suspected to be the origin of chytrids, and has exhibited asymptomatic infection, suggesting a long-term coexistence with the chytrids. Therefore, the skin microbiota of this resistant species warrant investigation, along with other factors that can affect the microbiota. Among the 149 newts sampled in their natural habitats in Hong Kong, China, putative antifungal bacteria were found in all individuals. There were 314 amplicon sequence variants distributed over 25 genera of putative antifungal bacteria; abundant ones included *Acinetobacter*, *Flavobacterium*, and *Novosphingobium* spp. The skin microbiota compositions were strongly influenced by the inter-site geographical distances. Despite inter-site differences, we identified some core skin microbes across sites that could be vital to *P. hongkongensis*. The dominant cores included the family Comamonadaceae, family Chitinophagaceae, and class Betaproteobacteria. Moreover, habitat elevation and host sex also exhibited significant effects on skin microbiota compositions. The antifungal bacteria found on these newts offer an important resource for conservation against chytridiomycosis, such as developing probiotic treatments for susceptible species.

Key words: 16S amplicon sequencing, antifungal bacteria, *Batrachochytrium dendrobatidis*, *Batrachochytrium salamandrivorans*, host–pathogen–microbiota coevolution

INTRODUCTION

Microbiota, which are communities of microorganisms, have been found to reside on or within animals (Hoffmann 2017). These microbiota can include symbionts that enhance the innate immunity of the host by educating the immune system (Chen & Tsao 2013; Naik *et al.* 2015). Additionally, they aid in combating

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infections through competition with pathogenic microbes for resources and space, as well as by producing antimicrobial compounds against invasive pathogens (Grice & Segre 2011; Chen & Tsao 2013). Research conducted on germ-free laboratory mice has demonstrated that the absence of skin microbiota compromises immunity, leading to abnormal populations of epidermal T cells. However, this immune deficiency can be alleviated by introducing commensal skin bacteria, such as *Staphylococcus epidermidis* (Chen & Tsao 2013; Naik *et al.* 2015). In humans, fecal microbiota transplant has emerged as a treatment for patients with recurrent *Clostridioides difficile* infections. This procedure involves using a fecal preparation from a healthy stool donor to repopulate the patient's guts with a balanced microbiota. This new microbial community competes with *C. difficile* for resources and space, effectively combating the infection (Cammarota *et al.* 2014). These cases highlight the crucial role that resident body microbes play in maintaining the overall health of the host, indicating a coevolutionary relationship between these organisms and their hosts (Naik *et al.* 2015; Colston & Jackson 2016). This hypothesis of coevolution is supported by the observation of phylosymbiosis, which reveals a significant cophylogenetic signal between the microbiota and their corresponding hosts (Brooks *et al.* 2016; Colston & Jackson 2016; Ross *et al.* 2019).

Amphibians, among vertebrates, represent the most endangered group, with 41% of all species facing the threat of extinction (IUCN 2021). The causes of amphibian population decline are numerous and often complex. Chytridiomycosis, in particular, has caused significant biodiversity loss, contributing to the decline of at least 501 amphibian species, with 90 already extinct and 124 experiencing a decline of over 90% in abundance (Scheele *et al.* 2019; Fisher & Garner 2020). An outbreak of chytridiomycosis in Panama resulted in the loss of 30 amphibian species, including three caudate species *Bolitoglossa colonnea*, *B. schizodactyla*, and *Oedipina parvipes* (Crawford *et al.* 2010). Before the identification of *Batrachochytrium salamandrivorans* (*Bsal*) in 2013, chytridiomycosis was believed to be caused solely by the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), leaving some dramatic amphibian population declines unexplained, including the local extinction of the fire salamander (*Salamanca salamandra*) in the Netherlands (Martel *et al.* 2013; Spitzen-van der Sluijs *et al.* 2013). It has been subsequently discovered that *Bsal* specifically targets salamanders and newts, while *Bd* can infect a wider range of amphibian species, including frogs, salamanders, and caecilians (Martel *et al.* 2013, 2014; Fisher & Garner 2020).

Both *Bd* and *Bsal* infect the amphibian skin, which is the organ responsible for gaseous exchange and osmoregulation, exhibiting specific adaptations to aquatic and terrestrial habitats (Varga *et al.* 2019; Fisher & Garner 2020). The amphibian skin, being mucosal, water-permeable, and in direct contact with a diverse and microbially rich environment, is susceptible to pathogen invasion (Varga *et al.* 2019). Similar to other vertebrates (Fredricks 2001; Martín-Platero *et al.* 2006; Lai *et al.* 2010; Martín-Vivaldi *et al.* 2010; Pérez-Sánchez *et al.* 2011; Naik *et al.* 2015), the microbiota is one of the vital innate immune defenses of the amphibian skin (Varga *et al.* 2019). Coevolution might have shaped a skin microbiota that confers higher resistance to infection in amphibians (Colston & Jackson 2016; Foster *et al.* 2017; Bates *et al.* 2018). In the context of conservation, research has primarily focused on the amphibian skin microbiota, particularly its implications for understanding chytrid pathogenesis and treatment. Previous studies have demonstrated interactions between the skin microbiota and *Bd* infection. For example, in the Sierra Nevada yellow-legged frog (*Rana sierrae*), *Bd* was found to disturb the skin bacterial community both in the wild populations and in controlled experiments (Jani & Briggs 2014). In the red-backed salamander (*Plethodon cinereus*), *Bd* changed their microbiota and caused high host mortality across different temperatures (Muletz-Wolz *et al.* 2019). *Bd* infection history also had a significant impact on the skin microbiota of the common midwife toad (*Alytes obstetricans*), resulting in reduced bacterial diversity in epizootic populations (Bates *et al.* 2018). On the other hand, the inoculation of anti-*Bd* bacterial isolates identified from resistant species has been shown to reduce morbidity, mortality, and symptom severity in the mountain yellow-legged frog (*Rana muscosa*) (Harris *et al.* 2009a), the western toad (*Anaxyrus boreas*) (Kueneman *et al.* 2016), and *P. cinereus* (Harris *et al.* 2009b) when infected with *Bd*.

Contrary to the intensive research associated with *Bd*, far less is known about the emerging fungal pathogen *Bsal*, including its genetic and phenotypic variations, virulence, and threats to global salamander and newt populations (Fisher & Garner 2020). Genetic evidence suggests that *Bd* originates in East Asia (O'hlanon *et al.* 2018; Fisher & Garner 2020). It is worth noting that *Bsal* has also only been found in East and Southeast Asia, apart from the epidemic in Europe (Martel *et al.* 2014; Laking *et al.* 2017; Yuan *et al.* 2018). Asia, often referred to as a "coldspot" of chytridiomycosis, exhibits a low prevalence of chytrids and lacks chytridiomycosis outbreaks, suggesting that certain Asiatic amphibian species may have

evolved natural resistance to *Bsal* or possess immune responses that limit the impact of the pathogen (Swei *et al.* 2011; Laking *et al.* 2017; Yuan *et al.* 2018). However, studies on amphibian skin microbiota in Asia are relatively scarce compared to Europe and North America. In European newt species, including the smooth newt (*Lisotriton vulgaris*), the great-crested newt (*Triturus cristatus*), and *S. salamandra*, *Bsal* infection has been associated with alterations in skin microbial composition (Bletz *et al.* 2018; Bates *et al.* 2019). *Bsal*-inhibiting bacteria have been identified from the skin microbiota of *S. salamandra*, suggesting that increasing their abundance could potentially slow down the progression of chytridiomycosis (Bletz *et al.* 2018).

In addition to chytrid fungal infection, the amphibian microbial community is affected by various host and environmental factors. The species identity of amphibians has been identified as the strongest predictor of the composition of a skin microbial community (Kueneman *et al.* 2014). This finding is supported by studies such as the one documenting distinct skin bacterial communities among different Panamanian frog species (Belden *et al.* 2015). Moreover, the effect of life history traits on skin microbial compositions has been observed in *A. boreas* (Prest *et al.* 2018) and the Cascades frog (*Rana cascadae*) (Kueneman *et al.* 2014, 2017) as they undergo significant changes from tadpole to adult stages. While the effect of host sex on the skin microbiota is not well documented in amphibian studies, its influence has been found in a plethodontid salamander, the yellow-eyed ensatina (*Ensatina eschscholtzii xanthoptica*) (Prado-Irwin *et al.* 2017). However, in other plethodontid species, skin microbiota structures are more influenced by geographical parameters such as elevation and habitat type rather than phylogenetic relatedness or life stages (Prado-Irwin *et al.* 2017; Bird *et al.* 2018; Muletz Wolz *et al.* 2018), as environmental factors often co-vary along the elevation gradient of a habitat (Hughey *et al.* 2017; Muletz Wolz *et al.* 2018). Similar findings have been reported in the cane toad (*Rhinella marina*), in which geographical location dictated the structure of the skin microbial community (Abarca *et al.* 2018). Additionally, co-evolution with predators might drive the recruitment and proliferation of toxin-producing bacteria in the skin microbiota of amphibians (Vaelli *et al.* 2020). A recent study reported that bacteria from the genera *Aeromonas*, *Pseudomonas*, *Shewanella*, and *Sphingopyxis* on the rough-skinned newt (*Taricha granulosa*) produce a deadly neurotoxin, tetrodotoxin (TTX), which could serve as a defense against predation (Vaelli *et al.* 2020). Nonetheless, the relative importance of different factors in shaping am-

phibian skin microbiota remains unclear and likely varies depending on the context.

The Hong Kong newt (*Paramesotriton hongkongensis*) (Fig. 1a) is a subtropical salamander listed as Near Threatened by the International Union for Conservation of Nature. It has a highly restricted distribution in southern China (Fu *et al.* 2013; Lau 2017; Lau *et al.* 2017; IUCN 2021). A recent survey conducted in 2018 found that *P. hongkongensis* had the highest incidence of *Bsal* presence on the skin, with over 50% of individuals testing positive for the pathogen. This was observed in a single population located in Wutongshan near Hong Kong, China. (Yuan *et al.* 2018). Given the low number of studies on the skin microbiota of amphibians, specifically salamanders, in Asia and its connection to the origins of *Bd* and *Bsal*, the findings from this study can offer important insights into the host–pathogen–microbiota coevolution within the region. Furthermore, the anti-chytrid bacterial communities found on the salamander skin in this chytrid-coldspot region can be a useful resource for amphibian conservation, such as providing targets for developing probiotic treatments against chytridiomycosis. The high *Bsal* prevalence and absence of host symptoms reported in the previous survey make *P. hongkongensis* a suitable system for these purposes (Yuan *et al.* 2018). Here we aimed to: (1) characterize the skin microbiota compositions and the core microbiota of *P. hongkongensis* populations, (2) investigate whether *P. hongkongensis* harbors putative antifungal bacteria, and (3) determine the host and environmental factors associated with the skin microbial community structure.

MATERIALS AND METHODS

Field sampling

We collected *P. hongkongensis* skin swabs ($n = 149$) and water samples ($n = 12$) from 15 locations in Hong Kong, China (Fig. 1b) between October 2019 and February 2020 (Table S1, Supporting Information) during their breeding season when the newts gathered in streams to reproduce. We captured each newt with gloved hands and kept it in an individual plastic bag. A sterile swab (FLOQSwabs, Copan, Italy) was used to stroke each individual in a single direction 30 times each on the ventral and dorsal sides and five times on each leg. We recorded the snout-to-vent length (SVL), total length (TL), weight, and sex, and calculated the body condition index (BCI, weight/SVL) for each individual. One milliliter of stream water was taken from each of the 12 locations. The samples were immediately kept on ice

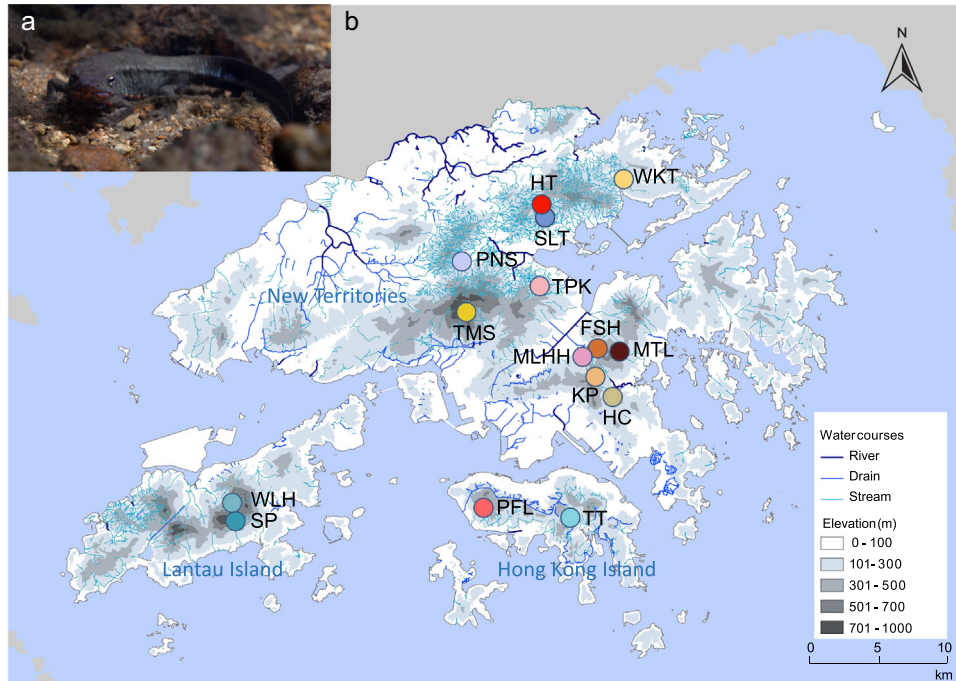


Figure 1 (a) *Paramesotriton hongkongensis* under water. Photo credit: Hon Shing Fung. (b) Map of Hong Kong showing New Territories, Lantau Island, and Hong Kong Island, with sampling sites indicated by colored spots. Elevation is represented by shades of gray. Stream system is shown by blue lines. Abbreviations of sampling sites: FSH, Fa Sam Hang; HC, Ho Chung; HT, Hok Tau; KP, Kowloon Peak; MLHH, Ma Lai Hau Hang; MTL, Mui Tsz Lam; PNS, Pak Ngau Shek; PFL, Pok Fu Lam; SLT, Sha Lo Tung; SP, Sunset Peak; TMS, Tai Mo Shan; TPK, Tai Po Kau; TT, Tai Tam; WLH, Wong Lung Hang; WKT, Wu Kau Tang.

in the field and transferred to the laboratory within 3 h. They were then stored at -80°C until DNA extraction. This study was approved by the University animal ethics committee (EC052/2021) and the Department of Health ([19-103] in DH/HT&A/8/2/6 Pt. 1).

DNA extraction, DNA library preparation, and sequencing

We extracted DNA from skin swabs and water samples using the E.Z.N.A. Tissue DNA Kit (Omega Bio-tek, USA) following the manufacturer's protocol under aseptic conditions. Four negative controls were included during DNA extraction. We also prepared mock communities (MCs) by pooling equimolar genomic DNA of multiple bacteria (Table S2, Supporting Information).

For high-throughput sequencing of the V4 region of the 16S rRNA gene to characterize the skin microbiota of *P. hongkongensis*, all samples, MCs, and negative controls were subjected to a two-step PCR (Cruaud *et al.* 2017; Huang *et al.* 2022) for library preparation. The first-step PCR was performed in a $30\text{-}\mu\text{L}$

reaction containing $6\ \mu\text{L}$ of 5X GoTaq Flexi Buffer (Promega, USA), $0.6\ \mu\text{L}$ of 10 mM dNTP Mix, $2.4\ \mu\text{L}$ of 25 mM MgCl_2 , $0.15\ \mu\text{L}$ of GoTaq G2 Flexi DNA Polymerase (Promega, USA), $1\ \mu\text{L}$ of DNA, $1.5\ \mu\text{L}$ each of 10- μM forward and reverse primers, $3\ \mu\text{L}$ of 10% dimethyl sulfoxide (DMSO) (Sigma, USA), $0.15\ \mu\text{L}$ of 20 mg mL^{-1} bovine serum albumin (BSA) (New England Biolabs, USA), and $13.7\ \mu\text{L}$ of ultrapure water. The forward and reverse primers comprise 515F (5'-GTGYCAGCMGCCGCGGTAA-3') (Parada *et al.* 2016) and 806R (5'-GGACTACNVTGGGTWCTAAT-3') (Apprill *et al.* 2015), respectively, a heterogeneity spacer, and a linker. Thermocycling conditions were 95°C for 2 min, followed by 25 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 5 min.

Products from the first-step PCR were purified and used as the template in the second-step PCR, in a $45\text{-}\mu\text{L}$ reaction consisting of $9\ \mu\text{L}$ of 5X GoTaq Flexi Buffer (Promega, USA), $0.9\ \mu\text{L}$ of 10 mM dNTP Mix, $5.4\ \mu\text{L}$ of 25 mM MgCl_2 , $0.225\ \mu\text{L}$ pf GoTaq G2 Flexi DNA Polymerase (Promega, USA), $18.5\ \mu\text{L}$ of purified DNA, $0.9\ \mu\text{L}$ each of 10- μM forward and 10- μM reverse

primers, 4.5 μL of 10% DMSO, and 4.675 μL of ultra-pure water. The forward and reverse primers consist of the linker, an i5 or i7 index, and the adapter sequence (Cruaud *et al.* 2017). Each sample had a unique combination of the i5 and i7 indices. Thermocycling conditions were 95°C for 2 min, followed by 10 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s, and a final extension of 72°C for 5 min. The PCR products from both PCR steps were purified with an E.Z.N.A. MicroElute Gel Extraction Kit (Omega Bio-tek, USA). The purified products from the second PCR step were quantified with the dsDNA HS Assay Kit and the Qubit 4 fluorometer (Invitrogen, USA), and pooled in an equimolar ratio to prepare a library multiplex. DNA was sequenced using NovaSeq (PE 250 bp; Illumina) by Novogene Corporation.

Sequence data processing

De-multiplexed 16S paired-end sequence reads were merged, trimmed to remove adapters, and quality-filtered. We merged the paired-end reads with `-fastq_mergepairs` in USEARCH v11.0.667 (Edgar 2010) and trimmed the primer sequences using CUTADAPT v2.4 (`max_error_rate = 0.15`) (Martin 2011). Quality assessment was performed with FastQC v0.11.8 (Andrews 2010) and `-fastq_eestats2` in VSEARCH v2.15.1 (Rognes *et al.* 2016). Only reads with lengths between 240 and 253 bp and expected errors per read less than one were retained with the VSEARCH `-fastq_filter`. Pre-processed reads were then de-replicated with VSEARCH `-derep_fulllength`. Chimeras and singletons, representing less than 0.0001% of total reads, were removed with USEARCH `-unnoise3` (Edgar & Flyvbjerg 2015; Edgar 2016b). The remaining sequences were clustered at a 99% similarity level using VSEARCH `-usearch_global` to generate amplicon sequence variants (ASVs). Taxonomy was assigned to each ASV to the lowest identifiable taxonomic level using the SINTAX algorithm of USEARCH (Edgar 2016a) with bootstrap support higher than 80%. The EzBioCloud 16S database was used as a reference for taxonomy assignment (assessed in July 2021) (Yoon *et al.* 2017). The same approach was applied to identify putative antifungal ASVs, using the anti-*Bd* bacteria database as a reference (inhibitory ASVs only; assessed in November 2021) (Woodhams *et al.* 2015), with a bootstrap cutoff of 99%. Only ASVs that could be identified at the genus or species level were considered to match the sequences from putative anti-*Bd* taxa. ASVs with unidentified taxonomy or determined as contaminants according to negative controls were removed (Table S3, Supporting Information). ASVs represented by less than 0.018% (a

threshold determined by MCs, as in Huang *et al.* (2021); Table S3, Supporting Information) of total reads in each sample were considered false positives and filtered out.

Data analysis

All statistical analyses were conducted using R version 4.1.0 (R Development Core Team 2021). The microbiota composition was represented by the relative read abundance (RRA) and weighted percentage of occurrence (wPOO) of each bacterial taxon in an individual, as well as frequency of occurrence (FOO) of each taxon in all swab samples. Taxa with an FOO exceeding 90% were considered as part of the core microbiota. Microbiota diversity was assessed by calculating the effective number of ASVs (hill number) for each sample (alpha diversity) and sampling site (gamma diversity) using the `hilldiv` package (Alberdi & Gilbert 2019). The hill number depended on the order of the diversity q (Hill 1973), where a higher q placed greater weight on abundant ASVs and resulted in a lower hill number. Differences in microbiota compositions between sites (beta diversity) were evaluated using both the pairwise Jaccard distance and Bray–Curtis dissimilarity index with the `vegan` package (Oksanen *et al.* 2022). Beta diversity was visualized using non-metric multidimensional scaling (NMDS) and principal coordinates analysis (PCoA) with the `phyloseq` package (McMurdie & Holmes 2013), and hierarchical clustering was performed using Ward's method with the `dendextend` package (Galili 2015). Pairwise permutational ANOVA using the `pairwiseAdonis` package (Martinez Arbizu 2020) was performed to test the differences in microbiota compositions between sites, with the Benjamini–Hochberg false discovery rate correction based on 999 permutations. The homogeneity of intra-group beta-dispersion was assessed using the `phyloseq` package (McMurdie & Holmes 2013). The contribution of each ASV to the microbiota composition variation was determined using the SIMPER (similarity percentage) with the Bray–Curtis dissimilarity using the `vegan` package (Oksanen *et al.* 2022). Statistical significance was checked using the Kruskal–Wallis test by ranks. Only ASVs contributing more than 0.01% were retained.

To examine the effect of environmental and host factors on skin microbiota composition (beta diversity), two statistical models were used. In the first linear model, the relationship between the geographical distance between sampling sites and microbiota similarity was tested. The pairwise Jaccard distance or Bray–Curtis dissimilarity was used as the response variable, and the geographical distance between sites was used as the explanatory

variable. Pairwise distances between sites separated by the sea were excluded in the model, that is, only pairwise distances between sites in the same region (New Territories, Hong Kong Island, or Lantau Island; Fig. 1b) were included. In the second linear mixed model, principal coordinate axis 1 was extracted from the Bray–Curtis dissimilarity or Jaccard distance matrix and used as the response variable. Elevation, BCI, and sex were included as fixed effects, and the sampling site and sampling month were included as random effects.

RESULTS

Total, putative anti-*Bd*, and core skin microbiota of *P. hongkongensis*

Using high-throughput sequencing of the V4 region of the 16S rRNA gene, we identified 9433 amplicon sequence variants (ASVs; Table S4, Supporting Information), belonging to 2605 bacterial taxa classified at the lowest possible taxonomic level. In total, 42 phyla, 128 classes, and 274 orders of bacteria were identified. Among the ASVs, 314 were found in the putative anti-*Bd* bacteria database (Woodhams *et al.* 2015) and were assigned to 25 genera (Table S5, Supporting Information), accounting for 9.7% of the total reads. On average, each *P. hongkongensis* individual harbored 21 putative anti-*Bd* ASVs, and all individuals had at least three putative anti-*Bd* ASVs. The most prevalent putative anti-*Bd* ASV was present in 86.6% of all individuals, while 114 putative anti-*Bd* ASVs were exclusively found in single individuals. Among the ASVs, two from *Acinetobacter*, three from *Flavobacterium*, and three from *Novosphingobium* exhibited the highest RRA and occurrence. Rarefaction analysis indicated sufficient sequencing depth for ASV identification (Fig. S1, Supporting Information).

The most abundant phyla were Proteobacteria (59.2%), Bacteroidetes (21.2%), and Verrucomicrobia (12%), collectively accounting for over 92% of the total reads. No other phyla had an RRA >1% (Fig. 2). Within Proteobacteria, the most abundant classes were Betaproteobacteria (44.8%), Gammaproteobacteria (6.5%), Alphaproteobacteria (6.4%), Cytophagia (1.2%), and Deltaproteobacteria (1%). Among Bacteroidetes, Sphingobacteriia (5.9%) and Flavobacteria (3.5%) were the most abundant classes, while Opitutae (6.2%) and Verrucomicrobiae (5.7%) dominated within Verrucomicrobia. The most abundant taxon was Comamonadaceae, a family in Betaproteobacteria, representing 33.3% of the total reads and present in every individual skin microbiota (Fig. 2). Five out of 16 ASVs, each accounting for >1% reads, were as-

signed to Comamonadaceae (Table S6, Supporting Information). Among the 14 taxa with RRA >1%, three were putatively anti-*Bd* (*Acinetobacter*, *Flavobacterium*, and *Novosphingobium*; Table S5, Supporting Information). The core microbiota comprised 13 bacterial taxa, representing 7.4% of the total reads. These taxa were primarily composed of species belonging to the Betaproteobacteria class (e.g. *Undibacterium* in order Burkholderiales) and the Alphaproteobacteria class (e.g. *Novosphingobium* in order Sphingomonadales) within the phylum Pseudomonadota. Additionally, the core microbiota included taxa from the Chitinophagia class (e.g. family Chitinophagaceae) and the Flavobacteriales class (e.g. *Flavobacterium* in Flavobacteriales) within the phylum Bacteroidota (Table S7, Supporting Information), two of which were putatively anti-*Bd* (*Flavobacterium* and *Novosphingobium*; Table S5, Supporting Information). All taxa that accounted for >1% of the total reads or were within the core microbiota belonged to one of the three most abundant phyla.

The six *Bd*/*Bsal*-positive samples showed a comparable number of putative anti-*Bd* ASVs (mean = 21.3, SD = 8.4) to the negative samples (mean = 21.4, SD = 11.8; Fig. 2). Among the positive samples, the *Bd*-positive sample had 36 putative anti-*Bd* ASVs, while the five *Bsal*-positive samples had an average of 18.4 (SD = 5) putative anti-*Bd* ASVs, respectively. In addition, we identified several ASVs from bacteria that are potentially capable of producing TTX in our dataset, including 1 ASV for *Aeromonas*, 20 ASVs for *Pseudomonas*, 2 ASVs for *Shewanella*, and 2 ASVs for *Sphingopyxis*. However, only ASV21 (*Aeromonas* sp.) was present in a majority of newt individuals (60%) and water samples (75%), and its RRA was highly variable across samples (mean = 0.4%, SD = 1.68%, range = 0–13.9%; Tables S8,S9, Supporting Information).

Microbiota diversity within and between sites

Microbiota composition and diversity differed among sites and between skin and water samples (Figs 2,3; Fig. S2 and Tables S10–S12, Supporting Information). Individual microbiota from Hok Tau (HT), Kowloon Peak (KP), and Wu Kau Tang (WKT) exhibited the highest ASV richness (at $q = 0$), while those from Tai Mo Shan (TMS) exhibited the lowest (Figs 1b,3a; Tables S13,S14, Supporting Information). HT and KP also showed the highest gamma diversity in microbial communities (Fig. 3b). As the value of q increased, both the alpha diversity (within each sample) and gamma diversity (within each site) of the newt skin microbiota from all sites decreased rapidly, reflecting highly uneven

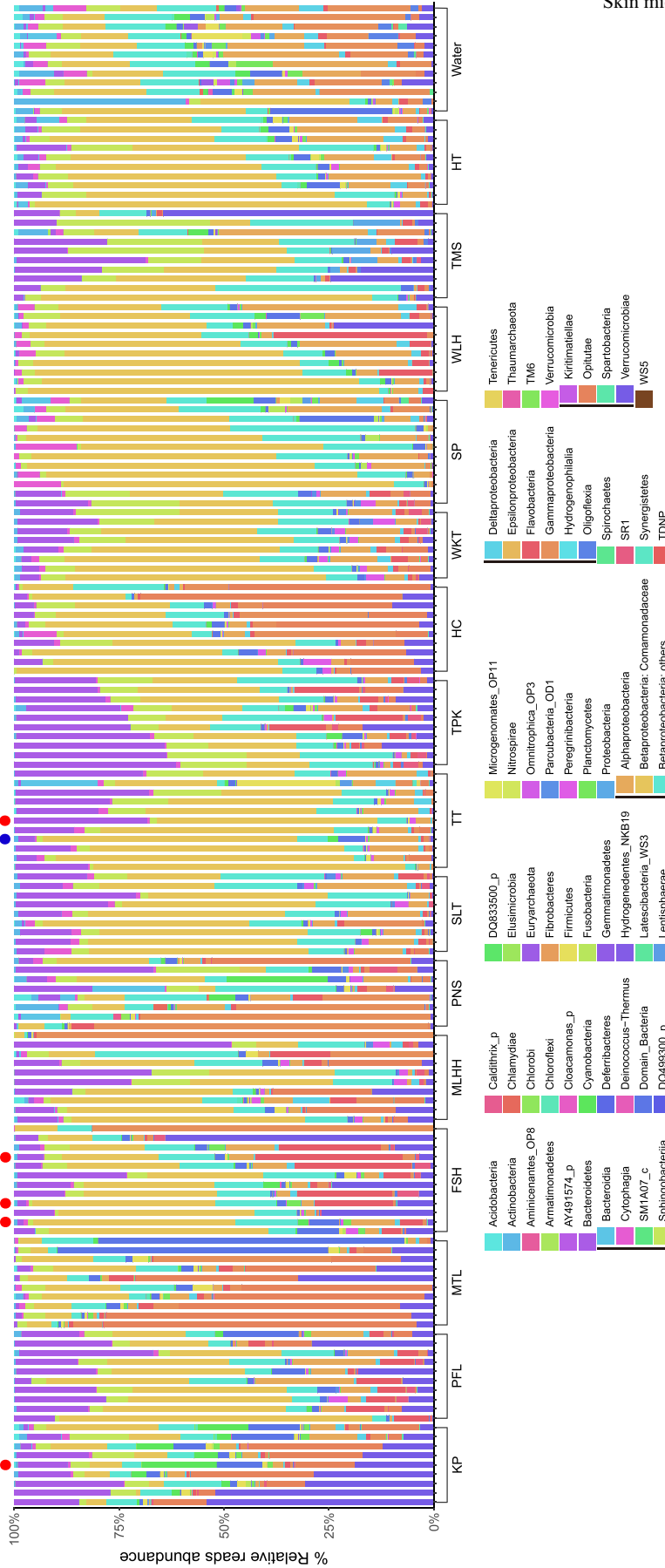


Figure 2 Skin microbiota compositions of *Paramesotriton hongkongensis* by sampling sites. Relative read abundances of bacterial taxa of each skin swab sample are shown. All taxa are shown at the phylum level except that the three most abundant phyla, Proteobacteria, Bacteroidetes, and Verrucomicrobia, are reported at the class level. Color-coded taxa in columns from left to right are presented in a top-down manner in each bar. The most abundant taxon, the family Comamonadaceae (phylum: Proteobacteria; class: Betaproteobacteria), is specifically indicated. *Bsal* and *Bd* positive samples are marked by a red dot and a blue dot, respectively, above the corresponding bar. Refer to Fig. 1 for the abbreviations of sampling sites.

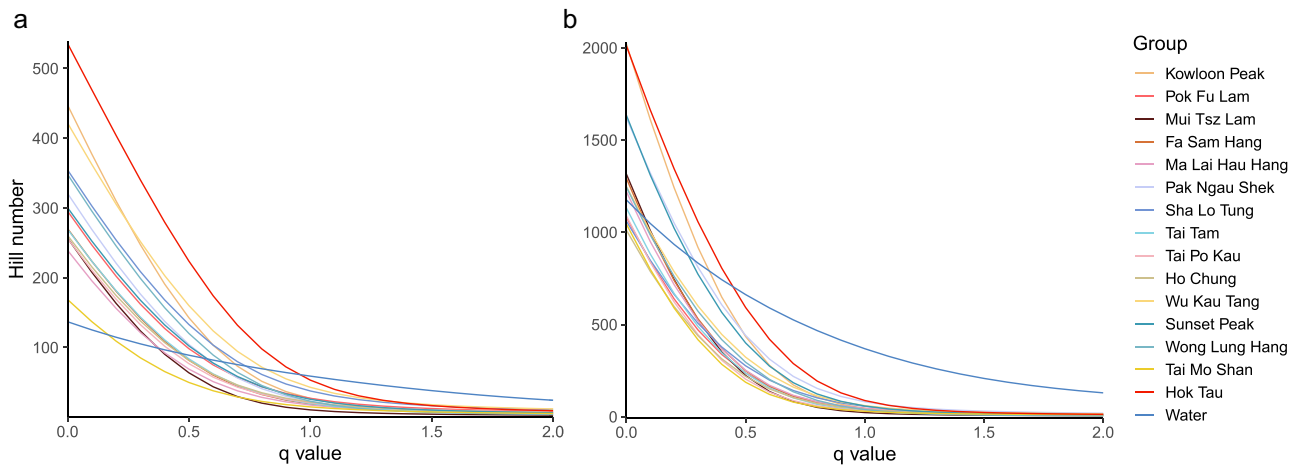


Figure 3 Alpha and gamma diversity of *Paramesotriton hongkongensis* skin microbiota from 15 sampling sites. (a) Alpha diversity—the mean effective number of amplicon sequence variants (ASVs) in an individual sample for each site and (b) gamma diversity—the total effective number of ASVs found in each site, when q ranges from 0 to 2 are shown.

relative abundances of bacterial taxa in the skin microbiota.

Microbiota composition analyses showed that the skin microbiota demonstrated site differences and were differentiated from water microbiota (hierarchical clustering, Fig. 4; NMDS and PCoA, Fig. S3, Supporting Information). Skin microbiota were well differentiated by site in the hierarchical clustering analysis based on both the Bray–Curtis dissimilarity (Fig. 4; Table S15, Supporting Information) and Jaccard distance (Fig. S4 and Table S16, Supporting Information), indicating site-specific bacterial compositions. The Bray–Curtis dissimilarity analyses revealed that skin microbiota from Sunset Peak (SP) and Wong Lung Hang (WLH) were highly similar to each other (Figs 1b,4). TMS had a relatively distinct skin microbiota composition from most sites according to the results from NMDS (Fig. S3a, Supporting Information) and PCoA (Fig. S3c and Table S17, Supporting Information). The Jaccard distance analyses presented similar patterns to the Bray–Curtis dissimilarity results in NMDS (Fig. S3b, Supporting Information) and PCoA (Fig. S3d and Table S18, Supporting Information). In Bray–Curtis dissimilarity analyses, bacterial communities in water samples were clustered as a distinct group, except for the one from SP, which clustered with SP skin microbiota samples (Fig. 4; Figs S3,S4, Supporting Information).

The skin microbiota composition was significantly different between all pairwise site comparisons (adjusted $P \leq 0.002$; Tables S19,S20, Supporting Information). The mean number of ASVs contributing to the top 10% dissimilarity between any two sites was 30.1 (SD = 8.7). Within the top 10% dissimilarity, seven ASVs were

present in more than 50% of the 105 pairwise comparisons (Table S21, Supporting Information), while 90 ASVs contributed to the dissimilarity of just one or two site pairs. Site-specific patterns were observed in the composition of newt skin microbiota (Figs 2,4; Fig. S4, Supporting Information). While the bacterial communities from most sites were dominated by Betaproteobacteria, those from Mui Tsz Lam (MTL) had a large proportion of Opiritae (39.3% RRA; Fig. 2). Among the samples from KP, Verrucomicrobiae was the most abundant class (25.8% RRA; Fig. 2). In half of the samples from Pak Ngau Shek (PNS), Gammaproteobacteria were present in the highest abundance (58% RRA; Fig. 2). Skin microbiota from SP and WLH, the two sites on Lantau Island, as well as TMS, the highest peak in Hong Kong, China (Fig. 1), formed relatively well-separated groups in NMDS (Fig. S3a,b, Supporting Information). Compared to other sites, bacterial communities from MTL, SP, and WLH had a particularly low abundance of Bacteroidetes (Fig. 2). Microbiota in water samples appeared to be more evenly distributed across taxa compared to skin microbiota (Fig. 3) and showed greater similarity to each other than to skin microbiota (Fig. 4; Figs S3,S4, Supporting Information).

Effects of environmental and host factors on skin microbiota

The pairwise distance between microbiota was positively correlated to the geographical distance between sites in terms of Bray–Curtis dissimilarity ($P < 0.001$,

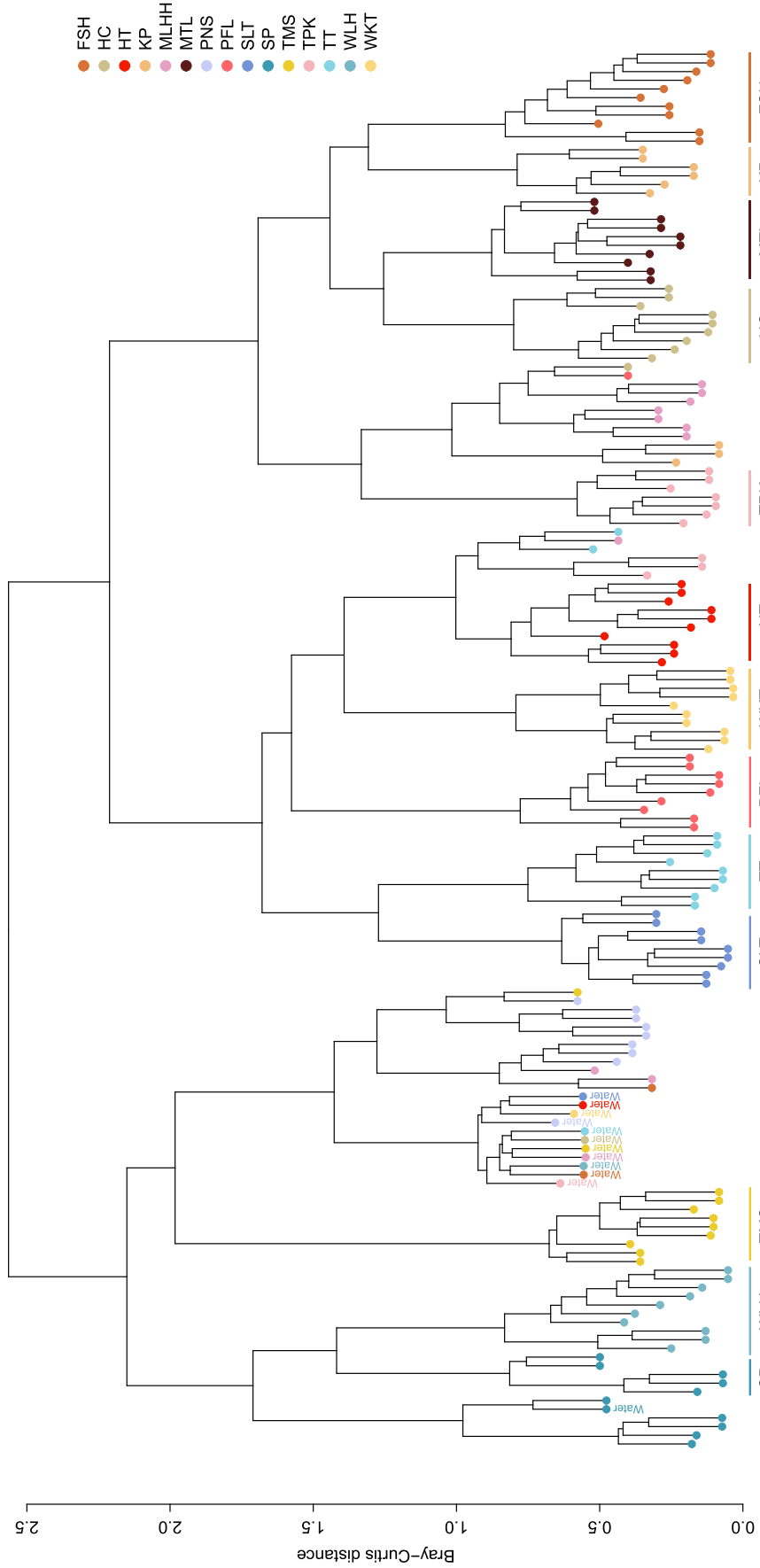


Figure 4 Hierarchical clustering of *Paramesotriton hongkongensis* skin microbiota by sampling sites based on the Bray-Curtis dissimilarity. Clusters with at least five samples from the same site are highlighted by the site name. Refer to Fig. 1 for the abbreviations of sampling sites.

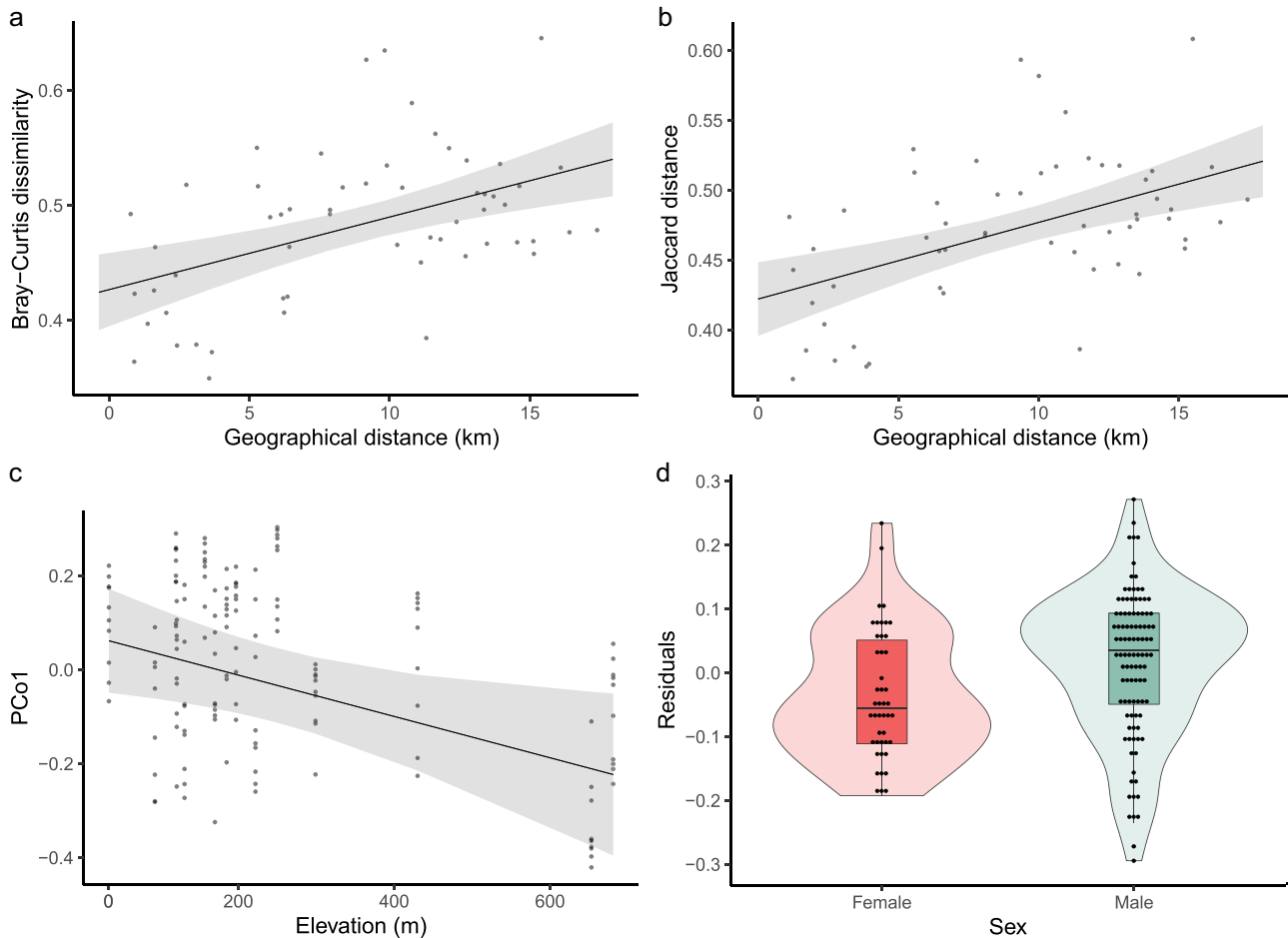


Figure 5 Effects of environmental and host factors on *Paramesotriton hongkongensis* skin microbiota. The (a) Bray–Curtis dissimilarity ($P < 0.001$, $R^2 = 0.21$) and (b) Jaccard distance ($P < 0.0001$, $R^2 = 0.23$) between the microbiota from different sites increased with the geographical distance between sites. (c) Microbiota composition (represented by principal coordinate axis 1 [PCo1] based on Bray–Curtis dissimilarity) varied with site elevation ($P = 0.02$). (d) The difference in microbiota composition between sexes ($P < 0.01$), shown as the differential residual distributions in the fitted model where sex was not included.

$R^2 = 0.21$; Fig. 5a) and Jaccard distance ($P < 0.0001$, $R^2 = 0.23$; Fig. 5b). The linear mixed model (Table 1) revealed that the skin microbiota composition changed with site elevation ($P = 0.02$; Fig. 5c) and showed significant differences between host sexes ($P < 0.01$; Fig. 5d).

DISCUSSION

Skin microbiota diversity and composition in *P. hongkongensis*

As the pandemic caused by chytrid fungi continues to threaten global amphibian diversity, Asia has gained increasing attention from the scientific community, as

it has been suggested as the origin of these pathogens (O’hanlon *et al.* 2018; Fisher & Garner 2020). This study is one of the few to investigate the skin microbiota in wild amphibian populations in Asia (Sabino-Pinto *et al.* 2016; Bletz *et al.* 2017b; Bataille *et al.* 2018; Yang *et al.* 2020; Mutnale *et al.* 2021). Our findings reveal that the diverse bacterial communities on the skin of *P. hongkongensis* were predominantly composed of Proteobacteria, Bacteroidetes, and Verrucomicrobia. The skin microbiota of amphibians, as is likely in the case with newts, may produce antimicrobial compounds that protect against harmful pathogens (Rebollar *et al.* 2020), which will be discussed in a later section. Additionally, amphibian microbiota may actively participate in nutrient cycling by breaking down the complex organic matter present on the

Table 1 Linear mixed model assessing the effects of environmental and host factors on the skin microbiota of *Paramesotriton hongkongensis* ($n = 149$)

Fixed effects	Effect	SE	<i>t</i>	<i>P</i>
(Intercept)	9.59×10^{-2}	9.36×10^{-2}	1.02	0.31
Elevation	-4.40×10^{-4}	1.82×10^{-4}	-2.42	0.02
BCI	-1.26×10^{-2}	4.25×10^{-2}	-0.30	0.77
Sex	6.52×10^{-2}	2.38×10^{-2}	2.74	0.01
Random effects				SD
Location				0.12
Month				0.03
Residual				0.16

Site elevation, body condition index (BCI), and sex were included as fixed effects. Sampling location and month were included as random effects. Significant effects are bolded.

amphibian's skin (Brunetti *et al.* 2023). Moreover, these microbes may interact with the host's immune system, potentially influencing the immune response of the amphibians (Küng *et al.* 2014). Their presence in the skin microbiota plays a crucial role in enhancing the overall microbial diversity, thereby significantly contributing to survival and well-being (Harrison *et al.* 2019). The compositions of the skin microbiota were significantly different across sampling sites, and most of them were highly differentiated from the streamwater microbiota present in the habitats of *P. hongkongensis*. The dissimilarity between skin microbial communities was found to be positively correlated with the geographical distance between sites. Moreover, among other environmental and host factors tested, elevation and host sex were identified as significant factors in determining the skin microbiota structure of the newts.

In congruence with many existing skin microbiota studies in amphibians, Proteobacteria was the most abundant bacterial phylum on *P. hongkongensis*, followed by Bacteroidetes (e.g. Bletz *et al.* 2017c; Muletz Wolz *et al.* 2018; Douglas *et al.* 2021). Comamonadaceae, a family in Pseudomonadota, was also commonly identified as an abundant family (McKenzie *et al.* 2012; Belden *et al.* 2015; Prado-Irwin *et al.* 2017; Bletz *et al.* 2017a, 2018; Jiménez *et al.* 2019; Walker *et al.* 2020; Walke *et al.* 2021). While Verrucomicrobia was occasionally found among the dominant phyla, its relative abundance rarely exceeded 3% (Belden *et al.* 2015; Bletz *et al.* 2017b, 2018; Walke *et al.* 2021), except in the case of the Japanese giant salamander (*Andrias japonicus*, 9%) and the larvae of wild Japanese fire belly newts (*Cynops*

pyrrhogaster, 20%) in Japan (Sabino-Pinto *et al.* 2016). However, a few phyla that are commonly found in high abundance on amphibian skin, such as Actinobacteria, Firmicutes, Acidobacteria, and Cyanobacteria (McKenzie *et al.* 2012; Belden *et al.* 2015; Prado-Irwin *et al.* 2017; Bletz *et al.* 2017a, 2018; Jiménez *et al.* 2019; Walker *et al.* 2020; Walke *et al.* 2021), were scarce (RRA < 1%) on *P. hongkongensis*. In a study on the skin microbial community structures of 89 frog species in Madagascar, Actinobacteria and Acidobacteria were found to occur in significantly higher relative abundance on terrestrial species compared to aquatic or semiaquatic species, which might explain their low abundance on *P. hongkongensis*, as it is primarily aquatic during the sampling period (Bletz *et al.* 2017a).

The core microbiota of *P. hongkongensis*: Potential for protection against chytrid fungal pathogens

Although there were large intraspecific variations in the skin microbiota across different sites, a core group of microbiota members was shared by most *P. hongkongensis* individuals, albeit with generally low relative abundances. The presence of a core microbiota, which maintains a relatively stable composition despite variations in environmental conditions experienced by the hosts, has been observed in many amphibian species, suggesting its functional importance. For example, the core microbiota structures on coqui frogs (*Eleutherodactylus coqui*) did not vary along the elevation gradient or with changes in

forest integrity (Hughey *et al.* 2017). The core microbiota of *P. hongkongensis* comprised bacterial species that were likely well adapted to reside on the host skin and perform vital functions for the host, thus establishing a symbiotic relationship (Prado-Irwin *et al.* 2017; Muletz Wolz *et al.* 2018). However, since our sampling was limited to the breeding season of the newts, the abundance of these core bacterial species may fluctuate temporally as the newts move from an aquatic to a terrestrial environment after the breeding season, which can result in changes in various relevant host traits, for example, the levels of skin moisture or reproductive hormones (Lau 2017; Lau *et al.* 2017; Bletz *et al.* 2017b). Seasonal changes in the abundance of core microbes, such as Chitinophagaceae, which was also detected in *P. hongkongensis*, have been reported in the North American wood frog (*Rana sylvatica*) (Douglas *et al.* 2021). Noteworthy, *P. hongkongensis* produces TTX (Yotsu *et al.* 1990), which is believed to act as a defense against predators, although the synthesis mechanism of this deadly neurotoxin is unknown. Further investigation of the TTX-producing ability of *Aeromonas* sp. found on many newt individuals can help us understand whether *P. hongkongensis* has developed a symbiotic relationship with certain toxin-producing bacteria to increase its fitness, as observed in another poisonous newt (Vaelli *et al.* 2020).

Furthermore, the core microbiota might play a functional role in enhancing host resistance against *Bd* infection. For example, based on bioinformatic and culture tests, it was predicted that *P. cinereus* has maintained a core microbiota in which bacterial members provided anti-*Bd* functions (Loudon *et al.* 2014). Multiple species of *Novosphingobium* and *Flavobacterium* were detected in the core microbes of *P. hongkongensis*. These genera have already been shown to exhibit anti-*Bd* properties (Harris *et al.* 2006), with the latter genus being widely distributed among amphibian species, such as *R. cascadae* (Roth *et al.* 2013), the green-eyed frog (*Lithobates vibicarius*) (Jiménez *et al.* 2019), and the four-toed salamander (*Hemidactylium scutatum*) (Lauer *et al.* 2008). Comamonadaceae, *Methylobacter*, and *Undibacterium* were also identified as core taxa on the wild Oriental fire-bellied toad (*Bombina orientalis*) in Japan (Sabino-Pinto *et al.* 2016), and the abundance of *Methylobacter* and Rhizobiales on wild *B. orientalis* was found to vary with individual *Bd* infection status in South Korea (Bataille *et al.* 2016, 2018). Although the functions of core microbiota on amphibian skin are not well understood, the overlapping skin microbiota composition between *P. hongkongensis* and other Asian amphibians studied suggests a potential protec-

tive role of their core microbes against chytrid fungal pathogens.

It may be reasonable to assume the putative anti-*Bd* bacterial taxa identified here, using the anti-*Bd* isolates database, could potentially possess anti-*Bsal* properties as well. This assumption is based on the fact that many of these bacteria can produce broad antifungal compounds (Woodhams *et al.* 2015; Muletz Wolz *et al.* 2018). Putative antifungal bacteria were found on *P. hongkongensis* in great diversity, with high prevalence but low abundance. Notably, eight ASVs from *Acinetobacter*, *Flavobacterium*, and *Novosphingobium* were particularly abundant and prevalent, indicating a putative symbiotic relationship with *P. hongkongensis* (Muletz-Wolz *et al.* 2017). Most of the putative anti-*Bd* microbiota had restricted distributions among newt individuals and showed dissimilar taxon identities. This pattern might have resulted from the colonization by putative anti-*Bd* bacteria acquired from distinct pools of microbes present in different locations (Muletz-Wolz *et al.* 2017). The estimated relative abundances of the putative anti-*Bd* bacteria in this study were generally much lower compared to those found in some other amphibian species, such as three *Plethodon* species in North America (Muletz Wolz *et al.* 2018) and six frog species in India (Mutnale *et al.* 2021). This observation suggests that rare taxa in a microbiota may play a functionally important role. Another possible explanation could be the failure to identify significant antifungal members present in the microbiota of *P. hongkongensis* using the anti-*Bd* bacteria database (Woodhams *et al.* 2015). This database was compiled by testing bacterial isolates from certain amphibian species using culture methods. As a result, it is unlikely to include rare or unculturable bacteria and host-specific microbial species on untested amphibians, especially those in Asia. However, it is worth noting that putative anti-*Bd* bacteria were detected in all 100% of the sampled newts. This finding indicated the potential resistance or even herd immunity against chytridiomycosis in *P. hongkongensis*. This resistance might be attributed to historical interactions with *Bd* and *Bsal* strains endemic to Asia, where *Bd* exhibited a high degree of diversity and has not been found to cause morbidity or mortality in local wild amphibian populations (Bataille *et al.* 2013).

Previous studies have suggested that an amphibian population can achieve herd immunity to *Bd* when around 80% of the individuals are protected by anti-*Bd* microbes, allowing the population to coexist with *Bd* (Woodhams *et al.* 2007; Muletz-Wolz *et al.* 2017). In the case of *P. hongkongensis* population in Wutongshan, all *Bsal*-positive individuals showed no symptoms despite

the high prevalence of the pathogen (Yuan *et al.* 2018). For the *P. hongkongensis* population in Hong Kong, China, the prevalence of *Bd* and *Bsal* was very low (Chen *et al.* 2023), with positive individuals being asymptomatic, supporting the idea of potential herd immunity achieved through antifungal skin microbes. However, in this study, no noticeable difference was observed in the skin microbiota between *Bsal*-positive and *Bsal*-negative individuals. Noteworthy, *Janthinobacterium* spp. (class Betaproteobacteria, phylum Proteobacteria) were detected on *P. hongkongensis*. The anti-*Bd* property of *Janthinobacterium lividum* has been well verified (Brucker *et al.* 2008). This bacterium has been shown to produce indole-3-carboxaldehyde and violacein, which can significantly inhibit *Bd* growth at low concentrations (Brucker *et al.* 2008). Moreover, the addition of *J. lividum* to amphibian skin can reduce both the morbidity and mortality caused by *Bd* infection (Becker *et al.* 2009; Harris *et al.* 2009a). In addition, numerous ASVs belonging to a few common anti-*Bd* genera, such as *Pedobacter* and *Pseudomonas*, were found on *P. hongkongensis*.

Geographic factors influencing the skin microbiota structure

One of our main findings was that the skin microbiota structure of newts was largely influenced by site, depending on environmental or host genetic factors. Previous studies have shown that the population genetic structure of *P. hongkongensis* aligns with the landscape topography, which cannot be fully explained by isolation by distance (Lau 2017). Based on this population structure, there were five genetic clusters of *P. hongkongensis*, one on Hong Kong Island (Pok Fu Lam [PFL], Tai Tam [TT]), one on Lantau Island (SP), and three on the New Territories (North: WKT; East: HC, MTL, KP; and Central: Tai Po Kau [TPK]), while an admixture of the New Territories North and East clusters was observed at PNS (Lau 2017). Consistent with the host population structure, the skin microbiota from the two sites (WLH and SP) on Lantau Island were similar to each other and distinct from other sites (Fig. 4; Fig. S4, Supporting Information). The geographical isolation of the Lantau Island populations from the mainland not only limited the gene flow between newts but likely also restricted the exchange of individual microbiota. However, this pattern was not observed between the two Hong Kong Island newt populations (PFL and TT). Their skin microbiota were more similar to those of the New Territories populations than to each other, implying that factors other than geographical isolation alone

might also influence microbiota structure. Microbiota differences in the New Territories can be partially explained by geographical and host genetic distances, for example, individuals from the New Territories East sites (i.e. HC, MTL, KP, and FSH) had mostly similar microbiota, but these factors could not account for all the observed differences. Therefore, there were instances where the microbiota from certain sites did not align with either the host population structure or geographical proximity. Overall, geographical distance appeared to have a larger influence, as microbiota within the same site strongly clustered together and did not mix with those from different sites within the same genetic population of newts (Fig. 4; Fig. S4, Supporting Information).

How amphibians acquire their skin microbes in each new generation and how their microbiota are shaped and maintained remain important questions in the field. Horizontal transmission happens when microbes are selected from the habitat to form a microbiota (Bright & Bulgheresi 2010). Members of the *P. hongkongensis* skin microbiota might come from the living environment, such as water, soil, and leaf litter (Muletz Wolz *et al.* 2018). The clear difference in the microbiota between skin and stream water implies that even though the skin of *P. hongkongensis* might initially contact all bacterial species present in the surroundings, only a subset of these species had managed to colonize and proliferate on the skin. Since the environmental conditions, for example, water quality (Kueneman *et al.* 2014; Krynak *et al.* 2016), soil, and pH (Fierer & Jackson 2006; Muletz Wolz *et al.* 2018), of various sites could be different, the composition of bacteria available from the environment for horizontal transmission could certainly vary in response to the site-specific conditions. Moreover, the environmental characteristics at each site could affect host traits, so the site-wise differences in microbiota could also reflect the influence of site-specific host traits. For instance, the production of antimicrobial peptides by the skin, a key component in amphibian immune defense, could be regulated by water surface area and conductivity, thus affecting the skin microbiota composition (Krynak *et al.* 2016).

Influence of elevation and host sex on the skin microbiota structure

In addition to geographical distance, elevation also had an effect on the skin microbial communities of newts, similar to previous observations that skin microbiota structures varied along elevational gradients from 0 to

875 m and 700 to 1000 m above sea level (Hughey *et al.* 2017; Muletz Wolz *et al.* 2018). The influence of elevation is complicated, and many environmental factors co-vary along the gradient, which might, in turn, affect the host traits as well as the growth and interactions of different bacteria on the host (Muletz Wolz *et al.* 2018). Furthermore, *Bd* prevalence was found to positively correlate with elevation, which might have led to a stronger selection for anti-*Bd* bacteria on surviving hosts at higher elevations if there have been recurring infections (Bresciano *et al.* 2015). Considering the impact of elevation, we recommend that future studies investigate the potential influence of environmental temperatures on the skin microbiota of newts.

We also found that the variations in the microbiota structures of *P. hongkongensis* were associated with host sex. Although it has been shown in Blanchard's cricket frog (*Acris blanchardi*) that sex interacted with latitude and water surface area in affecting the skin microbiota (Krynak *et al.* 2016), it has no effect on *E. eschscholtzii* (Prado-Irwin *et al.* 2017) or *R. sylvatica* (Douglas *et al.* 2021). In *P. hongkongensis*, females exhibited cannibalism on eggs and larvae, which caused a difference in the diets between the sexes (Fu *et al.* 2013). As diet can influence the skin microbiota (Antwis *et al.* 2014), the effect of sex might be an indirect indicator of the effect of diet. Alternatively, hormonal differences between the sexes might explain their microbiota divergence. A relationship between sexual differences in host-associated microbiota and sex hormone levels has been documented in birds and primates (Escallon Herkrath 2015; Mallott *et al.* 2020), but whether such a relationship exists in amphibian skin microbiota has not been investigated.

CONCLUSION

The skin microbiota of *P. hongkongensis* was diverse, with a group of core skin microbes potentially coevolving with, and providing essential functions for, the hosts. The bacterial community structure of *P. hongkongensis* was highly site-specific, which was likely attributed to geographical isolation and environmental characteristics instead of host population genetics, as demonstrated by the effect of elevation. Host sex also influenced the newt skin microbiota, possibly because of the differences in diet or hormonal levels between the two sexes. The high prevalence and low abundance of putative antifungal bacteria found on the skin, together with the low prevalence and dispersed distribution of chytrid infection in the newt population, suggest that *P. hongkongensis* has had a history of

exposure to and coexistence with the fungal pathogens in the region.

Using *P. hongkongensis* as a reference species, interrogation of the compositions and patterns of the skin microbiota structures of other amphibians in East Asia, where chytrids are proposed to be native, will enhance our understanding of host–pathogen–microbiota coevolution. Such knowledge can be applied to the conservation of *P. hongkongensis* as well as amphibian populations naïve to pathogenic chytrids. Given that the extant *P. hongkongensis* skin microbiota has likely been shaped by the coevolution with chytrids, it is a promising source for identifying and characterizing antifungal bacteria not yet known to us, and thereby developing novel probiotic treatments against chytridiomycosis. Further investigations into the factors governing the differences in the skin microbiota between sites and sexes could improve our understanding and our ability to develop effective probiotic treatments.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The sequencing data have been archived in the National Center for Biotechnology Information (NCBI) under the BioProject accession number PRJNA1113385.

REFERENCES

- Abarca JG, Zuniga I, Ortiz-Morales G *et al.* (2018). Characterization of the skin microbiota of the cane toad *Rhinella cf. marina* in Puerto Rico and Costa Rica. *Frontiers in Microbiology* **8**, 2624.

- Alberdi A, Gilbert MTP (2019). hilldiv: An R package for the integral analysis of diversity based on Hill numbers. *bioRxiv* 545665. <https://doi.org/10.1101/545665>
- Andrews S (2010). FastQC: A quality control tool for high throughput sequence data. Available from URL: <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>
- Antwis RE, Haworth RL, Engelmoer DJ, Ogilvy V, Fidgett AL, Preziosi RF (2014). Ex situ diet influences the bacterial community associated with the skin of red-eyed tree frogs (*Agalychnis callidryas*). *PLoS ONE* **9**, e85563.
- Apprill A, McNally S, Parsons R, Weber L (2015). Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquatic Microbial Ecology* **75**, 129–37.
- Bataille A, Fong JJ, Cha M *et al.* (2013). Genetic evidence for a high diversity and wide distribution of endemic strains of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* in wild Asian amphibians. *Molecular Ecology* **22**, 4196–209.
- Bataille A, Lee-Cruz L, Tripathi B, Kim H, Waldman B (2016). Microbiome variation across amphibian skin regions: Implications for chytridiomycosis mitigation efforts. *Microbial Ecology* **71**, 221–32.
- Bataille A, Lee-Cruz L, Tripathi B, Waldman B (2018). Skin bacterial community reorganization following metamorphosis of the fire-bellied toad (*Bombina orientalis*). *Microbial Ecology* **75**, 505–14.
- Bates KA, Clare FC, O’hanlon S *et al.* (2018). Amphibian chytridiomycosis outbreak dynamics are linked with host skin bacterial community structure. *Nature Communications* **9**, 693.
- Bates KA, Shelton JM, Mercier VL *et al.* (2019). Captivity and infection by the fungal pathogen *Batrachochytrium salamandrivorans* perturb the amphibian skin microbiome. *Frontiers in Microbiology* **10**, 1834.
- Becker MH, Brucker RM, Schwantes CR, Harris RN, Minbiole KP (2009). The bacterially produced metabolite violacein is associated with survival of amphibians infected with a lethal fungus. *Applied and Environmental Microbiology* **75**, 6635–38.
- Belden LK, Hughey MC, Rebollar EA *et al.* (2015). Panamanian frog species host unique skin bacterial communities. *Frontiers in Microbiology* **6**, 1171.
- Bird AK, Prado-Irwin SR, Vredenburg VT, Zink AG (2018). Skin microbiomes of California terrestrial salamanders are influenced by habitat more than host phylogeny. *Frontiers in Microbiology* **9**, 442.
- Bletz MC, Archer H, Harris RN *et al.* (2017a). Host ecology rather than host phylogeny drives amphibian skin microbial community structure in the biodiversity hotspot of Madagascar. *Frontiers in Microbiology* **8**, 1530.
- Bletz MC, Kelly M, Sabino-Pinto J *et al.* (2018). Disruption of skin microbiota contributes to salamander disease. *Proceedings of the Royal Society B* **285**, 20180758.
- Bletz MC, Perl RB, Bobowsk BT *et al.* (2017c). Amphibian skin microbiota exhibits temporal variation in community structure but stability of predicted Bd-inhibitory function. *The ISME Journal* **11**, 1521–34.
- Bletz MC, Vences M, Sabino-Pinto J *et al.* (2017b). Cutaneous microbiota of the Japanese giant salamander (*Andrias japonicus*), a representative of an ancient amphibian clade. *Hydrobiologia* **795**, 153–67.
- Bresciano J, Salvador C, Paz-Y-Mino C *et al.* (2015). Variation in the presence of anti-*Batrachochytrium dendrobatidis* bacteria of amphibians across life stages and elevations in Ecuador. *Ecohealth* **12**, 310–19.
- Bright M, Bulgheresi S (2010). A complex journey: Transmission of microbial symbionts. *Nature Reviews Microbiology* **8**, 218–30.
- Brooks AW, Kohl KD, Brucker RM, Van Opstal EJ, Bordenstein SR (2016). Phylosymbiosis: Relationships and functional effects of microbial communities across host evolutionary history. *PLoS Biology* **14**, e2000225.
- Brucker RM, Harris RN, Schwantes CR *et al.* (2008). Amphibian chemical defense: Antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *Journal of Chemical Ecology* **34**, 1422–29.
- Brunetti AE, Lyra ML, Bauermeister A *et al.* (2023). Host macrocyclic acylcarnitines mediate symbiotic interactions between frogs and their skin microbiome. *Iscience* **26**, 108109.
- Cammarota G, Ianiro G, Gasbarrini A (2014). Fecal microbiota transplantation for the treatment of *Clostridium difficile* infection: a systematic review. *Journal of Clinical Gastroenterology* **48**, 693–702.
- Chen G, Lau A, Wan B *et al.* (2023). Occurrence of pathogenic chytrid fungi *Batrachochytrium salamandrivorans* and *Batrachochytrium dendrobatidis* in the Hong Kong newt (*Paramesotriton hongkongensis*) and other wild and imported amphibians in a subtropical Asian region. *The Journal of Wildlife Diseases* **59**, 709–21.

- Chen YE, Tsao H (2013). The skin microbiome: Current perspectives and future challenges. *Journal of the American Academy of Dermatology* **69**, 143–55. e3.
- Colston TJ, Jackson CR (2016). Microbiome evolution along divergent branches of the vertebrate tree of life: What is known and unknown. *Molecular Ecology* **25**, 3776–800.
- Crawford AJ, Lips KR, Bermingham E (2010). Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. *PNAS* **107**, 13777–82.
- Cruaud P, Rasplus J-Y, Rodriguez LJ, Cruaud A (2017). High-throughput sequencing of multiple amplicons for barcoding and integrative taxonomy. *Scientific Reports* **7**, 41948.
- Douglas AJ, Hug LA, Katzenback BA (2021). Composition of the North American wood frog (*Rana sylvatica*) bacterial skin microbiome and seasonal variation in community structure. *Microbial Ecology* **81**, 78–92.
- Edgar RC (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**, 2460–61.
- Edgar RC (2016a). SINTAX: A simple non-Bayesian taxonomy classifier for 16S and ITS sequences. *bioRxiv* 074161.
- Edgar RC (2016b). UNOISE2: Improved error-correction for Illumina 16S and ITS amplicon sequencing. *bioRxiv* 081257.
- Edgar RC, Flyvbjerg H (2015). Error filtering, pair assembly and error correction for next-generation sequencing reads. *Bioinformatics* **31**, 3476–82.
- Escallon Herkrath C (2015). Reproductive physiology, avian malaria, and the cloacal microbiome in tropical Rufous-collared Sparrows (*Zonotrichia capensis*) (Dissertation). Virginia Tech, Blacksburg, USA.
- Fierer N, Jackson RB (2006). The diversity and biogeography of soil bacterial communities. *PNAS* **103**, 626–31.
- Fisher MC, Garner TW (2020). Chytrid fungi and global amphibian declines. *Nature Reviews Microbiology* **18**, 332–43.
- Foster KR, Schluter J, Coyte KZ, Rakoff-Nahoum S (2017). The evolution of the host microbiome as an ecosystem on a leash. *Nature* **548**, 43–51.
- Fredricks DN (2001). Microbial ecology of human skin in health and disease. *Journal of Investigative Dermatological Symposium Proceedings* **6**, 167–69.
- Fu VWK, Karraker NE, Dudgeon D (2013). Breeding dynamics, diet, and body condition of the Hong Kong newt (*Paramesotriton hongkongensis*). *Herpetological Monographs* **27**, 1–22.
- Galili T (2015). dendextend: An R package for visualizing, adjusting and comparing trees of hierarchical clustering. *Bioinformatics* **31**, 3718–20.
- Grice EA, Segre JA (2011). The skin microbiome. *Nature Reviews Microbiology* **9**, 244–53.
- Harris RN, Brucker RM, Walke JB *et al.* (2009a). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *The ISME Journal* **3**, 818–24.
- Harris RN, James TY, Lauer A, Simon MA, Patel A (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *EcoHealth* **3**, 53–56.
- Harris RN, Lauer A, Simon MA, Banning JL, Alford RA (2009b). Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. *Diseases of Aquatic Organisms* **83**, 11–16.
- Harrison XA, Price SJ, Hopkins K, Leung WT, Sergeant C, Garner TW (2019). Diversity-stability dynamics of the amphibian skin microbiome and susceptibility to a lethal viral pathogen. *Frontiers in Microbiology* **10**, 2883.
- Hill MO (1973). Diversity and evenness: A unifying notation and its consequences. *Ecology* **54**, 427–32.
- Hoffmann AR (2017). The cutaneous ecosystem: The roles of the skin microbiome in health and its association with inflammatory skin conditions in humans and animals. *Advances in Veterinary Dermatology* **8**, 71–83.
- Huang PY, Poon ESK, Chan LY *et al.* (2022). Dietary diversity of multiple shorebird species in an Asian subtropical wetland unveiled by DNA metabarcoding. *Environmental DNA* **4**, 1381–96.
- Huang P-Y, Poon ESK, Wong ATC, So IWY, Sung Y-H, Sin SYW (2021). DNA metabarcoding reveals the dietary composition in the endangered black-faced spoonbill. *Scientific Reports* **11**, 18773.
- Hughey MC, Pena JA, Reyes R, Medina D, Belden LK, Burrowes PA (2017). Skin bacterial microbiome of a generalist Puerto Rican frog varies along elevation and land use gradients. *PeerJ* **5**, e3688.
- IUCN (2021). *The IUCN Red List of Threatened Species*. Version 2021–3. Available online at from URL: <https://www.iucnredlist.org>

- Jani AJ, Briggs CJ (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *PNAS* **111**, E5049–58.
- Jiménez RR, Alvarado G, Estrella J, Sommer S (2019). Moving beyond the host: Unraveling the skin microbiome of endangered Costa Rican amphibians. *Frontiers in Microbiology* **10**, 2060.
- Krynak KL, Burke DJ, Benard MF (2016). Landscape and water characteristics correlate with immune defense traits across Blanchard's cricket frog (*Acris blanchardi*) populations. *Biological Conservation* **193**, 153–67.
- Kueneman JG, Parfrey LW, Woodhams DC, Archer HM, Knight R, Mckenzie VJ (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Molecular Ecology* **23**, 1238–50.
- Kueneman JG, Weiss S, Mckenzie VJ (2017). Composition of micro-eukaryotes on the skin of the cascades frog (*Rana cascadae*) and patterns of correlation between skin microbes and *Batrachochytrium dendrobatidis*. *Frontiers in Microbiology* **8**, 2350.
- Kueneman JG, Woodhams DC, Harris R, Archer HM, Knight R, Mckenzie VJ (2016). Probiotic treatment restores protection against lethal fungal infection lost during amphibian captivity. *Proceedings of the Royal Society B: Biological Sciences* **283**, 20161553.
- Küng D, Bigler L, Davis LR, Gratwicke B, Griffith E, Woodhams DC (2014). Stability of microbiota facilitated by host immune regulation: informing probiotic strategies to manage amphibian disease. *PLoS ONE* **9**, e87101.
- Lai Y, Cogen AL, Radek KA *et al.* (2010). Activation of TLR2 by a small molecule produced by *Staphylococcus epidermidis* increases antimicrobial defense against bacterial skin infections. *Journal of Investigative Dermatology* **130**, 2211–21.
- Laking AE, Ngo HN, Pasmans F, Martel A, Nguyen TT (2017). *Batrachochytrium salamandrivorans* is the predominant chytrid fungus in Vietnamese salamanders. *Scientific Reports* **7**, 44443.
- Lau A (2017). Conservation and population biology of the Hong Kong newt (*Paramesotriton hongkongensis*) (Thesis). University of Hong Kong, Hong Kong, China.
- Lau A, Karraker NE, Martelli P, Dudgeon D (2017). Delineation of core terrestrial habitat for conservation of a tropical salamander: The Hong Kong newt (*Paramesotriton hongkongensis*). *Biological Conservation* **209**, 76–82.
- Lauer A, Simon MA, Banning JL, Lam BA, Harris RN (2008). Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. *The ISME Journal* **2**, 145–57.
- Loudon AH, Woodhams DC, Parfrey LW *et al.* (2014). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *The ISME Journal* **8**, 830–40.
- Mallott EK, Borries C, Koenig A, Amato KR, Lu A (2020). Reproductive hormones mediate changes in the gut microbiome during pregnancy and lactation in Phayre's leaf monkeys. *Scientific Reports* **10**, 9961.
- Martel A, Blooi M, Adriaensen C *et al.* (2014). Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science* **346**, 630–31.
- Martel A, Spitzen-van der Sluijs A, Blooi M *et al.* (2013). *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *PNAS* **110**, 15325–29.
- Martin M (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. Journal* **17**, 10–12.
- Martinez Arbizu P (2020). pairwiseAdonis: pairwise multilevel comparison using adonis. R package version 0.4. Available from URL: <https://github.com/pmartinezarbizu/pairwiseAdonis>
- Martín-Platero AM, Valdivia E, Ruíz-Rodríguez M *et al.* (2006). Characterization of antimicrobial substances produced by *Enterococcus faecalis* MRR 10–3, isolated from the uropygial gland of the hoopoe (*Upupa epops*). *Applied and Environmental Microbiology* **72**, 4245–49.
- Martín-Vivaldi M, Pena A, Peralta-Sánchez JM *et al.* (2010). Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. *Proceedings of the Royal Society B: Biological Sciences* **277**, 123–30.
- Mckenzie VJ, Bowers RM, Fierer N, Knight R, Lauber CL (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *The ISME Journal* **6**, 588–96.
- Mcmurdie PJ, Holmes S (2013). phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **8**, e61217.
- Muletz-Wolz CR, Direnzo GV, Yarwood SA, Campbell Grant EH, Fleischer RC, Lips KR (2017). Antifungal bacteria on woodland salamander skin exhibit high

- taxonomic diversity and geographic variability. *Applied and Environmental Microbiology* **83**, e00186–17.
- Muletz-Wolz CR, Fleischer RC, Lips KR (2019). Fungal disease and temperature alter skin microbiome structure in an experimental salamander system. *Molecular Ecology* **28**, 2917–31.
- Muletz Wolz CR, Yarwood SA, Campbell Grant EH, Fleischer RC, Lips KR (2018). Effects of host species and environment on the skin microbiome of *Plethodontid* salamanders. *Journal of Animal Ecology* **87**, 341–53.
- Mutnale MC, Reddy GS, Vasudevan K (2021). Bacterial community in the skin microbiome of frogs in a coldspot of chytridiomycosis infection. *Microbial Ecology* **82**, 554–58.
- Naik S, Bouladoux N, Linehan JL *et al.* (2015). Commensal–dendritic-cell interaction specifies a unique protective skin immune signature. *Nature* **520**, 104–8.
- O’hanlon SJ, Rieux A, Farrer RA *et al.* (2018). Recent Asian origin of chytrid fungi causing global amphibian declines. *Science* **360**, 621–27.
- Oksanen J, Simpson G, Blanchet FG *et al.* (2022). Vegan: community ecology package, Version 2.6-2. *Vienna (Austria): R Foundation for Statistical Computing*. Available from URL: <https://github.com/vegandevs/vegan>
- Parada AE, Needham DM, Fuhrman JA (2016). Every base matters: Assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environmental Microbiology* **18**, 1403–14.
- Pérez-Sánchez T, Balcázar J, García Y *et al.* (2011). Identification and characterization of lactic acid bacteria isolated from rainbow trout, *Oncorhynchus mykiss* (Walbaum), with inhibitory activity against *Lactococcus garvieae*. *Journal of Fish Diseases* **34**, 499–507.
- Prado-Irwin SR, Bird AK, Zink AG, Vredenburg VT (2017). Intraspecific variation in the skin-associated microbiome of a terrestrial salamander. *Microbial Ecology* **74**, 745–56.
- Prest TL, Kimball AK, Kueneman JG, Mckenzie VJ (2018). Host-associated bacterial community succession during amphibian development. *Molecular Ecology* **27**, 1992–2006.
- R Development Core Team (2021). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
- Rebolla EA, Martinez-Ugalde E, Orta AH (2020). The amphibian skin microbiome and its protective role against chytridiomycosis. *Herpetologica* **76**, 167–77.
- Rognes T, Flouri T, Nichols B, Quince C, Mahé F (2016). VSEARCH: A versatile open source tool for metagenomics. *PeerJ* **4**, e2584.
- Ross AA, Rodrigues HA, Neufeld JD (2019). The skin microbiome of vertebrates. *Microbiome* **7**, 79.
- Roth T, Foley J, Worth J, Piovio-Scott J, Pope K, Lawler S (2013). Bacterial flora on Cascades frogs in the Klamath mountains of California. *Comparative Immunology, Microbiology and Infectious Diseases* **36**, 591–98.
- Sabino-Pinto J, Bletz MC, Islam MM *et al.* (2016). Composition of the cutaneous bacterial community in Japanese amphibians: Effects of captivity, host species, and body region. *Microbial Ecology* **72**, 460–69.
- Scheele BC, Pasmans F, Skerratt LF *et al.* (2019). Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science* **363**, 1459–63.
- Spitzen-Van Der Sluijs A, Spikmans F, Bosman W *et al.* (2013). Rapid enigmatic decline drives the fire salamander (*Salamandra salamandra*) to the edge of extinction in the Netherlands. *Amphibia-Reptilia* **34**, 233–39.
- Swei A, Rowley JJ, Rödder D *et al.* (2011). Is chytridiomycosis an emerging infectious disease in Asia? *PLoS ONE* **6**, e23179.
- Vaelli PM, Theis KR, Williams JE, O’connell LA, Foster JA, Eisthen HL (2020). The skin microbiome facilitates adaptive tetrodotoxin production in poisonous newts. *eLife* **9**, e53898.
- Varga JF, Bui-Marinos MP, Katzenback BA (2019). Frog skin innate immune defences: Sensing and surviving pathogens. *Frontiers in Immunology* **9**, 3128.
- Walke JB, Becker MH, Krinos A *et al.* (2021). Seasonal changes and the unexpected impact of environmental disturbance on skin bacteria of individual amphibians in a natural habitat. *FEMS Microbiology Ecology* **97**, fiaa248.
- Walker DM, Hill AJ, Albecker MA *et al.* (2020). Variation in the slimy salamander (*Plethodon* spp.) skin and gut-microbial assemblages is explained by geographic distance and host affinity. *Microbial Ecology* **79**, 985–97.
- Woodhams DC, Alford RA, Antwis RE *et al.* (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology* **96**, 595–595.

Woodhams DC, Vredenburg VT, Simon M-A *et al.* (2007). Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biological Conservation* **138**, 390–398.

Yang X, Hou X, Wei L *et al.* (2020). Characterization of skin symbiotic bacteria of sympatric amphibians in Southeastern China. *Asian Herpetological Research* **11**, 381–393B.

Yoon S-H, Ha S-M, Kwon S *et al.* (2017). Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *International Journal of Systematic and Evolutionary Microbiology* **67**, 1613.

Yotsu M, Iorizzi M, Yasumoto T (1990). Distribution of tetrodotoxin, 6-epitetradotoxin, and 11-deoxytetrodotoxin in newts. *Toxicon* **28**, 238–41.

Yuan Z, Martel A, Wu J, Van Praet S, Canessa S, Pasmans F (2018). Widespread occurrence of an emerging fungal pathogen in heavily traded Chinese urodelan species. *Conservation Letters* **11**, e12436.

SUPPLEMENTARY MATERIALS

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

Table S1 Detailed information of the *P. hongkongensis* individuals from which skin swabs were collected and the water samples between October 2019 and February 2020. Abbreviations of collection period: Jan, January; Feb, February; Sep, September; Oct, October; Nov, November; and Dec, December

Table S2 Species compositions of the mock communities (MC) 1 to 3. Three mock communities were prepared from four species with known taxonomic identities in phyla Proteobacteria and Firmicutes. A cross 'X' indicated that the species was included in the mock community. MC 1 to 3 were prepared by mixing a fixed amount (12 ng) of DNA from each of the species

Table S3 Read counts of taxa identified in individual mock communities and negative controls. The species used in the mock communities are indicated by green color. Non-zero read counts of amplicon sequence variants (ASVs) in negative controls are colored in gray. ASVs removed after treatment based on negative controls are labeled as 'Yes' in dark gray color

Table S4 Taxonomy of the bacterial amplicon sequence variants (ASVs) identified in the skin swabs and water samples

Table S5 Taxonomy of the bacterial ASVs identified with antifungal properties found in the *P. hongkongensis* skin microbiota

Table S6 Bacterial ASVs found in the *P. hongkongensis* skin microbiota with an overall relative read abundance (RRA) over 1% and the corresponding taxonomy. Family Comamonadaceae incorporates more abundant ASVs than all other taxa. *Putative antifungal ASVs

Table S7 Core skin microbiota of *P. hongkongensis* represented by taxa with a frequency of occurrence (FOO) over 90%. *Taxa containing putative antifungal ASVs

Table S8 Relative read abundance of the bacterial ASVs identified in individual *P. hongkongensis* skin microbiota and water microbiota. Potential TTX-producing bacteria are indicated by #

Table S9 Weighted percentage of occurrence of the bacterial ASVs identified in individual *P. hongkongensis* skin microbiota and water microbiota. Potential TTX-producing bacteria are indicated by #

Table S10 Bacterial phyla identified in the *P. hongkongensis* skin microbiota. The three most abundant phyla, Proteobacteria, Bacteroidetes, and Verrucomicrobia are reported at the class level. The most abundant taxon, the family Comamonadaceae (phylum: Proteobacteria; class: Betaproteobacteria) is specifically indicated. The relative read abundance (RRA), weighted percentage of occurrence (wPOO), and frequency of occurrence (FOO) of each taxon at each site and in all samples (overall) are shown

Table S11 Relative read abundance of the bacterial phyla identified in individual *P. hongkongensis* skin microbiota and water microbiota. The three most abundant phyla, Proteobacteria, Bacteroidetes, and Verrucomicrobia are reported at the class level. The most abundant taxon, the family Comamonadaceae (phylum: Proteobacteria; class: Betaproteobacteria) is specifically indicated

Table S12 Weighted percentage of occurrence of the bacterial phyla identified in individual *P. hongkongensis* skin microbiota and water microbiota. The three most abundant phyla, Proteobacteria, Bacteroidetes, and Verrucomicrobia are reported at the class level. The most abundant taxon, the family Comamonadaceae (phylum: Proteobacteria; class: Betaproteobacteria) is specifically indicated

Table S13 Statistical significance values (*P*-value) of pairwise comparisons of the bacterial alpha diversity (hill numbers) of *P. hongkongensis* skin microbiota between sites and between the skin microbiota at each site and water microbiota. Comparisons were performed by Kruskal-Wallis test, followed by a posthoc Dunn test with

Benjamini–Hochberg correction ($P < 0.05$). More weight is given to abundant amplicon sequence variances while the order of diversity q increases. Hill numbers of the orders of diversity $q = 0, 1$, and 2 are closely related to species richness, Shannon, and Simpson indices, respectively. Abbreviations: FSH, Fa Sam Hang; HC, Ho Chung; HT, Hok Tau, KP, Kowloon Peak; MLHH, Ma Lai Hau Hang; MTL, Mui Tsz Lam; PNS, Pak Ngau Shek; PFL, Pok Fu Lam; SLT, Sha Lo Tung; SP, Sunset Peak; TMS, Tai Mo Shan; TPK, Tai Po Kau; TT, Tai Tam; WLH, Wong Lung Hang; and WKT, Wu Kau Tang

Table S14 Diversity of *P. hongkongensis* skin microbiota compositions. The alpha and gamma diversities, indicated by hill numbers, represent the effective number of amplicon sequence variants at the individual level and site level, respectively. Beta diversity represents the difference in effective numbers among individual *P. hongkongensis* skin microbiota. More weight is given to abundant amplicon sequence variants while the order of diversity q increases. Hill numbers of the orders of diversity $q = 0, 1$, and 2 are closely related to species richness, Shannon, and Simpson indices, respectively

Table S15 Pairwise Bray–Curtis dissimilarity based on the relative read abundance of bacterial ASVs in *P. hongkongensis* skin microbiota between sites (0–1: similar to dissimilar)

Table S16 Pairwise Jaccard distance based on the relative read abundance of bacterial ASVs in *P. hongkongensis* skin microbiota between sites (0–1: similar to dissimilar)

Table S17 Bacterial ASVs found in the *P. hongkongensis* skin microbiota with a score larger than 0.2 or

smaller than -0.2 in the principal coordinates analysis (PCoA) for both principal coordinate 1 (PCo1) and PCo2 using the abundance-based Bray–Curtis dissimilarity

Table S18 Bacterial ASVs found in the *P. hongkongensis* skin microbiota with a score larger than 0.2 or smaller than -0.2 in the principal coordinates analysis (PCoA) for both principal coordinate 1 (PCo1) and PCo2 using the incidence-based Jaccard distance

Table S19 Pairwise PERMANOVA and SIMPER analyses of the *P. hongkongensis* skin microbiota from 12 sites using abundance-based Bray–Curtis dissimilarity

Table S20 Pairwise PERMANOVA and SIMPER analyses of the *P. hongkongensis* skin microbiota from 12 sites using incidence-based Jaccard distance

Table S21 Bacterial ASVs found in the *P. hongkongensis* skin microbiome that contribute to over 0.1% variation in over 50% of the pairwise comparisons of skin microbiota composition between sites in the SIMPER analysis using abundance-based Bray–Curtis dissimilarity

Figure S1 Rarefaction curves of all skin swab samples of *P. hongkongensis*.

Figure S2 Skin microbiota composition of *P. hongkongensis* by sampling sites shown in weighted percentage of occurrence.

Figure S3 Non-metric multidimensional scaling (NMDS) and principal coordinates analysis (PCoA) of *P. hongkongensis* skin microbiota from 15 sampling sites.

Figure S4 Hierarchical clustering of *P. hongkongensis* skin microbiota from 15 sampling sites based on Jaccard distance.

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