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HOST MICROBE INTERACTIONS

Composition of the Cutaneous Bacterial Community in Japanese Amphibians: Effects of Captivity, Host Species, and Body Region

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Abstract The cutaneous microbiota plays a significant role in the biology of their vertebrate hosts, and its composition is known to be influenced both by host and environment, with captive conditions often altering alpha diversity. Here, we compare the cutaneous bacterial communities of 61 amphibians (both wild and captive) from Hiroshima, Japan, using high-throughput amplicon sequencing of a segment of the 16S rRNA gene. The majority of these samples came from a captive breeding facility at Hiroshima University where specimens from six species are maintained under highly standardized conditions for several generations. This allowed to identify host effects on the bacterial communities under near identical environmental conditions in captivity. We found the structure of the cutaneous bacterial community significantly differing between wild and captive individuals of newts, *Cynops pyrrhogaster*, with a higher alpha diversity found in the wild individuals. Community structure also showed distinct patterns when comparing different species of amphibians kept under highly similar conditions, revealing an intrinsic host effect. Bacterial communities of dorsal vs. ventral skin surfaces did not significantly differ in most species, but a trend of higher alpha diversity on the ventral surface was found in Oriental fire-bellied toads, *Bombina orientalis*. This study confirms the cutaneous microbiota of amphibians as a highly dynamic system influenced by a complex interplay of numerous factors.

Keywords Microbiota · Anura · Caudata · Japan · 16S rRNA · Illumina sequencing

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Introduction

The integument of animals is the first level of protection against invasive pathogens from the environment, but represents more than a physical barrier. In vertebrates, a chemical defense is provided by the active compounds secreted by skin glands in combination with metabolites produced by microbes inhabiting the skin [1, 2]. The role of symbiotic skin bacteria in host immunity has been intensively studied [3, 4]. These microorganisms are known to contribute to and modulate the immune responses in the host [5, 6]. After its initial establishment, the microbiota of the skin is highly dynamic and throughout the entire life of the host, microbes are constantly acquired and lost [7].

In amphibians, the skin provides an incomplete physical barrier and active compounds within the mucosal environment play an important role in defense against pathogens [8]. Many of these compounds, such as alkaloids and antimicrobial peptides, originate from the host and are known to interact with and inhibit pathogens [9–11]. Other compounds originate from skin-associated bacteria, and are also known to

play a role in suppressing pathogens themselves and to interact synergistically with host-produced compounds [12–15].

As in humans and other vertebrates, the composition of the cutaneous bacterial communities on amphibians is dynamic, and influenced by multiple factors, including host species and environment [16–22]. Additionally, the skin is a heterogeneous structure, with differences in texture and density of glands among body regions [23], which can influence the composition of skin-associated bacteria [24].

To better understand how cutaneous microbial communities influence the health of their amphibian hosts, it is essential to characterize the composition and specificity of these communities. In the present study, we assessed variation of the skin bacterial communities of six species of amphibians (*Bombina orientalis*, *Bufo japonicus*, *Cynops pyrrhogaster*, *Echinotriton andersoni*, *Odorrana splendida*, and *Rana japonica*) from one of the largest amphibian breeding facilities worldwide. In this center, located in Hiroshima University in Japan, different species of amphibians are kept under highly standardized conditions for several generations, providing ideal and novel conditions for a comparative assessment of host effects on the microbial community structure because it removes the confounding effects of inhabiting different environments. In addition, this study provides information on the composition of the skin microbial community of Asian amphibians which are still poorly studied. We analyzed samples from this collection and from wild-caught specimens from Hiroshima University campus, to explore to which degree cutaneous bacterial communities differ in composition and diversity (1) between wild and captive conspecific amphibians, (2) between species kept under highly similar conditions in captivity, and (3) between dorsal and ventral body surface in captive amphibians.

Material and Methods

Sampling

During the first week of March 2015, 61 adult amphibians belonging to six species housed at the breeding facility of the Institute for Amphibian Biology, Hiroshima University, were sampled for skin bacterial communities: Oriental fire-bellied toads, *Bombina orientalis*; Japanese common toads, *Bufo japonicus*; Japanese fire-bellied newts, *Cynops pyrrhogaster*; Anderson's crocodile newts, *Echinotriton andersoni*; Amami Ishikawa's frogs, *Odorrana splendida*; and Japanese brown frogs, *Rana japonica*. This captive collection was initiated with wild individuals in 1994 (for *Rana japonica*) and currently is composed of wild caught to F2 generations for the amphibian species used here. For this study, we sampled 7–14 individuals per species, with typically only 1–2 specimens from a single cage to reduce pseudoreplicates (Table 1; exceptions were *B. orientalis* with three individuals from two cages each and *E. andersoni* with five individuals from one cage). Specimens in this collection are maintained for the purpose of other research and conservation breeding projects, and sufficient large sample sizes from independent cages could not be obtained in all cases for a single sex. Because exploratory analyses of our dataset did not yield any difference in composition or diversity of the bacterial communities between the sexes, we combined data from male and female individuals for analysis. Individuals from all species (with exception of breeding groups of *E. andersoni*) are kept in similar environmental conditions, which consist of plastic terraria (61 × 40 × 15 cm) with a gravel substrate of 2–3-cm depth. There is continuous flow of water from the upper side and a drainage pipe at 1-cm level which keeps the environment wet, thus preventing drying of their skin. Water of all cages comes from the same source. *E. andersoni* individuals can also be kept in the same gravel-substrate containers, but for breeding they are kept in breeding cage (90 × 90 × 50 cm) with a wet muddy bottom (a mixture of 70:30 v/v leaves and soil used as floor cover).

Additionally, nine *C. pyrrhogaster* from a shallow pond and adjacent ditch (ca. 10–20-cm water depth; approximately 50 × 20 m) within the Hiroshima University campus were sampled. Wild individuals were captured by dip netting or manually with gloved hands, sampled on the ventral surface as described below and released quickly, assuring the shortest handling time possible. All sampled individuals were adults.

Sampling technique was non-invasive and consisted of rinsing the sampling surface of the amphibian with 50 ml of autoclaved filtered doubled distilled water to remove transient bacteria and then rubbing the cleaned surface ten times with a synthetic cotton swab (MW113; Medical Wire & Equipment, Corsham, UK). Clean nitrile gloves were used to hold and swab each individual. Swabs were individually placed in sterile 1.5 ml centrifuge tubes and stored at −20 °C until DNA extraction.

Sample Processing

DNA was extracted from the swabs with MoBio Power Soil htp-96 extraction kits (MoBio Laboratories, Carlsbad, CA, USA) with the minor adjustments outlined in Kueneman et al. [20] and double centrifugation time to account for the available rotor speed. The V4 region [25] of the bacterial 16S rRNA gene was amplified and sequenced using the dual-index approach of Kozich et al. [26].

All PCRs were performed on a T1 thermal cycler (Biometra) in duplicate. Individual reactions (12.5 μl) were composed by 0.15 μl of Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 0.25 μl of each forward (515 F: 5′-GTGCCAGCMGCCGCGGTAA-3′) and reverse primer

Table 1 Summary of provenance of 61 adult amphibian individuals sampled from the breeding facility of the Institute for Amphibian Biology, Hiroshima University. Numbers refer to specimens for which ventral skin swabs were included after quality filtering; numbers for dorsal swabs in some cases differ slightly (see Fig. 1). Wild-caught individuals refer to specimens that had been maintained in all cases for more than 1 year in captivity, often for much longer

Species	Number captive-bred	Number wild-caught	Cages
Bombina orientalis	3 males, 7 females	0	4
Bufo japonicus	1 female (Hiroshima prefecture)	1 male, 5 females (Hiroshima, Hyogo, Kagoshima, Tottori prefectures), all >1 year in captivity	5
Cynops pyrrhogaster	2 females (Hiroshima prefecture)	4 males, 3 females (Hiroshima prefecture)	8
Echinotriton andersoni	0	11 unsexed adults (Okinawa and Amami Islands)	4
Odorrana splendida	5 males, 1 female, 1 unsexed specimen (F1 2012, Amami Island)	3 females (Amami Island), over 4 years in captivity	6
Rana japonica	5 males and 7 females (Hiroshima and Kagashima prefectures, and F1 and F2 crosses)	2 females (Hiroshima prefecture), all >1 year in captivity	7
Total	32	29	34

(806R: 5′-GGACTACHVGGGTWTCTAAT-3′) (10 μM), 0.25 μl of dNTP, 2.5 μl of buffer (as supplied with the polymerase; Thermo Scientific), 8.1 μl of H_2O, and 1 μl of template DNA. The protocol consisted on an initial denaturation step at 98 °C for 1 min, then amplification during 30 cycles at 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension of 5 min at 72 °C. After amplification, the two duplicate PCR products were combined (25 μl per sample) and visualized on 1 % agarose gels.

Samples were pooled for sequencing by collecting approximately equal amounts of each samples' PCR product and gel purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). The purified pool concentration was determined with the Broad-Range dsDNA kit on a Qbit 2.0 and the pooled samples were submitted for Illumina Miseq sequencing using paired-end 2 × 250 v2 chemistry at the Genome Center of the Helmholtz Center for Infection Biology in Braunschweig, Germany.

The sequences of the amplicon libraries were deposited in the NCBI short read database (Bioproject PRJNA320971).

Sequence Processing

All sequences were processed with Quantitative Insights Into Microbial Ecology (MacQIIME v1.9.1) unless otherwise stated [27]. Following the default settings of the Fastq-join, the raw forward and reverse 16S rRNA amplicons from each sample were paired [28, 29]. After the initial pairing, sequences underwent quality filtering to remove low-quality sequences. Sequences were additionally filtered by read length (250–253 base pairs; usegalaxy.org). The identification of chimeras was performed using usearch61 de novo based detection on a per sample basis (http://drive5.com/usearch/usearch_docs.html; [30]). Chimeric sequences, i.e., sequences originating from PCR artifacts and composed of partial sequences of different bacteria, were removed before picking operational taxonomic units (OTUs). Using an open reference OTU-picking strategy, sequences were clustered into OTUs at 97 % similarity (http://qiime.org/tutorials/open_reference_illumina_processing.html; [31]). First, the sequences were matched to the SILVA 119 database (24 July 2014; https://www.arb-silva.de) and the remaining sequences were de novo clustered at a 97 % similarity. The UCLUST [32] algorithm was used in both the reference-based and de novo clustering steps. The reference sequence for each OTU was chosen based on which sequence was most abundant for a given OTU. The taxonomy of the representative sequences was determined by aligning them with PyNAST [33] and assigning taxonomy using the RDP classifier [34] with the SILVA 119 taxonomy. A phylogenetic tree was built with FastTree [35] under default settings. Based on Bokulich et al. [36], a cut-off was made removing all OTUs with less than 0.005 % of the total reads, leaving 4,812,392 reads in the dataset. To standardize the number of reads across samples for our main analyses, all samples were rarefied to 1000 sequences per sample, and samples with fewer than 1000 sequences were removed.

Statistical Analyses

The overall data was divided in three sets to allow the following comparisons: (1) Comparisons between wild and captive individuals. These comparisons were restricted to *C. pyrrhogaster* and targeted the ventral skin bacterial community of nine captive and nine wild individuals. (2) Comparisons between species. These comparisons were made among five amphibian species that were kept under similar conditions for several generations. This analysis included the ventral skin samples from ten *B. orientalis*, seven *B.*

japonicus, nine *C. pyrrhogaster*, ten *O. splendida*, and fourteen *R. japonica*. (3) Comparison between body regions (i.e., ventral versus dorsal). These comparisons used the same individuals as the species comparison plus 11 *E. andersoni*.

Within each dataset, the data was divided into subgroups according to species and/or sample type (e.g., ventral swabs of *B. orientalis*, dorsal swabs of *B. orientalis*). The core bacterial community at the OUT level shared by at least 80 % of the samples in each sub-group was determined and all of the different cores merged. The datasets were then filtered for these merged core OTUs and the information on their proportion in each taxon visualized in bar plots.

Species richness was assessed using the Chao1 and OTU indices, and phylogenetic diversity was assessed using Faith's PD index. These indices were calculated within QIIME for all subgroups of the data using the entire datasets. These metrics were compared for each comparison group in R [37] version 3.3.2 with linear regression models and ANOVAs after confirming statistical normality of the dataset. A dissimilarity matrix among the samples was calculated for the samples within each comparison group outlined above using the Bray-Curtis and the unweighted UniFrac metric within QIIME [38]. These matrices were further analyzed in Primer 7 by non-metric multidimensional scaling (nMDS) and by performing a PERMANOVA to statistically analyze the three comparisons outlined above. If main effects were significant, subsequent pair-wise PERMANOVAs were completed. LEfSe analyses were performed on the Galaxy web-based interface (http://huttenhower.sph.harvard.edu/galaxy/) using standard parameters except the alpha value for the factorial Kruskal-Wallis test among classes (alpha = 0.01).

Core OTUs that were shared by 80 % of the samples per category were included in the comparisons represented by Venn diagrams, generated manually in CorelDraw X3 (Corel Corp.).

Results

Effect of Captivity Conditions

The cutaneous bacterial communities from captive *C. pyrrhogaster* were compared to those from a wild population at the same location. The 80 % core bacterial community in captivity was dominated by Xantomonadaceae (23.9 %) and Pseudomonaceae (18.9 %), while in the wild it was dominated by Comamonadaceae (44.9 %) and Verrucomicrobiaceae (19.2 %) (Fig. 1a, supplementary material, Fig. S1). Wild individuals had more diverse communities (Chao1 index; t test; P value = 0.044), and a tendency towards a greater phylogenetic diversity (Faith's PD index; t test; P value = 0.081), but similar OTU richness (t test: P value = 0.625) compared to captive individuals. The two groups analyzed were significantly different from each other in beta diversity (PERMANOVA: P value = 0.001) (Fig. 1b). The 80 % core of the communities from wild and captive individuals consisted of 35 and 23 OTUs, respectively, and 14 of these OTUs were shared between the two categories (Fig. 1c, supplementary material, Table S1). A LEfSe analysis revealed 107 taxa distinguishing the skin bacterial communities of wild and captive specimens. From these taxa, 69 and 38 occurred in higher proportions in wild and captive individuals, respectively. The phyla Acidobacteria, Actinobacteria, Fusobacteria, Tenericutes, and Verrucomicrobia were detected as more abundant for the wild individuals, while Spirochaetae was more abundant in the captive individuals. At the genus level *Acidothermus*, *Micrococcus*, *Marmoricola*, *Cloacibacterium*, *Sphingobacterium*, *Anaerotruncus*, *Bradyrhizobium*, *Ralstonia*, *Vogesella*, *Pseudomonas*, and *Opitutus* were significantly more abundant in the wild individuals, while *Ferruginibacter*, *Mucilaginibacter*, *Sphingomonas*, *Massilia*, *Thermomonas*, and *Spirochaeta* were significantly more abundant in captive individuals. Additionally, at the OTU level, 29 and 19 OTUs were more abundant in wild and captive individuals, respectively (supplementary material, Fig. S2).

Host Species Effect

Five amphibian species (*B. orientalis*, *B. japonicus*, *C. pyrrhogaster*, *O. splendida*, and *R. japonica*) kept under the same conditions at the breeding facility of Hiroshima University were analyzed. The 80 % core skin bacterial community was largely dominated by Flavobacteriaceae, followed by Moraxellaceae and Pseudomonaceae in *B. japonicus*, *O. splendida*, and *R. japonica*. In *B. orientalis* and *C. pyrrhogaster*, Pseudomonaceae was the most abundant family followed by unclassified Micrococcales and Flavobacteriaceae in the first species and Xanthomonadaceae and Comamonadaceae in the second (Fig. 2). ANOVA revealed differences in alpha diversity between the analyzed groups (P value < 0.001). Pair-wise tests between skin bacterial communities from the different species confirmed consistent significant differences for the number of OTUs, Chao1, and Faith's PD indexes for the pairs: *B. japonicus–R. japonica* and *O. splendida–R. japonica*, being *R. japonica* less OTU rich and alpha diverse in both cases (Table 2, Fig. 2). The bacterial communities clustered according to species and were all significantly different from each other in beta diversity (Fig. 3a, supplementary material, Table S2). At the 80 % core OTU level, *B. orientalis* had 41 OTUs,

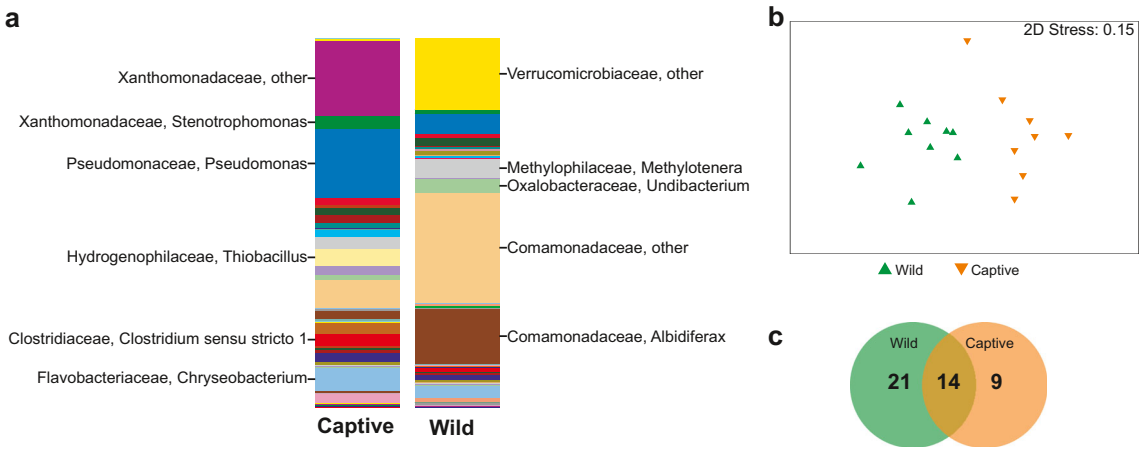

Fig. 1 **a** Composition of the 80 % core skin bacterial communities of wild and captive *Cynops pyrrhogaster* at the genus level. Most abundant genera are identified in the figure (full legend in supplementary material, Fig. S1). **b** Plot from a two-dimensional nMDS analysis, based on unweighted UniFrac distances between skin bacterial communities of wild and captive samples of *C. pyrrhogaster*. **c** Venn diagram showing numbers of 80 % core OTUs unique to and shared between wild and captive *C. pyrrhogaster*

B. japonicus had 43 OTUs, *C. pyrrhogaster* had 23 OTUs, *O. splendida* had 51 OTUs, and *R. japonica* had 32 OTUs. From these, six OTUs were shared among all species (Fig. 3b, supplementary material, Table S3). A LEfSe analysis revealed 16 taxa which distinguished the skin bacterial communities of the various species. Ten of these were associated with *B. orientalis*, one with *B. japonicus*, one with *C. pyrrhogaster*, two with *O. splendida*, and two with *R. japonica* (supplementary material, Fig. S3).

Body Region Effect

The dorsal and ventral surfaces of six amphibian species (*B. orientalis*, *B. japonicus*, *C. pyrrhogaster*, *E. andersoni*, *O. splendida*, and *R. japonica*) were compared. For all species,

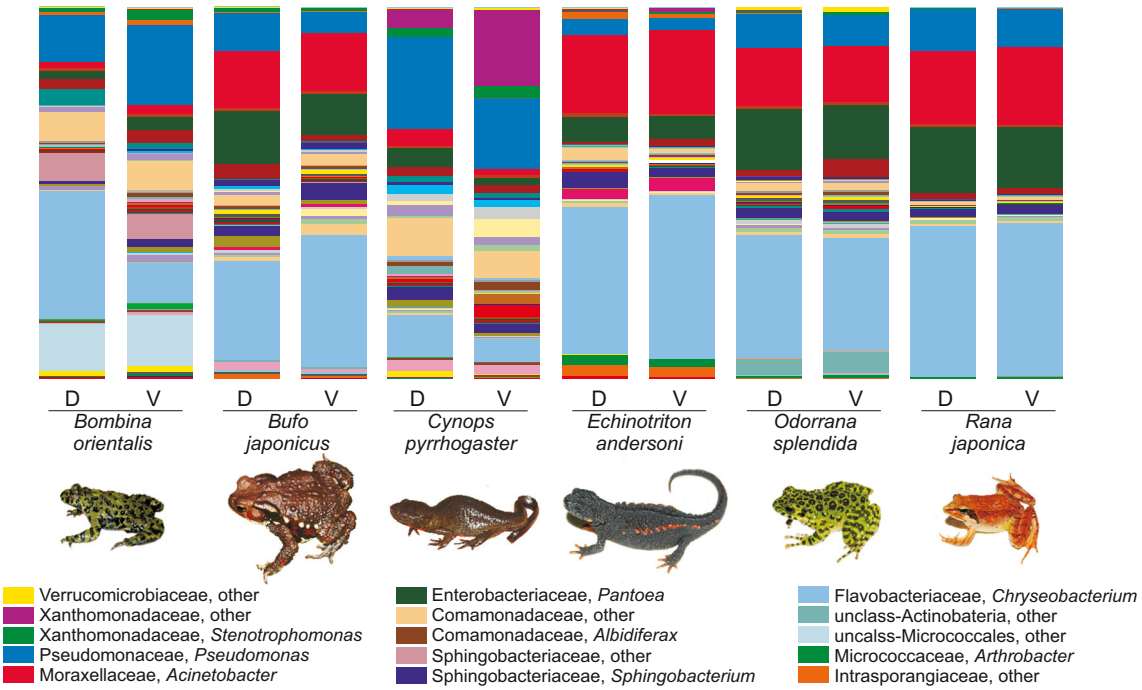

Fig. 2 Composition of the 80 % core skin bacterial communities of the analyzed species both dorsally and ventrally at the genus level. Most abundant bacterial genera are identified in the figure (full legend in supplementary material, Fig. S1). Unclass: group not classified bellow the order level. Other: group not classified bellow family level

Table 2 Comparisons of the alpha diversity values between species analyzed with ANOVA; P values are represented. Lower triangle reflects the differences in OTU numbers. Higher triangle reflects the differences in alpha diversity indexes: top value is related to the Faith's PD index; bottom value is related to the Chao1 index

	Bombina orientalis	*Bufo japonicus*	*Cynops pyrrhogaster*	*Odorrana splendida*	*Rana japonica*
Bombina orientalis		0.916	0.780	0.998	*<0.001*
		0.163	0.514	*0.004*	0.924
Bufo japonicus	0.999		0.999	0.729	*0.022*
			0.003	0.895	*0.013*
Cynops pyrrhogaster	0.652	0.619		0.516	*0.021*
				<0.001	0.938
Odorrana splendida	0.868	0.960	0.110		*<0.001*
					<0.001
Rana japonica	*0.005*	*0.009*	0.347	*<0.001*	

Significant P values are in italics. Absolute values are represented in Fig. 4

the 80 % core skin bacterial community of the dorsal and ventral surfaces were dominated by the same families and had similar abundances. Exceptions were found in *B. orientalis* and *C. pyrrhogaster*. On the ventral surface of *B. orientalis*, the abundance of Flavobacteriaceae was larger, while Pseudomonaceae and unclassified Micrococcales exhibited greater relative abundance dorsally. On the dorsum of *C. pyrrhogaster*, the abundance of Xanthomonadaceae was reduced when compared to the ventral surface, with Pseudomonaceae and Flavobacteriaceae being the most abundant families (Fig. 2). Differences in beta diversity were consistent across individuals as indicated by the clustering of bacterial communities according to body region in the nMDS plot (Fig. 4a, Table 3). For all species, the majority of core OTUs were shared between ventral and dorsal surfaces (Fig. 4b, supplementary material, Tables S4 to S9). With the exception of *B. orientalis*, no species showed differences in OTU richness or alpha diversity indices between dorsal and ventral cutaneous bacterial communities. In this species, all the metrics showed significant differences between the surfaces (Table 3), with larger values characterizing the ventral surface.

Discussion

Our study adds to the evidence characterizing the cutaneous bacterial communities of amphibians as being influenced by captivity, host species, and body region (e.g., [17, 20]). Bacterial communities of the skin of captive and wild Japanese amphibians were dominated by Gamma-Proteobacteria, Bacteroidetes and in some amphibian taxa Actinobacteria, which is in agreement with findings from amphibians in North America [17, 20], Central America [39, 40], and Europe [41]. Our study, herein, provides evidence that captive environment, body region, and host identity influence the community composition.

To explore how the captive environment at the Hiroshima amphibian breeding facility affects cutaneous bacterial communities, we focused on the comparison between wild and

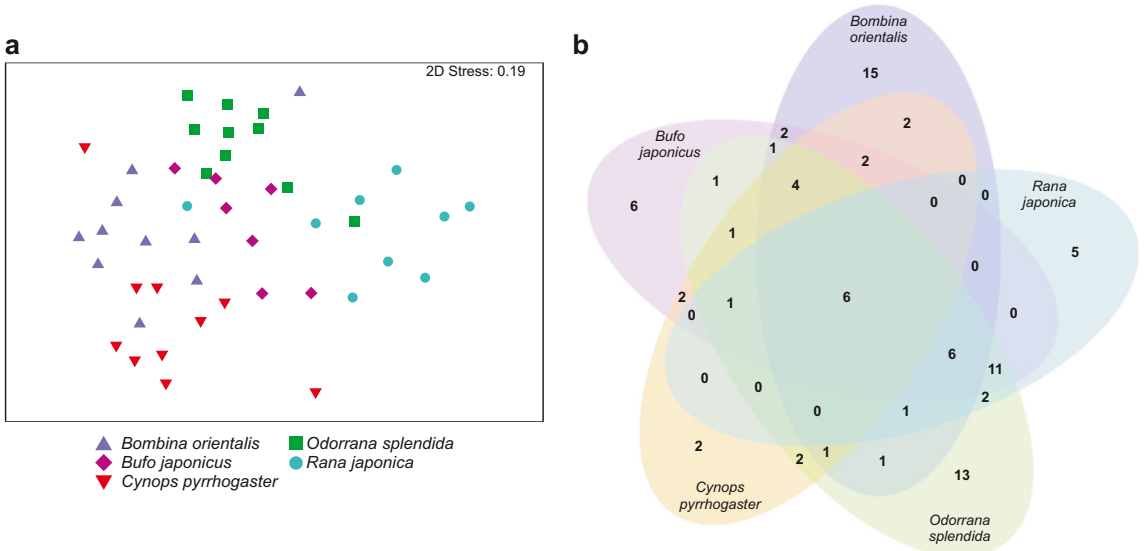

Fig. 3 **a** Plots from a two-dimensional nMDS analysis (unweighted UniFrac) representing the species-related differences in the composition of the skin bacterial communities. **b** Venn diagram showing number of 80 % core OTUs shared between the six analyzed species

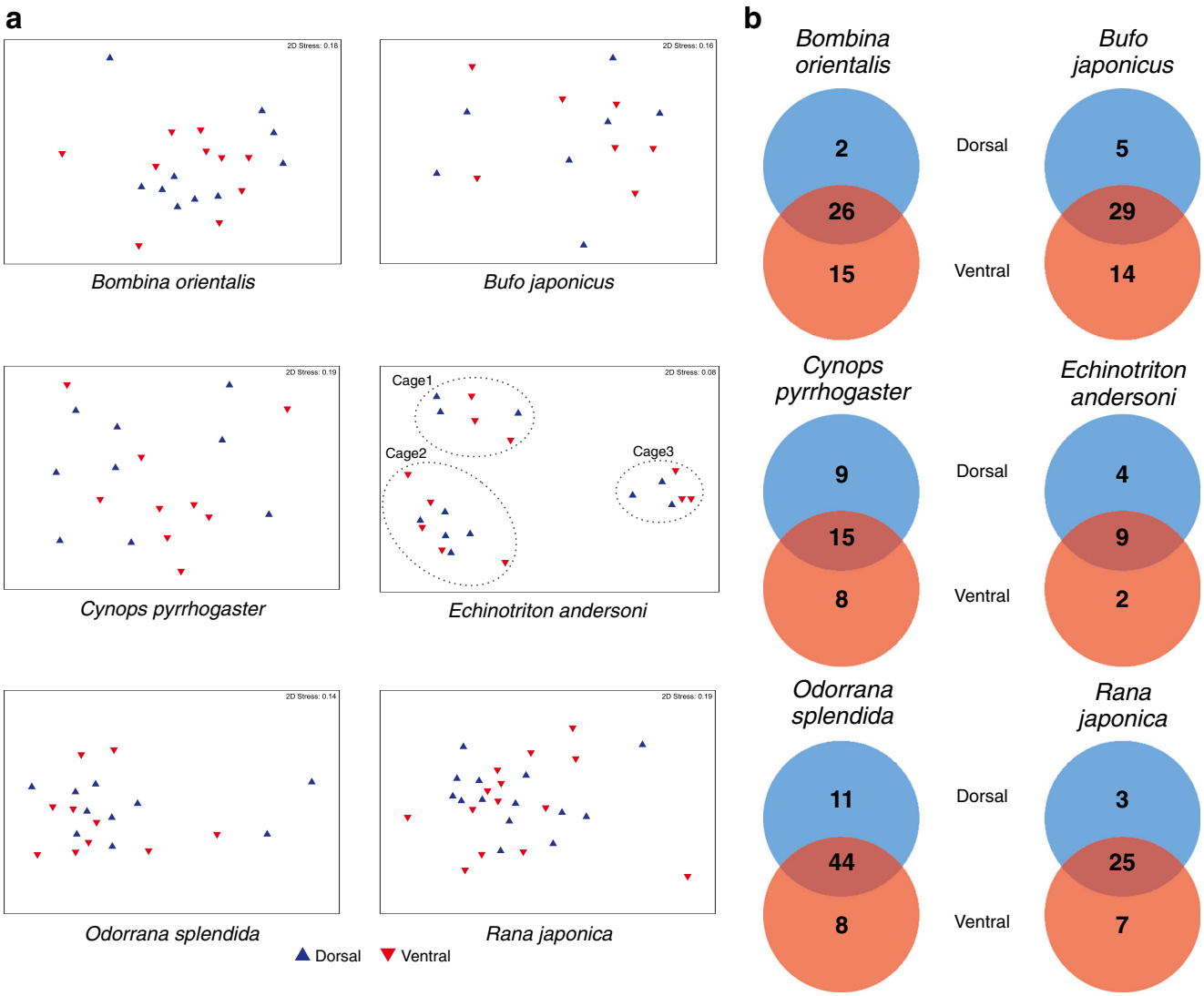

Fig. 4 **a** Plots from two-dimensional nMDS analyses (unweighted UniFrac) representing the body region-related differences in the composition of the skin bacterial communities. **b** Venn diagrams showing number of 80 % core OTUs shared between the dorsal and ventral body surfaces for the six analyzed species

captive *C. pyrrhogaster*. Some of the captive newts were direct descendants from the sampled wild population which is in close proximity (~300 m) to the breeding facility. Yet, the skin bacterial community between both groups was strongly divergent. We also found a decreased alpha diversity of cutaneous bacteria in captive newts, which agrees with previous studies

Table 3 Comparisons of the OTU richness, alpha diversity values (Faith's PD and Chao1) and structure (B-C: Bray-Curtis distance matrix; Un-Uni: unweighted UniFrac distance matrix) of the communities between dorsal and ventral surfaces analyzed with ANOVA

	OTU richness OTUs	Alpha diversity Faith's PD	Alpha diversity Chao1	Community structure B-C	Community structure Un-Uni
Bombina orientalis	*0.004*	*0.004*	*0.036*	0.063	0.347
Bufo japonicus	0.915	0.616	0.827	0.577	0.954
Cynops pyrrhogaster	0.626	0.851	0.176	0.127	0.291
Echinotriton andersoni	0.715	0.789	0.491	0.979	0.796
Odorrana splendida	0.777	0.545	0.649	0.510	0.999
Rana japonica	0.846	0.872	0.292	0.676	0.819

P values are represented. Significant P values are in italics

that consistently point towards a loss of alpha diversity in captivity [18, 19, 21, 24, 42]. This reduction in diversity may lead to a higher susceptibility of the hosts to diseases [12] and therefore needs to be considered for ex situ management of threatened amphibians, especially in projects that have as an objective of re-introducing individuals into the wild.

The breeding facility of the Institute of Amphibian Biology of Hiroshima University provides an exceptional setting for testing how the host factors influence skin-associated skin bacterial communities. Differences in cutaneous bacterial communities between species have regularly been found in syntopic wild amphibians (e.g., [17, 20]). However, in natural settings, confounding factors usually cannot be excluded, e.g., a treefrog and an aquatic frog might differ in skin microbiota either due to intrinsic host effects or due to their different microhabitat usage. In the Hiroshima breeding facility, most amphibians are kept under standardized conditions, i.e., in cages of the same size, same substrate, same water supply, same food, and largely at the same temperature. All specimens included in the present study had been kept under homogeneous conditions for several years. The skin bacterial communities of these individuals would be expected to be identical if the environmental factors were the sole driving forces acting on it, while species-specific communities would suggest that host factors are shaping the community composition. The identity and proportion of core genera were relatively constant between host species, suggesting the role of environmental factors. Chryseobacterium was the most abundant bacterial genus, except for the fire-bellied toad *B. orientalis* and the newt *C. pyrrhogaster* on which Pseudomonas and a taxon representing an unidentified Xanthomonadaceae genus were respectively most abundant. Still, we found significant differences in bacterial community structure between all five amphibian species compared (*B. orientalis*, *B. japonicus*, *C. pyrrhogaster*, *O. splendida*, and *R. japonica*). Despite some overlap in the nMDS plot, all individuals roughly clustered by species, reflecting the existence of intrinsic host effects in structuring the cutaneous microbial community. In the sixth species in our study, *E. andersoni*, specimens were kept under heterogeneous conditions, and it was therefore not included in the inter-species comparisons. In this species, individuals seem to cluster by tank (data not presented due to low sample size), reflecting once again the strong influence of environmental factors on the cutaneous microbiota [21].

Comparisons among bacterial communities of dorsal and ventral skin surfaces yielded no significant differences in five amphibian species (*B. japonicus*, *C. pyrrhogaster*, *E. andersoni*, *O. splendida*, and *R. japonica*) (Figs. 2 and 4, Table 3). However, in Oriental fire-bellied toads (*B. orientalis*), the ventral side had a higher species richness and phylogenetic diversity. This result is consistent with the analyses of Bataille et al. [24] in the same species, and could be related both to the different roughness of the dorsal and ventral sides, and to the toxin secretion (such as anti-microbial peptides) from the dorsal skin [43].

The composition and diversity of the bacterial communities of amphibians are determined by a multitude of factors, and disentangling these is a challenging task. Although amphibians at Hiroshima University are mainly kept for conservation of biological and genetic studies, the settings at this breeding facility allowed for an isolated testing of these factors, keeping other variables more constant than in natural settings. Simultaneous analyses of newts from the breeding center and their population of origin demonstrated how captive conditions changes and especially reduces the diversity of bacterial communities, probably due to overall reduced environmental complexity in the breeding tanks. Comparison among captive amphibians kept under highly similar conditions over a long time provided strong support for host-specific characteristics influencing cutaneous bacteria, and the differences between dorsal and ventral communities in only Oriental fire-bellied toads points to an effect of dorsal poison-secreting glands on the microbiota.

Less than a third of the 80 % core OTUs were shared between the groups in the wild-captive (shared OTUs = 32 %) and species (shared OTUs = 30 %) comparisons, while approximately two thirds where shared between body regions (shared OTUs = 62 %). Overall, as a testable hypothesis for future studies, we posit that external environmental factors (including those related to captive conditions) are as important as intrinsic host-related effects on influencing the composition and diversity of amphibian cutaneous microbiotas. Different parts of the amphibian body, on the contrary, usually bear similar cutaneous microbiotas, except in species where skin on different body parts produces secretions with distinct chemical properties.

Numerous other factors influencing amphibian-associated bacterial communities remain to be explored, such as temperature effects [44], the effect of pathogens on the community [40], and the emergence of opportunistic pathogenic bacteria in diseased and lesioned specimens. Most available studies, including the one presented here, focus on comparisons of the more abundant members of the bacterial communities, but future work on the rare OTUs is warranted as these can have fundamental functions for the host [45]. In general, the importance of the skin bacterial community to host health is well established [46]. An improved causal understanding of its composition and variation, and development of methods to maintain and manipulate it, can become

fundamental for conservation management of captive and wild amphibian populations.

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