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Gut and faecal microbial diversity in a Japanese lotic-breeding salamander, *Hynobius boulengeri* (Thompson, 1912)

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Gut microbiota can be influenced by several factors including host ecology and environmental conditions (Hong et al., 2011; Cornejo-Granados et al., 2017; Kohl et al., 2017; Alberdi et al., 2018; Hicks et al., 2018; Schmidt et al., 2019; Moeller et al., 2020). Previous studies on vertebrate gut microbiota focused mainly on mammals and birds (Pascoe et al., 2017) with less research done on amphibians. These studies have generally demonstrated that gut microbiota can have a variety of effects on their hosts, including on general health, the immune system, and nutrient absorption. In amphibians, studies have shown that the gut microbiome may be affected by environmental temperature (Fontaine et al., 2018; Zhu et al., 2021), season (Xu et al., 2020), and the process of metamorphosis (Kohl et al., 2013; Zhang et al., 2020; Yang et al., 2022). Amphibians are poikilotherm and many species are characterised by a biphasic life cycle with both terrestrial and aquatic stages, and these likely have different gut microbiomes or host-microbiome relationships than the homeothermic mammals and birds.

Salamanders of the genus *Hynobius* found in Japan are all endemic to the country, and it is likely that all of them have speciated within mainland Japan (Nishikawa, 2016). This radiation comprises both lotic- and lentic-breeding species (i.e., those breeding in flowing and still water, respectively; Sato, 1943). Their coexistence may be partially explained by interspecific differences in microhabitat, breeding, and feeding ecology. These differences in ecological traits may subsequently drive differences in gut microbiota, particularly when two sympatric salamander species have different breeding strategies. The gut microbiota of *Hynobius* salamanders has not been well investigated, with the exception of a single lentic-breeding species, *H. maoershanensis* Zhou et al., 2006 from southern China (Yang et al., 2022). In this study, we describe and compare the gut microbiota in the Odaigahara Salamander, *H. boulengeri*, based on samples from stomach, intestine, and faeces, to gain basic information of its gut microbiota diversity.

Hynobius boulengeri occurs in mountainous areas of the Kii Peninsula, in the southern part of Honshu Island, the main island of Japan. This species is a lotic breeder and is one of the largest species in the genus. Given the large size of adult individuals (136–194 mm; Nishikawa, 2021) and the faecal volume they produce, we expected that faecal samples would serve as a good proxy for assessing gut microbiota. Because the collection of faeces is non-invasive, this technique could be useful for microbiota surveys of both *in-situ* and *ex-situ* salamanders and might be especially useful for endangered species.

Materials and Methods

Five adult *H. boulengeri* were collected in Kamikitayama-mura, Yoshino-gun, Nara Prefecture (34.1778°N, 136.0915°E, elevation 1419 m) in May 2022. Each salamander was housed in a plastic container lined on the bottom with damp paper and kept in an incubator at a constant temperature of 14°C. Faecal samples were collected as soon as they were observed during daily checks over a one-month period. After this period, the salamanders were euthanized using methods approved by the Animal Experiment Guideline of Kyoto University. Their gastrointestinal tracts were then removed and opened, and the inner surfaces of stomach

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and intestines were swabbed with sterile cotton swabs. All samples were preserved in 70% ethanol and stored at −80°C in the freezer until DNA extraction.

To extract DNA, samples were mixed with 300 µl lysis buffer (0.1M Tris, 0.04 M EDTA), 25 µl 20% SDS, and 500 µl 0.2-mm glass beads in a 2.0-ml microtube. The microtubes were then shaken at 27 Hz for 5 min using Tissue Lyser II (Qiagen, www.qiagen.com). DNA was purified using a standard phenol-chloroform extraction method (Matsuki, 2006).

The V3-V4 region of the bacterial 16S rRNA gene was amplified with the 341F and 805R barcoded-primers (Klindworth et al., 2013) using a dual-index approach. The PCR mixture contained 6.4 µl of DNA-free water, 1 µl 10×PCR buffer for Blend Taq (Toyobo, www.toyobo-global.com), 0.1 µl of BlendTaq, 1.0 µl of 20 mM dNTPs, 0.5 µl of each primer (10 mM), and 0.5 µl of sample DNA. PCR-conditions were as follows: denaturation at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s (denaturation), 50°C for 30 s (annealing), and 72°C for 60 s (extension), and a final extension at 72°C for 10 min. Paired-end sequencing was performed on an Illumina MiSeq at the Bioengineering Lab (Sagamihara, Japan). We pre-processed raw sequence data into amplicon sequence variants (ASVs) with > 99% nucleotide identity using the *dada2* v1.26 package in R (Callahan et al., 2016) with default parameters. Raw reads were quality-filtered, dereplicated, and merged using the R functions *plotQualityProfile*, *filterAndTrim*, and a combination of *derepFastq* and *mergePairs*, respectively. The *makeSequenceTable* was employed to construct an ASVs table. Potential chimeras were removed using the *removeBimeraDenovo* function. Taxonomic classification of ASVs was based on Silva version 132 and Silva Species Assignment v132 (Callahan, 2018) using the *assignTaxonomy* function (for the details on the classification used, visit the website of SILVA taxonomy). ASVs classified as taxa of Archaea and Eukaryota and any unidentified taxa, were excluded, since we intended to focus on bacterial microbiota analysis in this study. We rarefied the number of sequences across all samples to 2565 reads using the *SRS* v0.2.3 package in R (Heidrich et al., 2021).

To characterize the bacterial assembles we measured α-diversity using indices, a version of Simpson's Diversity less sensitive to differences in sample size. We used the Wilcoxon Signed-rank Test, for pairwise comparisons of medians, using the *exactRankTests* v0.8-35 package in R (Hothorn and Hornik, 2022) to compare α-diversity between stomach and intestine samples, between stomach and faecal samples, and between intestine and faecal samples.

This is the first study on the microbial diversity in the gastrointestinal tract and in faeces of Japanese salamanders. In total, we obtained 670,017 raw reads from 14 samples, of which 172,522 passed filtering quality. Sample sequences were rarefied in the same length as the minimum one (HB7St:2565) based on a common procedure. A total of 771 ASVs were clustered using a sequence similarity of 99%. The ASVs obtained from the samples consisted of 15 phyla and 83 families.

To analyse the differences in bacterial community composition (ß-diversity) among sampling sites, we used the *vegdist* function in the *vegan* v2.6-4 package in R (Oksanen et al., 2022) to calculate a Bray-Curtis Dissimilarity Matrix. We conducted pairwise permutational multivariate analyses of variance (pairwise PERMANOVA) based on the matrix using a function in the *pairwiseAdonis* v0.4.1 package in R (Martinez, 2017) to test for the significant differences in community composition among sampling sites. The *p*-values were adjusted with a Bonferroni correction. A non-metric multidimensional scaling ordination (NMDS) plot was used to depict bacterial community structure. To explore at which classification level bacterial taxa cause differences in community structure, a Linear Discriminant Analysis Effect Size (LEfSe) was performed among sampling sites, using the *LEfSe* function in the *microbiomeMarker* package of R (Segata et al., 2011; Yang et al., 2022). Only bacterial taxa with LEfSE scores > 2 $\log_{10}$ are reported. The bacterial composition ratio was calculated based on the ASVs read numbers.

Results and Discussion

We found differences in α-diversity using indices (faeces: 0.98 ± 0.00, intestine: 0.95 ± 0.03, stomach: 0.94 ± 0.06). Pairwise comparisons showed that faecal samples had significantly higher α-diversity than those from stomach ($p < 0.05$) or intestine ($p < 0.05$). These results are similar to those of previous studies on lizards and frogs, which reported significant differences in α-diversity between sampling sites, with higher α-diversity observed in faecal samples than in intestinal samples (Kohl et al., 2017; Zhou et al., 2020).

The pairwise PERMANOVA results indicated that the faecal microbiota was significantly different from the stomach and intestine microbiota (Table 1).

Table 1. Result from a pairwise PERMANOVA to compare sampling sites. The *p*-values were adjusted using the Bonferroni correction.

Pairs	*df*	SumsOfSqs	*F*	R^2	*p*	*p* adjust
faeces vs. intestine	1	0.0087	2.2	0.24	0.0090	0.027
faeces vs stomach	1	0.0088	2.4	0.23	0.0060	0.018
intestine vs stomach	1	0.0059	1.5	0.17	0.1180	0.354

The result of NMDS (stress value = 0.074) indicated that faecal samples clustered separately from stomach and intestine samples, while the stomach and intestine samples did not exhibit clear separation from each other (Fig. 1A). Not only was α-diversity different, but the community composition in faeces was also significantly different from stomach and intestine samples.

The abundance of 27 bacterial taxa (from phylum to genus) varied among stomach, intestine, and faeces. Nine, seven, and 11 taxa had an elevated occurrence in stomach, intestine, and faecal samples, respectively. At the phylum level, the samples from the stomach had a higher level of Actinobacteria than the other samples. Proteobacteria and Firmicutes were detected with greater frequency in intestine and faeces, respectively. At the family level, the samples from the stomach had higher levels of *Micrococcaceae*, *Flavobacteriaceae*, and *Chitinophahales* than the intestines and the faeces, with samples from the intestine showing higher levels of *Bacteroidaceae* and *Nocardiaceae*, and samples from faeces with greater levels of *Ruminococcaceae*, *Rikenellaceae*, *Tannerellaceae*, Clostridiales vadin BB60 group and *Christensenellaceae* than the other samples. At the genus level, the samples from the stomach had a higher level of *Flavobacterium* than intestine and faeces, the samples from intestine had higher levels of *Bacteroides* and *Rhodococcus* than the stomach and the faeces, and the samples from faeces had higher levels of *Parabacteroides*, the Clostridiales vadin BB60 group, and *Alistipes* than the stomach and the intestine (Fig. 1B).

The dominant phyla present in stomach samples were Bacteroidetes (41.5%), Actinobacteria (21.5%), Proteobacteria (11.0%), Chloroflexi (10.0%), and Firmicutes (7.5%), which accounted for 91.5% of assigned phyla. Among intestine samples, the dominant phyla present there were Bacteroidetes (40.0%),

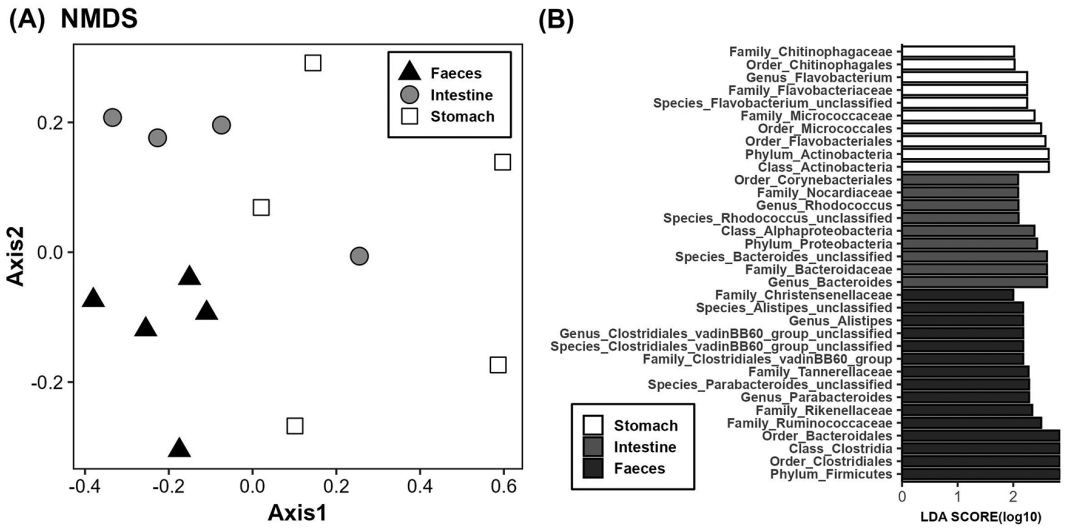

Figure 1. Analysis of bacterial community composition in *Hynobius boulengeri* from the Kansai Region of Japan, illustrating differences between sites in the body where bacterial samples were obtained. (A) NMDS plot of Bray-Curtis distances for microbiota samples from stomach (squares), intestine (circles), and faeces (triangles). One of the intestine samples could not be plotted due to a failure in the PCR. (B) Linear Discriminant Analysis score of selected bacterial taxa for stomach, intestine, and faecal samples.

Firmicutes (20.2%), Actinobacteria (19.4%), and Proteobacteria (14.5%), which accounted for 94.1% of assigned phyla. Among faecal samples, the dominant phyla present were Bacteroidetes (47.4%) and Firmicutes (46.7%), which accounted for 94.1% of assigned phyla (Fig. 2A). Gut and faeces microbiota in *H. boulengeri* were dominated by five phyla: Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, and Chloroflexi. The first three have been commonly identified in the intestine of urodeles (Walker et al., 2020; Wang et al., 2021; Yang et al., 2022). From the cloaca of the lentic breeding congener *H. maoershanensis*, the following bacterial phyla were detected in order of decreasing abundance: Proteobacteria, Bacteroidetes, Firmicutes, *Deferribacter*, and Actinobacteria (Yang et al., 2022). *Deferribacter* was found in *H. maoershanensis* (3.47 ± 9.32%) but was not detected in *H. boulengeri*.

In a family-level comparison of faeces from the lotic-breeding *H. boulengeri*, *Ruminococcaceae*, *Bacteroidaceae*, *Rikenellaceae*, *Tannerellaceae*, Clostridiales vadin BB60 group, and *Lachnospiraceae* were detected in this order of abundance, respectively (Fig. 2B), In the lentic-breeding *H. maoershanensis*'s cloaca (Yang et al., 2022), the following family were detected in order of decreasing abundance: *Flavobacteriaceae*, *Oxalobacteraceae*, *Pseudomonadaceae*, *Rikenellaceae*, *Deferribacteraceae*, and *Ruminococcaceae*. *Rlikenellaceae* and *Ruinococcaceae* were found in both species, but they have different composition rates. Unfortunately, we cannot discuss this difference between *H. boulengeri* and *H. maoershanensis* at this stage because we still do not have enough information as to what might cause such differences.

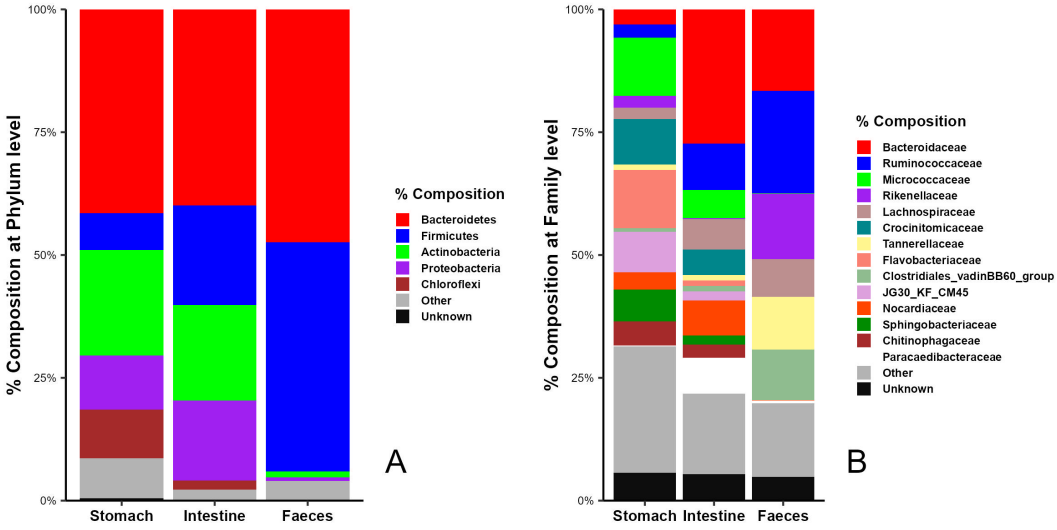

Figure 2. Percent composition of the bacterial microbiota in *Hynobius boulengeri* from the Kansai Region of Japan by phylum A and family B.

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