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PRIMARY RESEARCH PAPER

Cutaneous microbiota of the Japanese giant salamander (*Andrias japonicus*), a representative of an ancient amphibian clade

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Abstract *Andrias japonicus*, the Japanese giant salamander, is the second largest amphibian species in the world. The biology of this long-lived, fully aquatic salamander is still incompletely known, and studying the threats it experiences is important for conservation management. We used 16S amplicon sequencing to provide the first data on the composition and diversity of the cutaneous microbiome of this species. Skin bacterial communities of adult and larval giant salamanders were composed primarily of taxa belonging to the phyla Proteobacteria and Bacteroidetes, and, their community structure differed significantly from that of two other syntopic amphibians (*Cynops pyrrhogaster* and *Glandirana rugosa*). We also found differences between wild *A. japonicus* and captive individuals, with the latter having an increased bacterial diversity. The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) was detected only in captive individuals (40% prevalence), and did not correlate with a particular bacterial community structure. We identified eight bacteria that were significantly more abundant on *A. japonicus* compared to syntopic amphibians, one of which was *Janthinobacterium lividum*, a bacterial species known to exert *Bd*-inhibiting effects. Our study provides baseline data for future in-depth studies on the microbial ecology of cutaneous bacteria and the contribution of cutaneous bacteria to *Bd* resistance in giant salamanders.

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Introduction

Giant salamanders (family Cryptobranchidae) are an ancient group of amphibians known from approximately 160 million years ago (Gao & Shubin, 2003). Phylogenetically, they are the sister group to the Asian salamander family Hynobiidae, and the cryptobranchid–hynobiid clade is the sister group of all other extant salamanders (Roelants et al., 2007). These ancient groups have retained ancestral traits such as external fertilization, and much of cryptobranchid osteology has remained conserved since the Jurassic (Gao & Shubin, 2003).

As with many ancient amphibian clades, cryptobranchids are species poor. The family only contains three extant species, the North American hellbender [*Cryptobranchus alleganiensis* (Sonnini de Manoncourt and Latreille, 1801)], the Chinese giant salamander [*Andrias davidianus* (Blanchard, 1871)], and the Japanese giant salamander [*A. japonicus* (Temminck, 1836)], which are all fully aquatic and inhabiting clear stream habitats (Browne et al., 2011, 2012, 2014).

The three species of giant salamanders are of global conservation concern: *C. alleganiensis* and *A. japonicus* are classified Near Threatened, and *A. davidianus* is Critically Endangered (Browne et al., 2011). Habitat loss, degradation, and fragmentation through dams threaten all three species. Additionally, *A. davidianus*, is threatened by hunting for luxury food markets and for stocking salamander farms (Browne et al., 2011; Tapley et al., 2015), and *A. japonicus* is threatened by hybridization with introduced *A. davidianus* (Matsui, 2009). In *C. alleganiensis*, population declines and changes of age structure have been detected but their causes are not always well understood (Wheeler et al., 2003; Foster et al., 2009; Sabatino & Routman, 2009; Burgmeier et al., 2011; Hiler et al., 2013).

Whether diseases are impacting giant salamander populations is poorly known. Ranavirus-associated mortality has been observed in farmed *A. davidianus* (Geng et al., 2011) and abnormalities, such as fungal infections, skin wounds, tumors, and parasites (internal and external), have been reported in hellbenders (Wheeler et al., 2002; Hiler et al., 2005; Moser et al., 2008; Huang et al., 2010). Furthermore, the fungus *Batrachochytrium dendrobatidis* (Longcore, Pessier & D.K Nichols 1999) (*Bd*) has been detected on all three cryptobranchid species (Briggler et al., 2007, 2008; Goka et al., 2009; Bodinof et al., 2011; Souza et al., 2012; Zhu et al., 2014). This pathogen can infect amphibians of all three extant orders, Anura, Caudata, and Gymnophiona (Berger et al., 1998; Fisher et al., 2009; Gower et al., 2013; Pasmans et al., 2013), is responsible for the emerging infectious disease, chytridiomycosis (Longcore et al., 1999), and has caused severe amphibian declines and extinctions (Berger et al., 1998; Daszak et al., 1999; Lips et al., 2006). However, thus far no *Bd*-associated mortality of giant salamanders has been confirmed.

Recent research has drawn attention to the intricate link between chytridiomycosis and the functional composition of the so-called mucosome, i.e., the complete skin mucosal ecosystem containing interdependent host and microbial community factors (Woodhams et al., 2014). The amphibian skin hosts diverse bacterial communities (McKenzie et al., 2012; Kueneman et al., 2014; Belden et al., 2015; Sabino-Pinto et al., 2016). Bacteria found on amphibian skin provide important functions for the host. In particular, some cutaneous bacteria regularly found on the skin produce metabolites that inhibit *Bd* growth (Becker et al., 2009) and thereby mediate protection against chytridiomycosis (Harris et al., 2009; Becker & Harris, 2010; Bletz et al., 2013). Characterizing these bacterial communities has recently become possible through high-throughput sequencing techniques; however, almost no information of the bacterial communities associated with giant salamanders is currently available, with the exception of Nickerson et al. (2011) who provided a culture-based assessment of the microbiota of injured hellbenders, and Hernández-Gómez et al. (2016) who compared the microbiota of two hellbender subspecies as well as the microbiota of healthy and wounded skin.

Andrias japonicus, growing up to 1.5 m in length, is the second largest living amphibians in the world (Browne et al., 2012), and is distributed in western Honshu, Shikoku, and Kyushu islands, Japan (Matsui & Hayashi, 1992). It dwells mainly in mountain streams and rivers, but is sometimes also found more downstream (Tochimoto et al., 2007; Taguchi, 2009a).

The species is protected in Japan as cultural property ("National natural monument"), and numerous local conservation activities are occurring; however, baseline data for effective conservation are just emerging (Okada et al., 2008). Here, we provide the first survey of the cutaneous bacterial communities of this species, through amplicon-based sequencing of the bacterial 16S rRNA gene. We characterize and describe the cutaneous microbiota of the giant salamander, and investigate whether giant salamanders host unique commensal bacterial communities compared to two co-occurring amphibians, given their long independent evolutionary history and their distinct, purely aquatic life history. Furthermore, we analyze whether the cutaneous microbiota of *A. japonicus* differ among life stages and between *Bd*-positive and *Bd*-negative individuals. Our data bear relevance for conservation by identifying bacterial taxa naturally occurring on this species that could provide protection against chytridiomycosis, and by highlighting differences between captive and wild specimens that should be taken into account for captive breeding and reintroduction programs.

Materials and methods

Sampling procedure and sampling sites

Sampling was carried out in February 2015 at different sites in the Kyoto and Hiroshima Prefectures, Japan. Non-invasive swabs of the skin surface were taken. First, the sampling surface of the amphibian was rinsed with 50 ml of sterile water to remove transient bacteria. Subsequently the cleaned surface was rubbed (ten strokes) with a synthetic cotton swab (MW113; Medical Wire & Equipment, Corsham, UK). Clean nitrile gloves were worn to hold and swab each individual. Swabs were individually stored in sterile 1.5 ml centrifuge tubes and stored at −20°C until DNA extraction. Samples were taken separately from the dorsal and ventral surfaces of all adult and juvenile specimens to allow for comparison of these different body surfaces; however, most analyses herein focus on the ventral swabs only. The larvae sampled from the wild were too small to accurately sample body parts separately and were therefore swabbed across the entire body (dorsal plus ventral surface). Details of sampled individuals are as follows:

(1) Wild *Andrias japonicus*, their larvae, and other syntopic amphibians (*Cynops pyrrhogaster* (Boie, 1826) and *Glandirana rugosa* [Temminck and Schlegel, 1838)] and fish were sampled in a system of connected streams in Hiroshima Prefecture. Nine *A. japonicus* larvae (all first-year larvae of entirely black color and a small body size of ca. 4–6 cm total length), two *C. pyrrhogaster*, three *G. rugosa*, and one cyprinid fish [*Nipponocypris temminckii* (Temminck and Schlegel, 1846)] were collected at Misasa river (N34°35.701′/E132°47.683′). Three adults of *A. japonicus* were sampled at Mukunashi river (N34°35.961′/E132°48.937; N34°36.204′/E132°48.803′). Hiroshima Prefecture is located in the central part of the *A. japonicus* distribution. The species here inhabits upper and middle river sectors in the northern part of this prefecture. The sampling sites were in two small streams (1–5 m width and 0.2–1 m depth) where natural breeding nests of giant salamanders have been observed. One water sample was taken from the Misasa river to characterize the bacteria community of the aquatic environment from which wild *Andrias* were sampled.

(2) Captive *A. japonicus* were sampled at the conservation breeding center of the Japanese giant salamander in Hiroshima City Asa Zoological Park, including five adults, four 1-year-old larvae, four 2-year-old larvae, two 7-year-old subadults, and one 10-year-old subadult. In this center, the species has been bred almost every year since 1979 and there are hundreds of captive individuals, from hatchlings to over 30 years old (Kobara et al., 1980; Kuwabara et al., 1989). With attention to local genetic endemism, breeding has been conducted only using wild specimens from the Ota-gawa Riverine System in Hiroshima, and captive-bred ones with the same genetic background. The center has about 50 separate rearing water tanks with groundwater supply and three breeding water tanks supplied from a nearby stream and with groundwater, all located in an outdoor environment. All individuals were sampled from separate tanks and did not share any water in order to avoid pseudo-replication.

(3) Two adult hybrids (*A. japonicus* × introduced *A. davidianus*) in Kamo River, Kyoto Prefecture,

on 23 February 2015. One (total length: 780 mm, body weight: 3,500 g) was identified as F1 hybrid, and the second (total length: 825 mm, body weight: 3,700×*g*) was identified as F2 by genotyping with 15 microsatellite loci (Yoshikawa et al., 2011, 2012).

DNA extraction, PCR and sequencing

DNA was extracted from swabs with MoBio Power Soil htp-96 extraction kit (MoBio Laboratories, Carls, CA, USA) with the minor adjustments outlined in Kueneman et al. (2014) and doubled centrifugation time to account for the available rotor speed. The V4 region (Brosius et al., 1981) of the bacterial 16S rRNA gene was amplified and sequenced using the dual-index approach of Kozich et al. (2013).

All PCRs were performed in duplicate to account for possible PCR failure, which can occur when running single PCRs. PCRs for all samples included in this study were done with identical protocols and reagents. Individual reactions (12.5 µl) were composed of 0.15 µl of Phusion Hot Start II DNA Polymerase (Thermo Scientific), 0.25 µl of each forward (515F) and reverse primer (806R) (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 of 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, PCR products were combined (25 µl per sample), and visualized on 1% agarose gels.

Samples were pooled in approximately equal concentrations for sequencing, and gel purified with the QIAquick Gel Extraction Kit (Qiagen, Germany). The concentration of the purified pool was determined with the Broad Range dsDNA kit on a Qubit 2.0 and was submitted for Illumina Miseq sequencing using paired-end 2 × 250 v2 chemistry at the Helmholtz Center for Infection Research (*Glandirana*, *Cynops* and fish) or 2 × 300 v3 chemistry at the Analysis Center of Life Science, Natural Science Center for Basic Research and Development (ACOLS), Hiroshima University (all *Andrias* samples). The sequences of the amplicon libraries are deposited in the NCBI short read database (Bioproject PRJNA368738).

Sequence analysis

Unless otherwise noted, all sequence processing was conducted using Quantitative Insights Into Microbial Ecology (MacQIIME v1.9.1, Caporaso et al., 2011). Forward reads from each sample were quality filtered to remove low-quality sequences with the following criteria (QIIME defaults): any ambiguous base calls, less than 75% of read had consecutive base call with a quality score greater than 3, and more than three consecutive low-quality base calls. We used only forward reads as reverse reads typically suffer from lower quality (Kwon et al., 2013). Quality-filtered reads were trimmed to 250 base pairs on the usegalaxy.org platform to equalize the read lengths between the two different Miseq runs. Chimeras were identified on a per sample basis using usearch61 de novo-based detection within QIIME (Edgar et al., 2011), and subsequently removed from the quality-filtered fasta file. After all sequence filtering, 7,137,449 reads remained for analysis (53,571.65 ± 50,985.37 SD/samples; range: 2,094–289,838). The board range in sequencing depth resulted from combining two different illumina runs. These runs contained different total numbers of samples, leading to differences in coverage per sample. Sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using an open reference OTU-picking strategy (Rideout et al., 2014, http://qiime.org/tutorials/open_reference_illumina_processing.html). The GreenGenes 13_8 release (May 2013) was used as the reference database for initial reference-based OTU matching, and the UCLUST (Edgar, 2010) algorithm was used for de novo clustering steps. The most abundant sequence from each OTU was selected as a representative sequence and these representative sequences were aligned using PyNAST (Caporaso et al., 2010). Taxonomy was assigned using the RDP classifier (Wang et al., 2007) with the GG 13_8 taxonomy as the reference database. Lastly, a phylogenetic tree was built using FastTree (Price et al., 2010). To normalize the number of reads across samples for our main analyses, all samples were rarefied to 2,000 sequence reads per sample (Supplementary Fig. 1) (post filtering). Samples with fewer than 2,000 reads were removed.

Richness (number of OTUs and Chao1) and diversity (Faith's Phylogenetic Diversity and Shannon Diversity) were calculated for each sample. ANOVA

and Tukey's HSD tests were used to statistically compare alpha diversity values when greater than 3 samples per category were available.

Beta diversity among the samples was calculated for each comparison group using the Bray–Curtis metric and Weighted Unifrac metric within QIIME. These distance matrices were visualized by non-metric multidimensional scaling (NMDS) and analyzed with Permutational Multivariate Analysis of Variance (PERMANOVA) in Primer 7. Sample categories with less than 3 samples were excluded for analysis. If main effects were significant, subsequent pairwise PERMANOVAs were completed. Heterogeneity within sampled groups with respect to multivariate dispersions can influence interpretations of PERMANOVA results; therefore, we performed PERMDISP analyses to test for homogeneity of dispersions.To identify bacteria that were differentially abundant between sampling groups, we carried out Linear Discriminant Analysis Effect Size (LEfSe) analysis on the Galaxy web-based interface (http://huttenhower.sph.harvard.edu/galaxy/) using default parameters (LDA score > 2) except the alpha value for the factorial Kruskal–Wallis test among classes was set to be more stringent (alpha $= 0.01$). Rare OTUs (less than 5 reads across all samples) were removed prior to LEfSe analysis. The LefSe analysis was performed on the samples of wild amphibians from Hiroshima Prefecture with species as the class and life stage as the subclass to answer the question, which OTUs, if any, were differentially abundant between host species, consistently across life stages. BLAST searches using NCBI nucleotide BLAST web-based tool were performed on OTUs that were found to be differentially abundant in order to determine where else, if anywhere, they occur. An additional LEfSe analysis was completed to determine whether any OTUs were differentially associated with larval versus adult *Andrias*.

Functional predictions of selected OTUs were made using a bioinformatics approach with the published database of anti-fungal amphibian skin bacterial isolates (Woodhams et al., 2015). First, the 1,944 isolates within the published database trimmed to 250 bps, matching the region sequenced with Illumina and then were clustered into OTUs at 99% similarity (hereafter Woodhams-99 OTUs). These OTUs were categorized as "potentially inhibitory" if at least one of the isolates with the cluster was inhibitory. Next, the OTU sequences from the LEfSe-identified OTUs were clustered to the Woodhams-99 OTUs, allowing preliminary prediction of their anti-fungal potential.

Pathogen testing

We tested for the presence of *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*) in the sampled individuals, using quantitative real-time polymerase chain reaction (qPCR). qPCR reactions were performed according to Blooi et al. (2013). When available, we tested both dorsal and ventral swabs for each specimen, leading to a total of 81 swabs analyzed.

Results

General skin bacterial composition and structure of *Andrias japonicus*

Focusing on the samples of *Andrias japonicus* from Hiroshima Prefecture, the skin bacterial communities of adults were predominantly composed of Proteobacteria (69%), Bacteroidetes (14%), with minor proportions of Actinobacteria (7%), Firmicutes (5%), and Verrucomicrobia (2%) (other bacterial phyla represented by <1% of the reads). *A. japonicus* larvae had the same groups represented in their skin communities, but had a slightly reduced proportion of Proteobacteria (48%), Firmicutes (2%), and Actinobacteria (2%), and increased proportions of Bacteroidetes (34%) and Verrucomicrobia (9%). PCo analysis of all collected samples is presented in Supplementary Fig. 2.

Microbiota of *Andrias japonicus* in comparison to two co-occurring species

Species richness measured as the number of OTUs (OTU richness) was significantly larger in adults than larvae ($t = 3.232$, $P = 0.0093$); however, the Chao1 index was similar between adult and larval *A. japonicus* ($t = -0.095$, $P = 0.92$). Shannon diversity differed between adult and larval individuals ($t = 2.971$, $P = 0.020$), while phylogenetic diversity was not significantly different ($t = 1.662$, $P = 0.13$). Richness and diversity also differed among species

(Shannon: $F_{2,14} = 7.954$, $P = 0.004$; Faith's PD: $F_{2,14} = 4.091$, $P = 0.039$; Chao1: $F_{2,14} = 7.532$, $P = 0.006$; OTU Richness: $F_{2,14} = 7.032$, $P = 0.007$). In general, in comparison to *A. japonicus*, the cutaneous bacterial communities of Japanese fire-bellied newts, *Cynops pyrrhogaster*, were lower in richness and diversity (Tukey HSD, $P = 0.039$) while those of Japanese wrinkled frogs, *Glandirana rugosa*, exhibited slightly higher richness and diversity (Tukey HSD, $P = 0.076$) (Table 1; Supplementary Table 4 provides richness and diversity values based on a rarefaction depth of 10,000).

Distinct differences in community structure were detected among amphibian species and *A. japonicus* life stages sampled from Hiroshima streams (Fig. 1), despite overall small sample sizes (PERMANOVA: Bray Curtis: Pseudo-$F = 5.2778$, $P = 0.001$, Weighted Unifrac: Pseudo-$F = 5.3$, $P = 0.001$). Multivariate sample dispersion was not significantly different between groups (PERMDISP: $F_{2,12} = 4.9$, $P = 0.1$). In pairwise comparisons, significant differences were observed between larvae and adults of *A. japonicus* ($t = 2.2938$, $P = 0.005$), and between *A. japonicus* larvae and *G. rugosa* (Bray Curtis, $t = 2.3903$, $P = 0.005$). Adult *G. rugosa* and *A. japonicus* did not differ significantly (Bray Curtis, $t = 2.0938$, $P = 0.094$), but they did separate distinctly on the nMDS plot. Comparisons were not done between *C. pyrrhogaster* and other groups due to the sample size being less than three; however, the samples did separate on the nMDS plot. In addition, dorsal and ventral bacterial communities did not differ for the three wild adult individuals from Hiroshima (Pseudo-$F = 1.753$, $P = 0.115$) (Supplementary Fig. 3).

To identify bacterial OTUs specifically occurring on giant salamanders, a LEfSe analysis was carried out. Eight OTUs were found to be differentially more abundant on *A. japonicus* consistently across adult and larval individuals in comparison to the two other amphibian species (Table S1). These OTUs included, *Stenotrophomonas acidaminiphila*, *Janthinobacterium lividum*, *Sphingobacterium multivorum*, and one unidentified species each of the following genera *Arthrobacter*, *Chryseobacterium*, *Staphylococcus*, and each of the following families Pseudomonadaceae and Enterobacteriaceae. Most of these were rather rare, making up less than 1% of the community, except for *Sphingobacterium multivorum* which made up on average 2% of the reads in *A. japonicus* individuals. All of these bacterial OTUs were also found in comparable proportions in *A. japonicus* specimens from the Asa Zoo breeding center, and from Kyoto. They were not exclusive of *A. japonicus*; all of these OTUs were found in at least one non-*Andrias* sample, albeit often in very low quantities (1–2 reads). Additionally, these OTUs were mapped to the amphibian skin microbe database (Woodhams et al., 2015), and four of the eight OTUs, including the *S. multivorum* OTU, where identified as potentially inhibitory OTUs (Supplementary Table 1).

Table 1 Comparison of diversity indices of cutaneous bacterial communities of giant salamanders and the two other amphibians

Sample category	Sample size	Chao1 index	OTU richness	Faith's PD	Shannon index
A. japonicus larvae (Hiroshima)	9	570.0 ± 146.9	284.2 ± 69.7	28.08 ± 7.22	5.84 ± 0.69
A. japonicus adults (Hiroshima)	3	564.4 ± 57.6	364.7 ± 15.5	33.19 ± 3.33	6.77 ± 0.36
Cynops pyrrhogaster (Hiroshima)	2	375.5 ± 124.3	225.0 ± 52.3	23.57 ± 0.48	4.46 ± 0.69
Glandirana rugosa (Hiroshima)	3	876.8 ± 248.5	471.7 ± 127.8	39.63 ± 7.76	7.25 ± 0.93
A. japonicus × *A. davidianus* (Kyoto)	2	442.9 ± 139.2	244.5 ±92.6	21.03 ± 8.75	4.83 ± 1.77
A. japonicus larvae 1–2 years (captive)	8	865.5 ± 136.3	365.3 ± 36.7	35.76 ± 4.53	5.83 ± 0.31
A. japonicus subadults 7–10 years (captive)	4	981.3 ± 130.8	416.3 ± 50.5	41.46 ± 5.16	6.35 ± 0.52
A. japonicus adults (captive)	5	1,207.7 ± 145.9	459.0 ± 23.6	44.79 ± 3.72	6.63 ± 0.14
Wild Stream Water	1	544.91	346	27.48	6.70
Captive Tank Water	12	840.63 ± 114.85	366.41 ± 29.95	35.75 ± 2.42	5.88 ± 0.29

Only ventral swabs considered. Values are mean ± SD

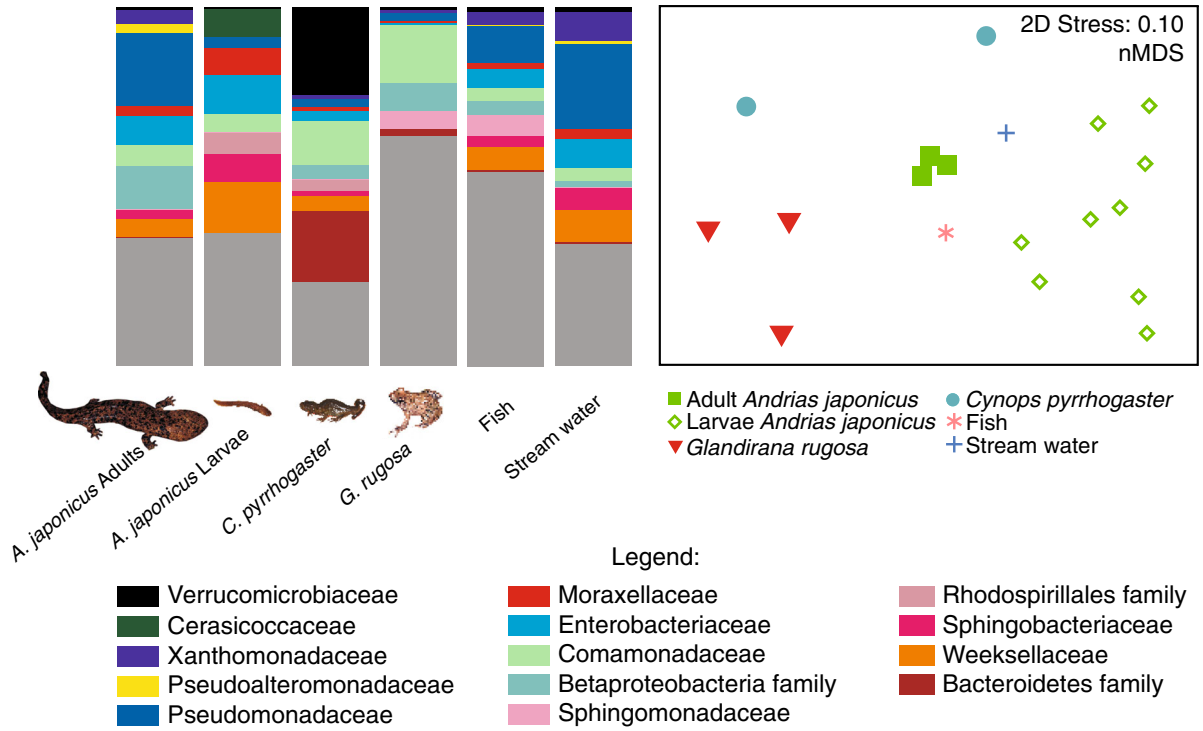

Fig. 1 Composition and structure of ventral skin bacterial communities on Japanese giant salamanders (*Andrias japonicus*) from a river system near Hiroshima, Japan. Taxonomic summary plots are at the family level. Most abundant bacterial families are depicted in color, with the *gray bar* representing all other taxa. The ordination is derived from a two-dimensional nMDS analysis (Bray–Curtis distance) depicting the host-related differences in the structure of the skin bacterial communities (ventral swabs only). For comparison, data for two syntopic amphibian species, one cyprinid fish (*Nipponocypris temminckii*), and from pond water are also shown. Taxonomic summary plots with full coloring of all taxa are presented in Supplementary Fig. 5

Comparison of skin microbiota of wild and captive individuals

The cutaneous microbial communities of giant salamanders from Hiroshima (wild *A. japonicus*), Asa Zoo (captive *A. japonicus*), and Kyoto (wild hybrids) clustered distinctly (Fig. 2), and community structure of captive and wild individuals from Hiroshima was significantly different (PERMANOVA: BC: Pseudo-$F = 8.3036$, $P = 0.005$, WUF: Pseudo-$F = 13.07$, $P = 0.001$; Kyoto samples not considered due to low sample size). Multivariate sample dispersion was not significantly different between groups (PERMDISP: $F_{2,16} = 0.153$, $P = 0.85$). Richness and phylogenetic diversity was distinctly higher in captive individuals (Table 1) (Faith's PD: $t = -4.5612$, $P = 0.007$; Chao1: $t = -8.7848$, $P = 0.0001$; OTU Richness: $t = -6.8089$, $P = 0.0005$), while Shannon diversity was not statistically different between wild and captive giant salamanders ($t = 0.628$, $P = 0.585$).

Comparison of skin microbiota across age classes

Our sampling of captive individuals at the Asa Zoo breeding center was comprised of individuals from different age categories. We excluded subadults of 7 and 10 years from analysis due to low sample sizes, and found that the ventral and dorsal skin bacterial community structure varied significantly between the remaining age categories of 1 year, 2 years, and adults (Vent PERMANOVA: Bray Curtis, Pseudo-$F = 1.9086$, $P = 0.039$ Weighted Unifrac: Pseudo-$F = 1.291$, $P = 0.256$, Dors PERMANOVA: Bray Curtis Pseudo-$F = 3.8515$, $P = 0.006$ Weighted Unifrac: Pseudo-$F = 5.7826$, $P = 0.014$, Fig. 3). For ventral communities, this was driven by the bacterial

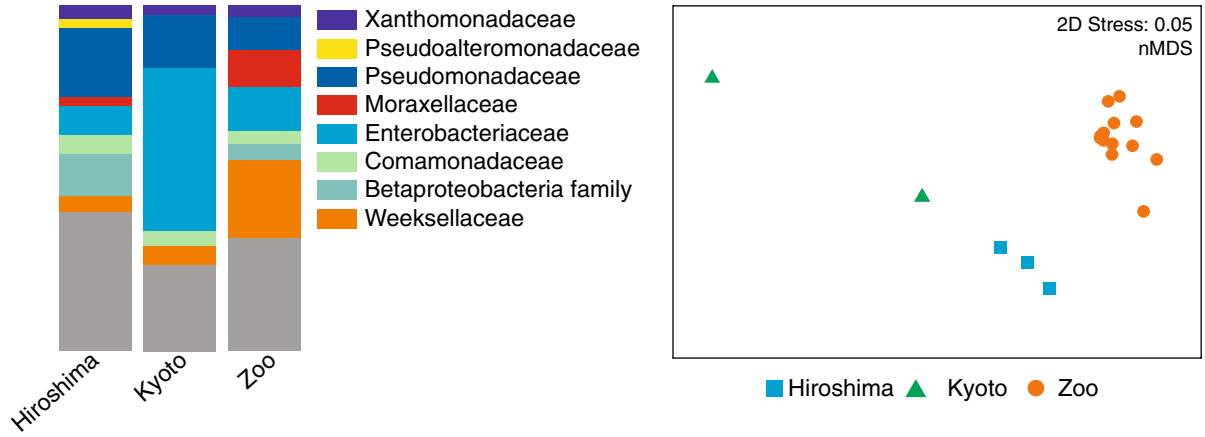

Fig. 2 Composition and structure of ventral skin bacterial communities on giant salamanders from different provenances: streams in Hiroshima prefecture (adult *Andrias japonicus*), a stream in Kyoto Prefecture (adult *A. japonicus x A. davidianus* hybrids), and the captive breeding center at Asa Zoo, Hiroshima (adults and juveniles of *A. japonicus*). Taxonomic summary plots are at the family level. Major taxa in the barplots are depicted in color, with the *gray bar* representing all other taxa. The ordination is derived from a two-dimensional nMDS analysis (Bray–Curtis distance) depicting the locational differences in the structure of the skin bacterial communities (ventral swabs only). Taxonomic summary plots with full coloring of all taxa are presented in Supplementary Fig. 5

communities of adults differing from those of 1-year juveniles ($t = 1.7813$, $P = 0.024$). Adults and 2-year juveniles ($t = 1.3751$, $P = 0.092$), and 1- and 2-year juveniles ($t = 0.9036$, $P = 0.968$) were not significantly different in pairwise comparisons. For dorsal communities, those of adults differed from those of 1-year juveniles ($t = 1.9933$, $P = 0.039$) and 2-year juveniles ($t = 2.2624$, $P = 0.022$). Dorsal communities of 1- and 2-year juveniles were not significantly different ($t = 0.98981$, $P = 0.426$). Multivariate sample dispersion was not significantly different between age groups with respect to the ventral samples (PERMDISP: $F_{2,10} = 1.9284$, $P = 0.444$), but was for the dorsal samples ((PERMDISP: $F_{2,10} = 10.093$, $P = 0.022$). We also found that dorsal and ventral bacterial communities of

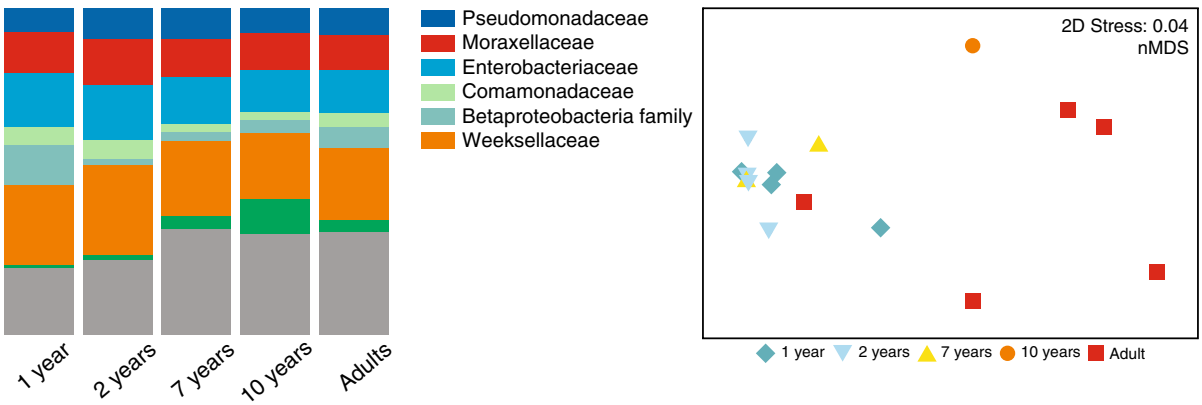

Fig. 3 Skin bacterial communities on Japanese giant salamanders (*Andrias japonicus*) from Asa Zoo, Hiroshima, across different age categories. Taxa plot presents community composition of the ventral skin surface for each age class at the family level. Major taxa are depicted in color, with the *gray bar* representing all other taxa. The ordination is derived from a two-dimensional nMDS analysis (Bray–Curtis distance) representing the age-associated differences in skin bacterial community structure for dorsal skin surfaces. Taxonomic summary plots with full *coloring* of all taxa are presented in Supplementary Fig. 5

breeding center individuals differed significantly (Pseudo-$F = 3.1361$, $P = 0.02$) (Supplementary Fig. 3).

Bd and *Bsal* screening and effects of *Bd* on bacterial community structure

No individuals were found positive for *Bsal*. For *Bd*, all samples analyzed from the wild (five larvae and three adults from Hiroshima Prefecture) tested negative, whereas 10 out 24 individuals from Asa Zoo breeding center tested positive. With only one exception (load: 989.2 zoospores per swab), loads were consistently low, ranging from 1.0 to 45.1 zoospores per swab, with an average of 10.77±14.23(SD). Bacterial community structure of *Bd*-positive and *Bd*-negative captive *A. japonicus* from the Asa Zoo breeding center was not significantly different for ventral or dorsal swabs (Vent PERMANOVA: Bray Curtis, Pseudo-$F = 1.2212$, $P = 0.211$, Weighted Unifrac, Pseudo-$F = 2.1914$, $P = 0.03$ (Supplementary Fig. 4) and Dors PERMANOVA: Pseudo-$F = 1.4125$, $P = 0.184$, Pseudo-$F = 1.6641$, $P = 0.153$).

Discussion

Our study provides the first exploration of the cutaneous microbiota of a unique amphibian, the Japanese giant salamander. This species is divergent in multiple traits from other amphibians whose skin microbiota have been examined (e.g., McKenzie et al., 2012; Kueneman et al., 2014, 2015; Bataille et al., 2015; Vences et al., 2015; Sabino-Pinto et al., 2016). Giant salamanders are fully aquatic, very long-lived and large-sized, with complex parental care, and have a long independent evolutionary history. We found the general composition of their cutaneous microbiota to be similar to that of other studied amphibians, with a predominance of Proteobacteria, Bacteroidetes, and Actinobacteria. Nevertheless, this microbiota showed a well-expressed host specificity and beta diversity differed between *Andrias* and the two other sympatric amphibian species. Giant salamanders may possess a unique mucus composition or a particular suite of antimicrobial peptides (Li et al., 2015) in comparison to other species, and these compounds could act as a structuring force on the resident microbial community (Rollins-Smith, 2009; Fraune et al., 2010). However, thus far antimicrobial peptides have only been indirectly detected via RNA-seq in *A. davidianus*, and the expression of these compounds on the skin surface has not been investigated.

We also identified a number of bacteria that were differentially abundant on giant salamander skin compared to the other two amphibian species. While the relative abundance of these OTUs was relatively low, they were common and more abundant on the skin of both larvae and adults from the Hiroshima Prefecture, and most were also present in giant salamanders from Asa Zoo and Kyoto. The existence of such OTUs present on larvae and adults is remarkable considering the extreme differences in size and general appearance among the life stages of giant salamanders (all of the first-year larvae of *A. japonicus* sampled from the wild measuring 4–6 cm total length, with totally smooth and blackish skin, versus adults of body sizes >100 cm and a highly wrinkled skin). While the function of the rare biosphere is quite unknown, rare taxa can at times been functionally important and keystone members of the community (Gobet et al., 2011; Sogin et al., 2011; Pedrós-Alió, 2012; Shade et al., 2014). The affinity of these bacteria for giant salamanders and their absence/lower abundance on the other two amphibians likely suggests host filtering, i.e., the mucosal environment of the giant salamander provides an environment more suitable for these taxa. BLAST searches suggest that these bacteria (if correctly identified by their 16S sequences) are also found in soil and water; therefore, they may colonize the mucosal environment from external sources rather than being vertically transmitted symbionts between parent and offspring. However, we cannot differentiate between these alternative hypotheses. While adult and wild *Andrias* shared some OTUs, overall their bacterial communities differed in beta diversity, which is likely a result of drastic differences in the mucosal environment on these different life-history stages. For example, immune system characteristics are not fully developed in larval amphibians in comparison to adults, which could alter the microbes' ability to colonize the skin environment (Holden et al., 2015). The observed differences in beta diversity between age classes could also be a result of differences in immune system maturation.

Previous studies have demonstrated the influence of captivity on host microbiota, including those on the amphibian skin (e.g., Antwis et al., 2014; Becker et al., 2014; Kohl et al., 2014; Loudon et al., 2014; Bataille

et al., 2015; Sabino-Pinto et al., 2016). Our results mirror other studies in finding that captive and wild individuals differ in their microbial community structure. Such a result makes sense in the context of the different environmental and ecological conditions that would characterize wild and captive settings. In many published cases, captivity led to a reduction in microbiota diversity and richness (Bataille et al., 2015), which stands in contrast to our finding of an increased richness in specimens from the Asa Zoo breeding center and similar Shannon diversity values between wild and captive samples. A similar pattern, however, was documented in *Atelopus zeteki* (Dunn, 1933) (Becker et al., 2014). This increased richness in captivity might be explained by the special setup of the breeding center that mimics natural conditions with a constant flow of groundwater and stream water and thereby might lack several of the artificial conditions in indoor aquaria. The alpha diversity of the water from salamander tanks also appeared greater than that of the water from the natural stream (data not shown); however, with only one sample from the wild stream habitat, any overall conclusions are limited. Additionally, in captivity, it is possible that the host skin secretions are lacking specific factors that may control the microbial communities and therefore without these selective factors more microbes are able to colonize. Such difference in host skin secretions could be related to dietary differences of captive individuals. Larvae in the breeding center are fed with frozen bloodworms and frozen shrimps, whereas adults and juveniles are fed with frozen shrimps, frozen fishes (horse mackerels, cods, Atka mackerels, and others), and live fishes (loaches), which do not fully mimic their natural diet. If host defenses are reduced the amphibian may be more at risk to disease, which should be taken into consideration when reintroduction is an intended outcome. On the other hand, if microbial diversity is greater, there is the potential for increased microbial defenses against possible pathogens.

Japanese giant salamanders are threatened by habitat loss and fragmentation, degradation with concrete stream banks, small artificial dams inhibiting upstream migration, and decline of prey resources (Wakabayashi et al., 1976; Kuwabara et al., 1980; Okada et al., 2008; Taguchi, 2009a, b; Taguchi & Natuhara, 2009). Furthermore, the introduction of *A. davidianus* into Japan in the 1970s (Ikoma, 1973) caused hybridization between the native *A. japonicus*, and even back-crosses between the hybrids and native and introduced species. In the Kamo River in the Kyoto Prefecture, the Kinki area, 57% of metamorphs and 71% of larvae of the giant salamanders collected were confirmed as Chinese giant salamanders or hybrids (Matsui, 2009). This introduction is of concern because of its direct effects in replacement of the native species, but could also be the source of new pathogens.

However, so far no disease-related declines of *Andrias* populations have been recorded from Japan. This is particularly relevant given the presence of *Bd* in this species, as reported herein and in previous studies (Goka et al., 2009). A commensal association appears to have been formed between specific haplotypes of the fungus and *A. japonicus* (Goka et al., 2009). Our qPCR analysis method was not able to discern among different *Bd* strains and therefore, it remains untested which *Bd* strain is present on the Japanese giant salamanders sampled for this study. Obtaining *Bd* isolates from *Andrias* will be important in order to understand what lineage of *Bd* [*BdGPL*, *BdCape*, *BdCH* (Farrer et al., 2011), *BdBrasil* (Schloegel et al., 2012), and *BdAsia* (Bataille et al., 2013)] is present, its virulence and the possible threats it imposes. *Bd*-positive individuals were only found in the captive individuals. While only a small number of individuals were sampled in the field, it is of relevance that these were all uninfected. This apparent difference in *Bd* infection status could be associated with sample size, but it is also possible that captive individuals become more susceptibility to infection as a result of stress (Peterson et al., 2013), altered microbiota (Becker & Harris, 2010; Loudon et al., 2014), or reduced host immune defenses (Venesky et al., 2012; Woodhams et al., 2014). Furthermore, Jani & Briggs (2014) found that *Bd* can alter microbial composition. Our data did not follow this pattern as they did not show any differences between *Bd*-positive and negative individuals; however, infection intensities were rather low in the positive individuals, which could explain the lack of community differences. Additionally, we do not know the prior *Bd*-history of the "negative" individuals; therefore, we cannot know what (if any) influence *Bd* could have posed on these microbial communities previously. It remains to be tested whether any of the bacteria found on the skin of *A. japonicus* might confer resistance against chytridiomycosis. Our predictive analyses using the

published amphibian anti-fungal bacteria database (Woodhams et al., 2015), showed that four of the *Andrias*-specific OTUs matched with inhibitory OTUs within the database, which provides preliminary evidence that cutaneous microbiota on *Andrias* may have anti-fungal properties. In this context it is worth mentioning that one of these eight bacterial OTUs—*Janthinobacterium lividum*—has previously been found to reduce *Bd* infection by production of the metabolite violacein (Becker et al., 2009; Harris et al., 2009). In fact, the 16S sequence of the *J. lividum* of Japanese giant salamanders fully matches that found on the skin of North American salamanders (Table S2).

More recently, a new species of chytrid fungus, *Bsal* (Martel A., Blooi M., Bossuyt, F. Pasmans F., 2013), has been described, and is responsible for declining salamander populations in Europe (Martel et al., 2013). Several Asian salamanders have the ability to clear *Bsal* infection, and it has been hypothesized that the origin of this pathogen is Asia (Martel et al., 2014). We found no indication of *Bsal* infection in any of the tested amphibians, which could either be related to the absence of this pathogen from the sites sampled or to pathogen resistance in *A. japonicus*.

The data presented herein on the cutaneous bacterial communities of giant salamanders corroborated several effects with statistical significance, such as host effects as well as the effects of captivity. We acknowledge that sample sizes in our study are comparatively low which is associated with difficulties in locating and catching adult giant salamanders (as in other recent studies; e.g., Hernández-Gómez et al., 2016), and concerns of disturbing multiple individuals and habitat of these threatened animals by intensive collecting activity. However, our sampling design for testing host effects largely excluded confounding factors such as season and location because all specimens were found in the same day and in the same river system. Furthermore, in the captive sampling, we took great care to avoid pseudo-replication by only sampling a single individual per tank. We are therefore convinced that the results presented herein represent true biological patterns and a solid basis for future more in-depth studies on the composition, dynamics, and function of the microbiota associated with this fascinating salamander.

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