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Metagenomic analysis between free-living and cultured *Epinephelus fuscoguttatus* under different environmental conditions in Indonesian waters

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ABSTRACT

In this study, we analyzed and compared feces of free-living and cultivated fish species, *Epinephelus fuscoguttatus* under different environmental conditions in Indonesian waters. Metagenome analysis was performed using Illumina MiSeq sequencing of the whole metagenomic DNA isolated from fish feces samples. The analysis covered both prokaryotic and eukaryotic DNA. Feces samples from mariculture fish revealed a highly stable distribution of several orders of bacteria when compared to samples from free-living fish, which were highly diverse and dominated by *Vibrionales*, *Pseudomonales*, *Rhizobiales* and non-classifiable *Alphaproteobacteria*. The eukaryotic content of the samples was dominated by residues of the host and nine additional fish species that formed a portion of the diet. Investigations on functional annotations for predominant bacterial taxa, using Gene Ontology enrichment, revealed a number of functions related to DNA metabolic processes, especially DNA repair, as well as antibiotic response in the free-living fish species.

1. Introduction

Indonesia with its myriad of islands is located within the coral triangle, an area with marine biodiversity that exceeds that of all other places on earth (Carpenter and Springer, 2005; Hutomo and Moosa, 2005). Fishery has always been an important economic factor for the country, based on the natural richness of highly valued fish species and ideal aquaculture conditions (Pet-Soede et al., 1999). However, population growth and rapid economic development has led to the depletion of fishing stock due to over exploitation. In addition, the growth in aquaculture related activities has resulted in an increase in wastewater production. This wastewater is poorly treated and discharged into the environment (Dsikowitzky et al., 2011) resulting in an immensely negative influence on marine ecosystems of Indonesian coastal waters and its inhabitants (Rinawati et al., 2012). Principally, in the region of Jakarta Bay, Northern Jakarta, a booming coastal megacity with over nine million inhabitants, that has been affected by increasing pollution load caused by untreated wastewater originating from households and industry (van der Meij et al., 2009, 2010; Siregar and Koropitan, 2013). Likewise, capture fisheries on Jakarta Bay have an influence on the local fish communities and their environment (Dsikowitzky et al., 2011; Cooper et al., 2009; Eng et al., 1989; Nordhaus et al., 2009). The consequences of these factors on the microbiome and especially the metagenome of fish are still unclear.

The microbiome of various fish species has been recently investigated by a number of studies. Recent findings have detected a core microbiome that appears to be common for certain fish species (Roeselers et al., 2011; Givens et al., 2015). Furthermore, Xia et al. (Xia et al., 2014) revealed the influence of starvation on the microbiome of fish by using a combined analysis on the microbiome and the functions of the intestinal tract. They recorded 33 phyla, 66 classes, 130 orders and 278 families of the intestinal microbiome using 16S amplicon sequencing and revealed that *Proteobacteria* (48.8%), *Firmicutes* (15.3%) and *Bacteroidetes* (8.2%) appeared as the predominant taxa. Specifically, *Proteobacteria* and *Firmicutes* have been widely reported as the characteristically predominant taxa for fish intestinal and gut microbiomes (Sullam et al., 2012; Sevellec et al., 2014; Xing et al., 2013). In contrast, the elucidation of functional annotations for the metagenomic samples of fish gut has only recently begun, resulting in a dearth of studies at this point in time (Xing et al., 2013). Furthermore, the microbiome of mariculture and free-living fish, and particularly a comparison between fish from these two different environments are yet to be studied. The brown-marbled grouper *Epinephelus* (*E.*) *fuscoguttatus* (order Perciformes, family Serrinadae) is a benthic marine fish species, widely distributed over the Indo-Pacific region and represents an important Indonesian fish species. *E. fuscoguttatus*, is a protogynous hermaphrodite and begins its lifecycle as female and changes its sex to male at later ages (Sugama et al., 2012). It is cultured in hatcheries, as well as extensively sourced from the wild. Adults or large juveniles are marketed directly and small juveniles are grown out to market size. In contrast to the difficult conditions for culturing, the species

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represents an important commercial fish species in Indonesia. However, the destruction of seagrass beds, vital for juveniles, and coral reefs, as well as intensive fishing has led to a near threatened status on the red list for this species (Cornish). In this study we collected 12 samples of *E. fuscoguttatus*, living under free-living and mariculture conditions, from Pulau Seribu, a chain of islands located to the North of Jakarta Bay. This area, stretching 45 km in the North into the Java Sea, consists of 110 islands and is part of the Thousand Island Marine National Park. We extracted total DNA from fish feces and sequenced it with Illumina MiSeq technology, using a whole metagenomic shotgun sequencing approach. We aimed at the identification and comparison of the taxonomical composition of the microbiome, particularly the predominant taxa and biodiversity, elucidating the functional components of these bacterial taxa, as well as the eukaryotic components within the fecal samples, providing information on food components and potential residues of parasitic sequences. (See Table 1.)

2. Results

2.1. Clustering of bacterial taxa for different environmental conditions

In total 12 samples, distributed over two environmental conditions, five free-living ($n = 5$) and seven mariculture ($n = 7$) samples were used to analyze bacterial composition. To test for the presence of two distinct environmental conditions, the samples were clustered, using a hierarchical clustering method with average linkage method (Supplementary Fig. S1). The clustering shows two distinct clusters representing the two different environmental conditions and one outlier (free2). While the first cluster consists only of samples from mariculture, the other consisted of free-living samples, except the outlier free2, and two mariculture samples (mari2, mari3). Using the statistical test Adonis, provided by R package vegan (Oksanen et al., 2016), we confirmed the composition of these clusters with a p-value of 0.034. As a result the samples free2, mari2 and mari3 which were statistically insignificant were excluded from further analysis.

2.2. Elucidation of bacterial communities under different environmental conditions

The annotated bacterial sequences of the remaining nine taxa revealed 16 different phyla, in decreasing order of abundance: *Proteobacteria* (71.00%), *Firmicutes* (23.71%), *Spirochaetes* (3.76%), *Actinobacteria* (1.13%), *Bacteroidetes* (0.40%), *Tenericutes* (0.07%), *Fusobacteria*, *Cyanobacteria*, *Deinococcus-Thermus*, *Verrucomicrobia*, *Acidobacteria*, *Lentisphaerae*, *Chlorobi*, *Aquificae*, *Chlamydiae* and *Synergistetes* (<0.01%).

Whereas, all samples were dominated by four bacterial phyla: *Proteobacteria* (71.00%), *Firmicutes* (23.71%), *Spirochaetes* (3.76%) and *Actinobacteria* (1.13%). The distribution of these predominant bacterial phyla exposed major differences between taxonomic level (Fig. 1a). While free-living samples revealed an asymmetrical structure of bacterial communities, dominated by one or two bacterial taxa per sample, the bacterial communities within the mariculture samples were more symmetrically distributed. On the free-living side, samples free3 and free5 were dominated by *Vibrionales* (free3: 88.77%) and *Bacillales* (free5: 75.89%), whereas free1 had two bacteria dominating: *Rhizobiales* (29.25%) and one unclassified bacteria (50.24%) at the order level. This unclassified annotation belonged to one taxa, annotated to *Proteobacteria* at phylum level. A more detailed annotation could not be achieved. However, the mariculture samples showed a more balanced distribution of bacteria. Most of the bacterial taxa appeared in a similar proportions. For each mariculture sample *Actinomycetales* (mean: 2.18%, sd: 0.41%), *Burkholderiales* (mean: 10.23%, sd: 0.59%), *Pseudomonadales* (mean: 24.10%, sd: 1.56%) *Enterobacteriales* (mean: 15.52%, sd: 6.36%) and *Rhizobiales* (mean: 5.23%, sd: 0.43%) occurred in a similar distribution. Further analysis, calculating a log2 fold change to determine significant differences between the samples, revealed a significant difference for *Vibrionales*, *Rhizobiales* and *Bacillales* over-represented in free-living samples and a significant difference for *Enterobacteriales* in the mariculture samples (Supplementary Fig. S2). While each free-living sample is dominated by one of the three bacteria *Vibrionales*, *Rhizobiales* and *Bacillales*, they have very low abundance for *Enterobacteriales* (mean: 1.72%, sd: 1.45%). In contrast the mariculture samples show a high stable occurrence for *Enterobacteriales* (mean: 15.52%, sd: 6.36%). Thereby the log2 fold change analysis support the findings of a more balanced distribution of bacteria in the mariculture samples.

On analyzing rare phyla, each sample revealed occurrences for *Bacteriodetes* (mean free-living: 0.19% vs. mean mariculture: 0.58%) and *Tenericutes* (mean free-living: 0.05% vs. mean mariculture: 0.10%) (Fig. 1b) with no significant differences between the two environmental conditions. In addition, sample free1 also showed abundance for

Table 1
Top 10 enriched GO terms per environmental condition. The enriched GO term were ordered by p-value and for every environmental condition 10 most enriched were used for functional analysis. In addition the distance to root of the ontology and number of matched Pfam identifier are listed.

GO term	Description	# annotated Pfam identifier	z-Score	p-Value	Level
Free-living					
GO:0006298	Mismatch repair	39	8.36	3.4e−14	8
GO:0006281	DNA repair	39	5.63	7.5e−08	6
GO:0006974	Response to DNA damage stimulus	39	5.29	3.3e−07	5
GO:0006835	Dicarboxylic acid transport	16	5.02	1.7e−06	9
GO:0031532	Actin cytoskeleton reorganization	8	4.67	4.9e−06	6
GO:0046677	Response to antibiotic	27	4.45	1.2e−05	4
GO:0044237	Cellular metabolic process	403	3.95	0,00003	2
GO:0008152	Metabolic process	429	3.87	4.2e−05	1
GO:0009636	Response to toxic substance	27	4.07	4.9e−05	3
GO:0042493	Response to drug	7	3.89	0.00009	3
Mariculture					
GO:0006259	DNA metabolic process	3	1.56	0.35	5
GO:0090304	Nucleic acid metabolic process	3	1.09	0.76	4
GO:0050896	Response to stimulus	3	1.02	0.85	1
GO:0044237	Cellular metabolic process	8	884	0.12	2
GO:0046483	Heterocycle metabolic process	4	833	0.12	3
GO:0034641	Cellular nitrogen compound metabolic process	4	784	0.13	3
GO:0050789	Regulation of biological process	3	719	0.14	2
GO:0006725	Cellular aromatic compound metabolic process	4	735	0.14	3
GO:0006139	Nucleobase-containing compound metabolic process	3	643	0.15	3
GO:1901360	Organic cyclic compound metabolic process	4	655	0.16	3