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Short Communication

The predominant gut microbiota in the grass puffer, *Takifugu alboplumbeus*, captured in both river and marine environments

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Abstract: The grass puffer, *Takifugu alboplumbeus*, a euryhaline fish species, was collected from both river and marine environments, and the gut microbiota of these specimens was examined using clone library analysis and qPCR technology. The results indicated that *Aliarcobacter* sp. constituted 27.3-96.9% of the three 16S rDNA libraries for river pufferfish and 40.6-86.8% of the three libraries for saltwater pufferfish, indicating that this bacterium is the dominant organism in both river and saltwater pufferfish. Furthermore, *Brevinema* sp., *Mucinivorans* sp., *Mycoplasma* sp., *Pseudomonas mosselii*, and unclassified members of Desulfovibrionaceae family were detected in both river and saltwater pufferfish at frequencies of 50-83%. In contrast, *Ilumatobacter fluminis*, *Ilumatobacter* spp., *Nitrincola* sp., *Tropicibacter alexandrii*, and unclassified members of the Microthrixaceae and Mycoplasmataceae families, as well as the Mollicutes class, were detected only from river pufferfish, while *Vibrio* spp. were detected only in two out of three libraries of saltwater pufferfish. However, qPCR for Vibrionaceae showed that the abundance of Vibrionaceae in the gut of river pufferfish was significantly lower than in saltwater pufferfish, although neither was the predominant bacteria. These results indicate that river and saltwater pufferfish have different gut microbiota. This suggests that the differences in the gut microbiota between river and saltwater pufferfish may be related to the differences in salt tolerance of the gut bacteria, as well as the differences in the environmental microbiota of river and marine waters.

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Introduction

Euryhaline fish species, including salmonids, eels, mullets, and tilapias, demonstrate a remarkable ability to flourish across a broad spectrum of salinities. It is thought that the composition of the gut microbiota in these fish is profoundly shaped by the salinity of the surrounding water during their migratory journey alongside their hosts (Sullam et al., 2012). Previous studies employing culture-dependent methods have shown that the gut microbiota of salmonids (Yoshimizu and Kimura, 1976), gray mullet, *Mugil cephalus* (Hamid et al., 1978), and redbelly tilapia, *Coptodon zillii* (Sakata et al., 1980) undergo changes that are correlated with the salinity of their environmental water. For instance, Yoshimizu and Kimura (1976) documented a shift in the dominant bacterial species within the gut microbiota of salmonids during their development, transitioning from *Aeromonas* and Enterobacteriaceae to *Vibrio* and *Pseudomonas*. These results underscore the significant impact of salinity on the gut microbiota of fish, which can be attributed to varying salt tolerance among the constituent bacteria.

Conversely, recent investigations employing culture-independent methods have unveiled the predominance of bacterial phyla such as Alphaproteobacteria, Actinobacteria, Betaproteo-bacteria, and Gammaproteobacteria in the gut microbiota of coastal fish (Tanaka et al., 2012). Notably, the vast majority of these bacteria were not previously discerned utilizing culture-dependent techniques. However, there are only a few reports on the dynamics of these bacteria in migratory fish that traverse between marine and river environments. The coastal species, grass puffer, *Takifugu alboplumbeus,* exhibits a unique behavior of transitioning from

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Table 1. Abundance of total bacteria and Vibrionaceae in six grass puffer specimens captured in river (R1-R3) and marine waters (S1-S3).

Specimen	Total bacteria (cells/g)	Vibrionaceae (copies/g)
R1	2.0×10^{10}	8.5×10^{2}
R2	3.4×10^{9}	3.6×10^{2}
R3	1.2×10^{10}	9.8×10^{3}
S1	5.3×10^{9}	9.9×10^{4}
S2	7.8×10^{9}	2.3×10^{6}
S3	5.6×10^{9}	1.3×10^{7}

seawater to river water (Kato et al., 2010). Consequently, the primary objective of this study is to conduct a comparative analysis of the predominant gut microbiota of pufferfish captured in both river and marine environments to better understand the ecology of the gut microbiota of fish.

Materials and Methods

Fish specimens: Three grass puffers (R1-R3) weighing between 44.0 and 52.7 g were collected by fishing in the Take River, Yokosuka, Kanagawa, which is located 640 m from the river mouth. The temperature and salinity of the river water were 19.7˚C and 0.5 ppt, respectively. Additionally, three grass puffers (S1-S3) weighing between 15.1 and 30.1 g were caught in the saltwater at Nagai Port, Yokosuka. The temperature and salinity of the saltwater at Nagai Port were 21.2˚C and 31.9 ppt, respectively.

All fish specimens were euthanized by ice cooling immediately after collection, and treated as follows: the gut contents were obtained aseptically by dissection and squeezing extrusion. Aliquots of each gut sample were stored at -80°C prior to use and then analyzed. Separately, bacterial cells in aliquots of each gut sample were stained with 4′, 6-diamidino-2-phenylindole (DAPI) to determine the total number of bacteria, using a BX50 fluorescence microscope (Olympus, Tokyo, Japan), as described by Porter and Feig (1980).

Construction and analysis of 16S rDNA- and Vibrionaceae-libraries: DNA was extracted from the gut content using the FastDNA SPIN Kit for Soil (MP Biomedicals, CA, USA). The 16S rDNA was amplified by polymerase chain reaction (PCR) using the universal primers 20F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and r2L (5′-CAT CGT TTA CGG CGT GGAC-3′) (Hiraishi, 1992); the sequences of Vibrionaceae-specific DNA were amplified by PCR using the Vibrionaceae-specific primers VIB-F (5′-CTA CTT GGA GGT TGT GGC CT-3′) and VIB-R (5′-GCT GGC AAA CAA GGA TAAG-3′) (Chen et al., 2022). The resulting amplicons were cloned into the pGEM T-Easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* DH5α to obtain both 16S rDNA- and Vibrionaceae-libraries. DNA sequences of clone inserts were analyzed according to Hiraishi (1992) and identified using EZBioCloud (Yoon et al., 2017). Representative sequences from this study have been deposited into the DDBJ/GenBank/EMBL databases under Accession Numbers LC779535 to LC779541.

Real-time PCR: The abundance (copies/g) of Vibrionaceae in the gut sample of the grass puffer was estimated by qPCR using the Vibrionaceae-specific primer set, VIB-F, and VIB-R, according to Chen et al. (2022).

Results and Discussions

The abundance of total bacteria and Vibrionaceae in grass puffer gut: Table 1 shows the total numbers of bacteria in gut contents stained with DAPI, as follows: R1-R3 specimens caught in river water of the Take River, 3.4×10^{9}-2.0×10^{10} cells/g; S1-S3 specimens caught in seawater at Nagai Port, 5.3×10^{9}-7.8×10^{9} cells/g. The total number of bacteria, ranging from 3.4×10^{9} to 2.0×10^{10} cells/g, was found to be similar to those of coastal fish, including pufferfish, as previously described (Chen et al., 2022). The abundance of Vibrionaceae was 3.6×10^{2} –9.8×10^{3} copies/g in river pufferfish and 9.9×10^{4} -1.3×10^{7}

Table 2. Distribution (no. of clones) of bacterial taxa in six libraries constructed from gut contents of grass puffer, captured in river and marine waters.

Class	Related taxa (accession no.; identity, %)	R1	R2	R3	S1	S2	S3
Acidimicrobiia	*Ilumatobacter fluminis* (AB360343; 97.0-97.4)	0	8	0	0	0	0
	Ilumatobacter spp.	0	5	0	0	0	0
	Unclassified Microthrixaceae family	0	5	0	0	0	0
Alphaproteobacteria	*Tropicibacter alexandrii* (MH596855; 97.4-98.0)	0	4	0	0	0	0
Bacteroidia	*Mucinivorans* sp. (HM630238; 92.9-93.6)	6	0	0	2	2	0
Desulfovibrionia	Unclassified Desulfovibrionaceae family	9	0	0	4	6	0
Epsilonproteobacteria	*Aliarcobacter* sp. (FN650333; 90.0-93.3)	79	27	95	41	56	92
Gammaproteobacteria	*Nitrincola* sp. (AY567473; 90.0-91.0)	0	14	0	0	0	0
	Pseudomonas mosselii (AF072688; 99.6-100)	0	2	0	8	20	10
	Vibrio spp.	0	0	0	12	0	1
Mollicutes	*Mycoplasma* sp. (M23939; 92.2-93.0)	11	0	0	25	3	0
	Unclassified Mycoplasmataceae family	7	0	0	0	0	0
	Unclassified Mollicutes class	0	6	0	0	0	0
Spirochaetia	*Brevinema* sp. (DQ340184; 94.5-97.5)	1	4	2	6	1	0
Others*		0	24	1	3	1	3
Total		113	99	98	101	89	106

copies/g in saltwater pufferfish. This result indicates that the abundance of Vibrionaceae in gut contents of R1–R3 specimens is considerably lower than in the S1-S3 specimens.

Gut microbiota of pufferfish: Table 2 shows the distribution of bacterial species across the six 16S rDNA-libraries. *Aliarcobacter* sp. (FN650333) constituted a substantial proportion, ranging from 27.3 to 96.9%, in river pufferfish, and from 40.6 to 86.8% in saltwater pufferfish. These findings underscore the dominance of this bacterium within the gut microbiota of river and saltwater pufferfish. In addition, sequences of *Brevinema* sp. (DQ340184), *Mucinivorans* sp. (HM630238), *Mycoplasma* sp. (M23939), *Pseudomonas mosselii* (AF072688) and unclassified members of Desulfovibrionaceae family were detected in both river and saltwater pufferfish at frequencies of 50-83%, suggesting that these species are less susceptible to the migration of the pufferfish from saltwater to river water. On the other hand, *Ilumatobacter fluminis* (AB360343), *Ilumatobacter* spp., *Nitrincola* sp. (AY567473), *Tropicibacter alexandrii* (MH596855), and unclassified members of the Microthrixaceae and Mycoplasmataceae families, as well as the Mollicutes class, were uniquely identified in river pufferfish. In contrast, *Vibrio* spp. was exclusively detected in the S1 and S3 libraries derived from saltwater pufferfish.

Table 3 shows the species distribution of the family Vibrionaceae within six Vibrionaceae–libraries. *Vibrio harveyi* (BCUF01000119) and *V. tasmaniensis* (AJ514912) were detected across all six libraries. *Vibrio cidicii* (LOMK01000001), *Vibrio hispanicus* (AY254039), and *V. orientalis* (ACZV01000005) were commonly found in both river and saltwater pufferfish, with frequencies ranging from 33-67%. In contrast, *Photobacterium aestuarii* (JF751050), *V. alfacsensis* (JF316656), *V. halioticoli* (BAUJ01000001), and *V. toranzoniae* (HE978310) were consistently identified in all three libraries for saltwater pufferfish, while *V. mangrovi* (FXXI01000024) and *V. ponticus* (AJ630103) were exclusively detected in river pufferfish.

In this study, we analyzed the gut microbiota of grass puffer collected from both river and saltwater environments using two sets of primers targeting 16S rDNA and Vibrionaceae. Total numbers of bacteria were in the range of 10^9 -10^{10} cells/g in both R1-R3 and S1-S3 specimens, with no significant difference observed. Within the 16S rDNA libraries, *Aliarcobacter* sp. (FN650333) dominated in both river and saltwater pufferfish, constituting 27.3-96.9% of the gut microbiota, making it the most prevalent taxon. Members of the genus *Vibrio* were found to constitute

Table 3. Distribution (no. of clones) of bacterial species belonging to the Vibrionaceae family in six libraries constructed from gut contents of grass puffer, captured in the river and marine waters.

Related species (accession no.: identity, %)	R1	R2	R3	S1	S2	S3
Photobacterium aestuarii (JF751050; 97.8-100)	0	0	0	1	11	17
Vibrio alfacsensis (JF316656; 99.6-100)	0	0	0	2	1	1
Vibrio cidicii (LOMK01000001; 99.6-100)	4	2	0	3	1	0
Vibrio halioticoli (BAUJ01000001; 99.6-100)	0	0	0	5	16	18
Vibrio harveyi (BCUF01000119; 98.7-100)	11	14	33	4	12	7
Vibrio hispanicus (AY254039; 100)	2	1	0	1	1	0
Vibrio mangrovi (FXXI01000024; 98.7-100)	27	24	6	0	0	0
Vibrio orientalis (ACZV01000005; 99.1-100)	0	0	1	8	0	0
Vibrio ponticus (AJ630103; 99.5-100)	3	3	1	0	0	0
Vibrio tasmaniensis (AJ514912; 99.1-100)	6	8	2	13	7	8
Vibrio toranzoniae (HE978310; 99.1-100)	0	0	0	4	2	2
Vibrionaceae family	0	0	4	12	3	2
Total	53	52	47	53	54	55

up to 0.9-11.9% of the gut microbiota in saltwater pufferfish, but were below the detection limit (approximately 1%) in river pufferfish. Zhao et al. (2020) examined the gut microbiota of Chinook salmon, *Oncorhynchus tshawytscha,* raised in both freshwater and saltwater environments and found that the genera *Photobacterium*, *Cetobacterium, Intestinibacter, Bacillus, Brevinema*, and *Romboutsia* were predominant in freshwater salmon, whereas the genera *Aliivibrio*, *Photobacterium*, *Pelomonas, Vibrio*, and *Mycoplasma* were predominant in saltwater salmon. Some researchers, moreover, revealed an increase in the abundance of *Vibrio* spp. within the gut microbiota of Nile tilapia, *Oreochromis niloticus,* and Atlantic salmon, *Salmo salar,* following their transfer from freshwater to saline environments (Zhang et al., 2016; Dehler et al., 2017). Conversely, Lai et al. (2020) examined the gut microbiota of marine medaka, *Oryzias melastigma*, which had been acclimated from seawater to freshwater. They reported that *Vibrio* spp. constituted 55.8% of the gut microbiota in marine medaka, but this decreased to 6.8% in freshwater medaka, with *Pseudomonas* spp. becoming the dominant bacteria, accounting for 51.2%. Taken together, these results suggest that *Vibri*o spp. are predominant in the gut of saltwater fish, and significantly lower in freshwater fish. The present result shows that the abundance of Vibrionaceae in the gut of river pufferfish was also significantly lower than in saltwater pufferfish, although neither was the predominant bacteria. In this connection, Chen et al. (2022) reported that the total number of bacteria (10^9-10^{11} cells/g) in guts of the coastal fish including pufferfish, is relatively constant, while the abundance of *Vibrio* spp. (10^5-10^{10} copies/g) varies greatly, suggesting that *Vibrio* spp. are not always dominant in some fish specimens. This phenomenon may be true in this study.

Thus, this study revealed that the gut microbiota of river grass puffers differs from that of saltwater puffers. Previous studies on salmonids and tilapia have reported that the salt tolerance of gut bacteria isolated from fish in saltwater is higher than that of those in freshwater (Yoshimizu and Kimura, 1976; Sakata et al., 1980). Furthermore, considering that the majority of gut bacteria are derived from the environment, the significant differences in bacterial communities between river and marine environments cannot be ignored (Cahill, 1990). These reports suggest that the differences in the gut microbiota between river and saltwater pufferfish may be related to the differences in salt tolerance of the gut bacteria, as well as the differences in the microbiota in river and marine waters. However, since most of the gut

bacteria of pufferfish are difficult to culture, it is necessary to investigate the salt tolerance of gut bacteria using culture-independent techniques such as molecular biological approaches.

Finally, Kato et al. (2010) reported that grass puffer migrates from seawater to river water, stay for an average of 3.6 hours, and return to the sea within the same day. In addition, Kato et al. (2010) experimentally placed grass puffer in a freshwater tank (salinity 0 ppt) and found that they were able to survive for 2 days but not for more than 4 days. These results strongly suggest that the changes in the gut microbiota of the grass puffer occurred in a short period, considering that the grass puffer spends only a few days at most in river water. Future observations at the laboratory level will be necessary.

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