High-throughput Sequencing Reveals the Dominance of *Shewanella* Species in the Intestinal Microbiota of Barbour's Seahorses (*Hippocampus Barbouri*)

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Abstract

In this study, taxonomic classification of intestinal microbiota from healthy Barbour's seahorses (*Hippocampus barbouri*) was determined as it plays an important role in host nutrition and immunity. Genomic DNA was extracted from the intestinal mucus samples of eleven Barbour's seahorses, which were subjected to high-throughput sequencing of bacterial 16S rRNA genes targeting V1-V3 regions. The results revealed that the most abundant operational taxonomic units (OTUs) were affiliated to the genus *Shewanella*. Our findings may provide baseline data for further studies in order to explore the potential implications of intestinal microbiota to seahorses in terms of health status, development, growth, and survival.

Introduction

There has been considerable attention paid to the intestinal microbiota because of its importance to host metabolism, immunity, and infection response (Pérez et al. 2010, Ni et al. 2014). Although these findings have been mostly shown to impact human and mammalian health, intestinal microbiota also play a significant role in fish health (Butt and Volkoff 2019). However, conventional characterization of intestinal microbiota has depended on cultivation-based techniques, thereby limiting our understanding of its importance to only those microorganisms that can be cultured (Ito et al. 2019). In fact, due to the low culturability (often <2%) of bacteria from different environments, culture-independent techniques have been used to provide a more comprehensive picture of fish microbiota (Huber et al. 2004). For instance, the advent of high-throughput sequencing technologies, such as Illumina or Oxford Nanopore, has led to significant advances in understanding bacterial community composition and structure in diverse environments (Straub et al. 2020). This has paved the way to reduce cost, time, and complexity to explore the taxonomic diversity and abundance of bacterial communities within the intestinal ecosystem of fish species. We therefore used this technology to explore the bacterial composition within the intestinal mucus of Barbour's seahorse (*Hippocampus barbouri*), with particular emphasis on the most dominant species.

Materials And Methods

Eleven healthy *Hippocampus barbouri* were collected from coral reefs off the coast of Cantiasay Island in Northeastern Mindanao, Philippines. Samples were immediately transported to our laboratory, where they were kept in an aquarium previously disinfected and under controlled aeration. Seahorses were then sacrificed within 24h prior to dissection to remove dietary contents (Ortega et al. 2021). The abdominal region was incised from lateral side using sterile materials (Tanu et al. 2011). Intestinal mucus was collected and homogenized in sterile saline solution. Genomic DNA was then extracted from the intestinal mucus of six female Barbour's seahorses and five male Barbour's seahorses. Samples were pooled according to their origin and HiPurA™ DNA Purification Kit (HIMEDIA; Mumbai, India) was used for DNA extraction, according to the manufacturer's instructions with slight modifications. Universal primers targeting the V1-V3 regions (5'-AGAGTTTGTATCCTGGCTAG-3') (5'-ATTACCGCGGCTGCTGG-3') were used

for PCR amplification of 16S rRNA genes, whose purified products were sent to Macrogen Inc. (Seoul, South Korea) for high-throughput sequencing on the Illumina MiSeq platform. Raw data were processed using the Quantitative Insights Into Microbial Ecology (QIIME 2) pipeline described by Bolyen et al. in 2019. At 97% sequence similarity of 16S rRNA genes, operational taxonomic units (OTUs) were initially defined. The weighted UniFrac test was then applied to determine whether two or more communities have the same structure (Lozupone et al. 2007). Subsequently, OTUs were clustered at 99% sequence similarity to define a core set of representative sequences for phylogenetic analyses. A heatmap was generated showing the relative abundance of OTUs assigned to the *Shewanellaceae* family across the samples, which were classified using the EzBioCloud database (Yoon et al. 2017). Phylogenetic analysis was performed by using MEGA version 6.0, as previously described (Balcázar et al. 2010).

Results And Discussion

A total of 66,547 sequence reads were collected from the two samples of seahorses' intestinal tissue. After quality filtering, random subsampling was performed to ensure that all sequence libraries contained the same number of sequences for α - and β -diversity comparisons. The number of OTUs was estimated at a level of 97% 16S rRNA gene sequence similarity, which resulted in 45 and 41 OTUs in intestinal samples from female and male seahorses, respectively. The species diversity and richness estimators (Shannon and Chao1) showed relatively similar values; however, the weighted UniFrac test (sensitive to abundances of taxa) revealed that while these intestinal samples have similar memberships, the relative abundances of each OTU are different.

Bacterial communities within the seahorse intestinal ecosystem were dominated by the phylum *Proteobacteria*, mainly represented by sequences affiliated with the family *Shewanellaceae* (92.9%) in female Barbour's seahorses, whereas sequences affiliated with the families *Shewanellaceae* (34.2%), *Pseudomonadaceae* (17.5%), *Chromatiaceae* (15.1%) and *Aeromonadaceae* (12.1%) were found in male Barbour's seahorses (Figure 1a). Sequences affiliated with the phyla *Bacteroidetes* (represented by the family *Flavobacteriaceae*) and *Firmicutes* (represented by the family *Planococcaceae*) were also found but their relative abundance was low (<2.0%).

Although previous studies have demonstrated that bacterial communities within the fish intestinal ecosystem are dominated by different families belonging to the phylum *Proteobacteria* (Egerton et al. 2018, Huang et al. 2020), sequences affiliated with the family *Shewanellaceae* were found to be highly dominant in the intestinal samples from both groups. Several factors may affect the diversity and abundance of the intestinal microbiota, such as diet, trophic level, season, habitat, and sex (Clements et al. 2007, Dhanasiri et al. 2011, Hovda et al. 2012, Cordero et al. 2015, Etyemez and Balcázar 2015, Etyemez-Büyükdavci et al. 2018).

Because the sequences belonging to the family *Shewanellaceae* were abundant, a phylogenetic analysis was carried out to establish their taxonomic affiliation. Sequences clustered at 99% sequence similarity and represented by OTU 1, OTU 2, OTU 96, and OTU 126 were found in the intestinal samples from both

groups, with OTU 1 being the most abundant in female Barbour's seahorses and OTU 1 and OTU 2 in male Barbour's seahorses (Figure 1b). Sequence similarity calculations based on a neighbor-joining analysis revealed that these OTUs grouped with known *Shewanella* species (Figure 1c). The closest described relative of OTU 1 was *S. baltica* NCTC 10735^T (98.5%), whereas OTU 2 was closely related to *S. basaltis* J83^T (99.6%) and *S. ulleungensis* MMS16-UL253^T (99.1%). Likewise, OTU 126 was related to *S. denitrificans* OS217^T (96.5%) and *S. maritima* D4-2^T (96.2%), whereas OTU 96 was closely related to unclassified *Shewanella* sp. MR-7 (99.5%).

Shewanella species are ubiquitously distributed in nature and have been usually isolated from surface freshwater and the deepest marine trenches (Satomi 2014, Lemaire et al. 2020). As they are natural inhabitants of marine waters, the presence of *Shewanella* species within the seahorse intestinal ecosystem may be influenced by their surrounding environment, in which they obtain their food. The wide distribution of *Shewanella* species in the aquatic environment is due to their unparalleled capacity to respire compounds found in nature and their capacity to thrive at low temperatures (Hau and Garlnick 2007). Moreover, bioactive compounds have been found in *Shewanella* spp., which may help increase the cytotoxic activity of the host immune response. Some strains have also been proposed as candidates for antibiotic synthesis (Leonardo et al. 1999, Kizhakkekalam et al. 2020). As a consequence, the availability of cutting-edge technologies and tools, sequenced genomes, rapid growth, and robust metabolism makes them ideal candidates for biotechnological applications (Amiri-Jami et al. 2006, Lemaire et al. 2020, Chakraborty et al. 2021).

In conclusion, the present study shows that high-throughput sequencing of 16S rRNA genes provides a reliable method for identifying the composition and structure of bacterial communities within the seahorse intestinal ecosystem. It should be noted that a normal intestinal microbiota may improve biological processes that benefit the host, including nutrition and immune system modulation (Maynard et al. 2012, Xing et al. 2013). Although these *Shewanella* species were found in apparently healthy seahorses, further studies are needed to isolate and characterize these species. Moreover, the information at hand may provide baseline data for further research in order to determine the potential implications to health status, development, growth, and survival of other seahorse species.

Declarations

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Author contributions

RCMHO: Conceptualization, methodology, investigation, formal analysis, visualization, writing - (both original draft and review and editing), funding acquisition. SRMT: Conceptualization, methodology,

supervision, funding acquisition. JGTM: Conceptualization, methodology, supervision. JLB: Methodology, investigation, formal analysis, visualization, supervision, writing - (review and editing).

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Data availability

All sequences obtained in this study are available and will be submitted to GenBank at the NCBI.

Consent to Participate

Not applicable.

Consent for Publication

All authors consent to the publication of this article.

Conflict of Interest

The authors declare no competing interests.

Ethical Statement

Legal requirements from the BFAR national office under a project “Seahorses and Pipefishes with pharmaceutical potentials in Mindanao” was acquired and granted and their guidelines for the care and use of animals have been followed.

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Figures

Figure 1

(a) Relative abundance of dominant bacterial families found in the intestinal samples from female and male Barbour's seahorses. **(b)** Heatmap of OTUs assigned to the *Shewanellaceae* family, which were compared with their abundances in each sample. The color intensity (log2 scale) in each panel shows the percentage of each OTU. **(c)** Phylogenetic dendrogram of selected OTUs with the most closely related *Shewanella* species, based on 16S rRNA gene sequences and constructed by the neighbor-joining method. Bootstrap percentages (>70%) based on 1000 replications are shown at branch nodes. Bar, 0.01 estimated substitutions per site.