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Culturable gut bacteria of Ikan Batak (*Neolissochilus sumatranus* Weber & de Beaufort, 1916) collected in Toba Samosir, Indonesia

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Abstract. *Dinoto A, Handayani R, Setianingrum N, Julistiono H. 2020. Culturable gut bacteria of Ikan Batak (*Neolissochilus sumatranus *Weber & de Beaufort, 1916) collected in Toba Samosir, Indonesia. Biodiversitas 21: 4483-4488.* Ikan Batak (*Neolissochilus sumatranus* Weber & de Beaufort, 1916) is one of the fish species that is rarely found in water and have the status of endangered species. In consequence, the loss of endemic fish may contribute to the loss of microorganisms that inhabits the fish as a host. The studies on microorganisms associated with *N. sumatranus* are very limited. Therefore, the purpose of this study was to isolate and identify the culturable bacteria isolated from the gut of *N. sumatranus*. Sampling of *N. sumatranus* was carried out in a river within Toba Samosir area which flows to Lake Toba. Fish gut content was collected for isolating microorganisms using three media, including MRS, 10X diluted MRS, and MRS supplemented with 1% bile salt. Thirteen isolates were successfully isolated and identified based on 16S rRNA. This study revealed various species of gut bacteria recovered from *N. sumatranus* based on BLAST analysis. The isolates showed closest relationship to species *Bacillus subtilis* (3 isolates), *Bacillus tequilensis* (2 isolates), *Tumebacillus ginsengisoli* (6 isolates), *Klebsiella pneumoniae* (1 isolate), and *Lactobacillus pentosus* (1 isolate) with the similarity ranging at 98.7 to 100%. All 16S rRNA gene nucleotides of isolates have been submitted to GenBank. This study also described the isolates that have a very close relationship with *Bacillus tequilla* and *Bacillus subtilis*. Further identification is challenged to obtain a big picture of the diversity of microorganisms and the functionality in the digestive ecosystem of *N. sumatranus* for their conservation and bioprospecting of microbial-based aquaculture.

Keywords: *Bacillus*, gut bacteria, Ikan Batak, *Klebsiella*, *Lactobacillus*, *Neolissochilus sumatranus, Tumebacillus*

INTRODUCTION

Ikan Batak (*Neolissochilus sumatranus* Weber & de Beaufort, 1916) is an endemic fish of North Sumatra, especially in Lake Toba, and has been categorized as endangered (vulnerable) based on the IUCN Red List (Barus et al. 2014). Nowadays, most studies of *N. sumatranus* were focused on the phenotypic analysis, ecology, and behavior of fish (Purba et al. 2013; Roesma et al. 2019). Unfortunately, information on microbiological association of *N. sumatranus* is very limited, thus the association between microorganisms and fish adaptation and survival remains unclear. In addition, potential uses of microorganisms originated from endemic fish are also unexplored.

The presence of microbial communities in fish gut is associated with the ecology and physiology of the host. The particular importance with respect to host diet and digestion showing the functional relationships between fish intestinal communities and diet (Clements et al. 2014). The fish gut microbiota also plays critical roles in epithelial renewal and maturation, and regulate immune responses (Xiong et al. 2019). The structure and composition of gut microbiota, metabolic capacity, gut content, and enzyme activity are influenced by host trophic level (Liu et al. 2016). The meta-analysis study indicated that the trophic level, species habitat salinity, and possibly taxonomy variation is strongly correlated with the gut microbiota composition in fishes (Sullam et al. 2012; Wong and Rawls 2012).

Microbiota composition in several fish species in marine and freshwater has been reported previously. Phyla Proteobacteria, Bacteroides, Actinobacteria, Firmicutes, and Fusobacteria are commonly found in the gastrointestinal tract of freshwater fish (Romero et al. 2014). Fish bacteria could be distinguished with reference to dietary habits. Herbivorous fishes are inhabited mostly by cellulose-degrading bacteria species of *Clostridium*, *Leptotrichia*, and *Citrobacter*, while the carnivorous fishes are dominated by *Cetobacterium* spp. and protease-producing bacteria like *Halomonas* spp. (Liu et al. 2016). Study of the commercial warmwater fish showed the presence of *Cetobacterium somerae*, *Plesiomonas shigelloides*, *Fusobacterium mortiferum*, and *Aeromonas* spp. (Larsen et al. 2014). In addition, fish habitat also affects the variation in strain level. Comparative genomes of fish pathogenic *Aeromonas* isolated from different locations demonstrated the distribution of mobile elements, which is dependent on the host and geographic origin (Tekedar et al. 2019). In our previous study of fish-originated bacteria in Indonesia, *Weissella paramesenteroides* was successfully isolated from Indonesian eels (*Anguilla bicolor*), in which this bacterium has potential properties of antimicrobials (Dinoto et al. 2018). Other local fishes are suggested to be microbial resources with unique properties.

Although, several reports about microorganisms inhabiting the fish gut have been published, no report is available on gut microbes in *N. sumatranus*. The purpose of this study was to isolate and identify the gut bacteria of *N. sumatranus*. This study challenges the further description to obtain a big picture of the diversity of microorganisms and the functionality in the digestive ecosystem of *N. sumatranus* for their conservation and bioprospecting of microbial-based aquaculture.

MATERIALS AND METHODS

Sample collection and bacterial isolation

Sampling was conducted near Desa Bonandolok, Kecamatan Balige, Kabupaten Toba Samosir, North Sumatra province, Indonesia in February 2018. The fish was caught by fishnet in the river about three kilometers from preserved area for Ikan Batak (*Neolissochilus sumatranus* Weber & de Beaufort, 1916) at Mual Sirambe Nauli. Only one fish was successfully collected in this river which is suggested to be out from the local conservation onto Lake Toba through the river stream. Gut content of fish was collected aseptically and the fish body was preserved in ethanol for the purpose of identification (Figure 1). Identification of fish was carried out in Museum Zoologicum Bogoriense (MZB) - LIPI (with professional support of Dr. Renny Kurnia Hadiaty). Fish gut content was inoculated into enriched broth media of de Man Rogosa and Sharpe broth (MRS) (Oxoid) in, ten times diluted MRS (1/10 MRS), and MRS supplemented with 1% (b/v) bile salts (MRS+bile) and incubated at room temperature for 48 days. Isolation of bacteria was conducted using serial dilution of 0.85% NaCl solution onto the plate, consisting of agar media: MRS, 1/10 MRS, and MRS+bile). A single colony was collected and preserved in the glycerol medium at -80°C deep freezer until use.

Colony PCR amplification

Colony PCR of purified bacterial isolate was performed using universal 16S rDNA primers, 27f (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492r (5´-GGTTACCTTGTTACGACTT-3´). The PCR reaction (50 µL) consisted of 25 µL GoTaq Green Master Mix (Promega), 1 µL 1492r primer (10 pM), and 1 µL 27f primer (10 pM), and a tiny amount of single bacterial colony. The PCR reaction was performed for 30 cycles using Takara Thermal Cycler Dice (Takara Co. Inc.) with the following conditions: 94°C for 5 min of pre-denaturation, 94°C for 1 min of denaturation, 55°C for 1 min of annealing, 72°C for 1 min of elongation and 72°C for 5 min of final extension. Visualization of PCR products was performed in gel electrophoresis agarose 1% with GelRed® Nucleic Acid Gel Stain (Biotium). The 1 Kb DNA ladder (Geneaid) was used to determine the size of PCR amplicon in the agarose gel. The sequencing of the 16S rRNA (ribosomal ribonucleic acid) gene was conducted by First BASE Laboratories.

Phylogeny analysis

The 16S rRNA sequences were subsequently aligned with the multiple alignment method using Clustal X version 2.0 and BLAST (Basic Local Alignment Search Tool) analysis was performed with the database available at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) on July 11, 2020. Clustal X version 2.0 software with neighbor-joining method of 1000 bootstraps was used to construct the phylogenetic trees.

RESULTS AND DISCUSSION

One sample of fish was successfully collected in the sampling area for one hundred meters along the river. A small fish (8 cm in length) was identified as *Neolissochilus sumatranus.* This study confirmed the presence of this species in Toba Samosir, North Sumatra, even though it was relatively rare. *N. sumatranus* was reported previously as endemic fish in North Sumatra freshwater, particularly in some of the rivers around the Asahan river and Lake Toba (Barus et al. 2014).

Figure 1. Sampling location at the river flowing to Lake Toba, North Sumatra, Indonesia (*top*) and a sample of *Neolissochilus sumatranus* (*bottom*)

In the preliminary step, the fish gut content was enriched in broth media. We observed visually the high density of cell cultures in the tube after 48 days of incubation. Based on plate counts, the total cell number of enriched media was about 10^6 to 10^7 cfu/ml. Actually, this total cell number was not representing bacterial population in the gut of *N. sumatranus*. The cell numbers suggested that gut bacteria could grow in the enriched media under aerobic conditions before the isolation process. A total of thirteen isolates with single colony were obtained after three times subculturing. Only isolates that grow well and demonstrated distinct morphological appearances were investigated further for taxonomical studies. PCR amplification of all isolates was successfully conducted showing the pattern at about 1500 bp on the electrophoresis gel. As results, the contig sequences obtained from all isolates were about 1397-1419 bp in length, and they were sufficient to compare with the collection of rRNA sequences of the NCBI GenBank.

The BLAST analysis demonstrated that the similarity percentages of all isolates were 98.65 to 100% (Table 1). The phylogenetic analysis of 1000 bootstraps showed that 13 isolates are clustered in four different clades (Figure 1), including the clades of *Bacillus* spp. (5 isolates), *Tumebacillus* spp. (6 isolates), *Lactobacillus* sp. (1 isolate), and *Klebsiella* sp. (1 isolate) with the confidence level (over 500 bootstraps). The obtained nucleotide sequences were deposited in NCBI GenBank (ncbi.nlm.nih.gov) and assigned the following accession numbers of isolates as follows: MT4 (MT758358), MT6 (MT758359), MT9 (MT758360), MT10 (MT758361), BT2 (MT758362), BT3 (MT758363), BT4 (MT758364), DT1 (MT758365), DT3 (MT758366), DT6 (MT758367), DT7 (MT758368), DT8 (MT758369), and DT9 (MT758370). The profiles of the bacteria in the gut of *N. sumatranus* were previously not clearly known. As long as we knew, this study documented for the first time diversity of bacteria in the gut of *N. sumatranus*.

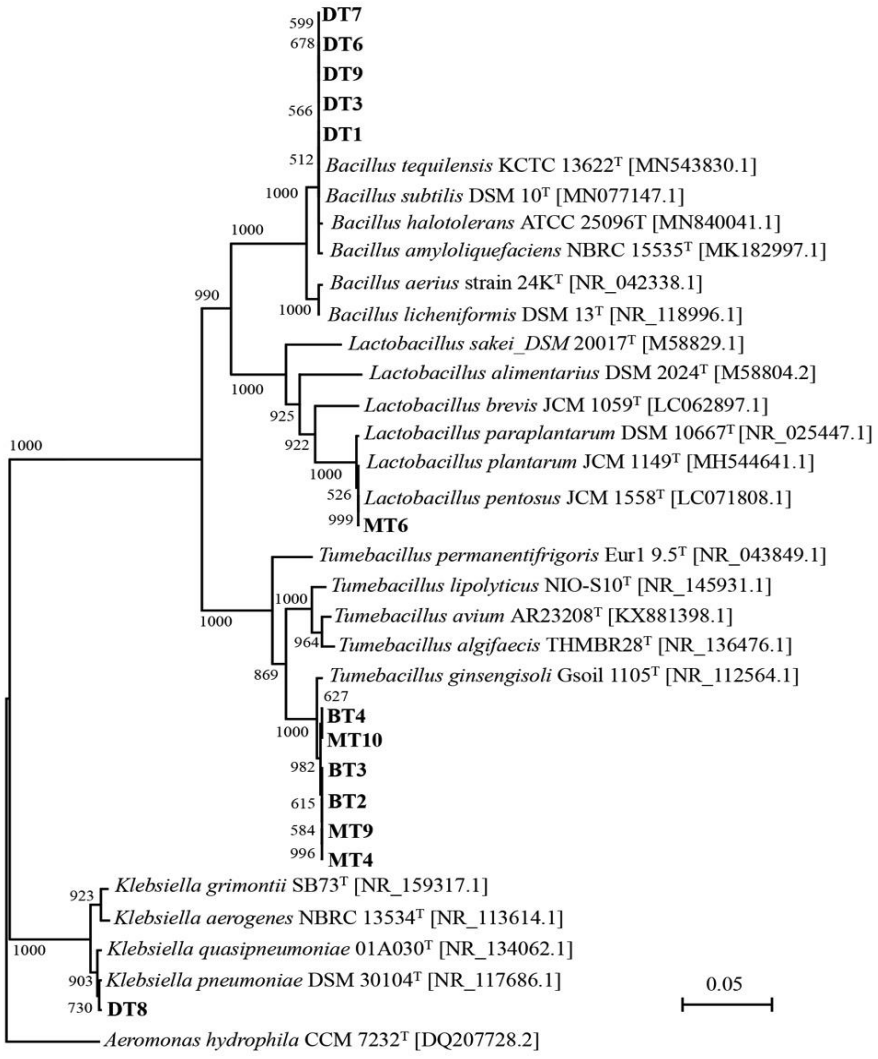

Figure 2. Phylogenetic tree of 16S rRNA sequences of isolates obtained from the gut of *Neolissochilus sumatranus*. Phylogenetic tree was constructed based on 16S rRNA gene sequencing by neighbor-joining method. Distances were estimated according to the Kimura two-parameter model with bootstrap percentages after 1000 simulations. *Aeromonas hydrophila* CCM 7372T was used as an outgroup

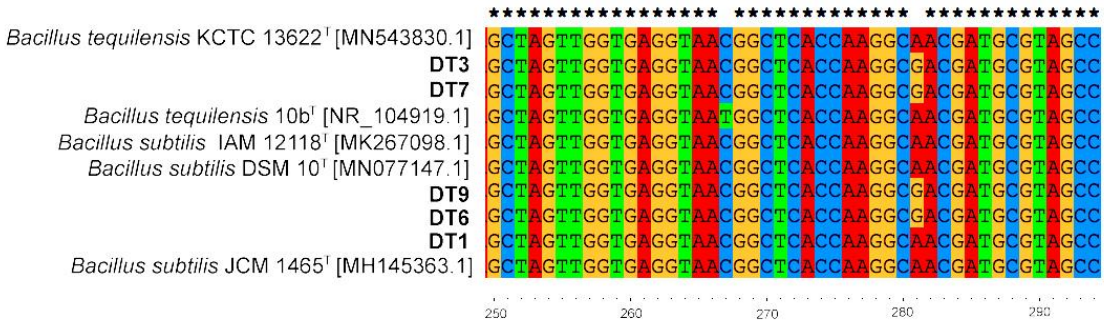

Figure 3. The 16S rRNA sequences alignment of isolates obtained from the gut of *Neolissochilus sumatranus* with representing type strain sequences of *Bacillus tequilensis* and *Bacillus subtilis* using clustalX

Table 1. BLAST results of 16S rRNA gene of isolates obtained from gut of *Neolissochilus sumatranus*

Isolate no.	Sequence length (bp)	Closest relationship [Accession No]	Similarity (%)
MT4	1408	*Tumebacillus ginsengisoli* Gsoil 1105^T [NR_112564.1]	98.72
MT6	1418	*Lactobacillus pentosus* JCM 1558^T [LC071808.1]	100.00
MT9	1415	*Tumebacillus ginsengisoli* Gsoil 1105^T [NR_112564.1]	98.73
MT10	1397	*Tumebacillus ginsengisoli* Gsoil 1105^T [NR_112564.1]	98.78
BT2	1419	*Tumebacillus ginsengisoli* Gsoil 1105^T [NR_112564.1]	98.80
BT3	1415	*Tumebacillus ginsengisoli* Gsoil 1105^T [NR_112564.1]	98.80
BT4	1408	*Tumebacillus ginsengisoli* Gsoil 1105^T [NR_112564.1]	98.65
DT1	1397	*Bacillus subtilis* DSM 10^T [MN077147.1]	100.00
DT3	1401	*Bacillus tequilensis* KCTC^T 13622^T [MN543830.1]	99.93
DT6	1411	*Bacillus subtilis* DSM 10^T [MN077147.1]	99.86
DT7	1397	*Bacillus tequilensis* KCTC^T 13622^T [MN543830.1]	99.93
DT8	1406	*Klebsiella pneumoniae* DSM 30104^T [NR_117686.1]	99.79
DT9	1400	*Bacillus subtilis* DSM 10^T [MN077147.1]	99.93

Total of six isolates (MT4, MT9, MT10, BT2, BT3, and BT4) of bacterial collection had the closest relationship of 16S rRNA gene with *Tumebacillus ginsengisoli* (similarity of 98.65 to 98.80%) (Table 1). Cells of *T. ginsengisoli* were observed microscopically as Gram-positive bacteria, aerobic, and non-motile rods. The type strain of this species, *T. ginsengisoli* Gsoil 1105^T (= KCTC 13942^T, =DSM 18389^T), was origin of the soil at a ginseng field in Pocheon Province, South Korea (Baek et al., 2011). The presence of *T. ginsengisoli* was interesting in the ecological perspective. Other species within genera *Tumebacillus* were reported on the aquatic environment. *Tumebacillus flagellatus* was isolated from cassava wastewater (Wang et al. 2013), whereas *Tumebacillus lipolyticus* was isolated from surface water (Prasad et al. 2015). *Tumebacillus avium* was previously reported in the gut of cinereous vulture, *Aegypius monachus*, however, the presence of this species in the gut of aquatic animals has not reported yet (Sung et al. 2018). This species is actually having broad distribution. *Tumebacillus* strain BK434 with a close relationship (99%) with related to *T. avium* was the origin of roots (Carper et al. 2020). Accumulated knowledge of bacterial distribution in broad habitats and their adaptation is very limited. However, we assumed that *T. ginsengisoli* in the gut of fish as reported in this study is related to the linkage of the soil and water ecosystem, where *N. sumatranus* inhabiting freshwater environments.

This present study reported five isolates having similarities to the bacterial genus *Bacillus* (99.86 to 100%). Three isolates (DT1, DT6, and DT9) were closest to *B. subtilis.* Another two species (DT3 and DT7) had the closest relationship with species *Bacillus tequilensis* (Table 1). Even isolate DT3 and DT7 having similarity with *B. tequilensis* (99.93%), BLAST of 16S rRNA gene sequences demonstrated very close relationship to *B. subtilis* (99.86% similarity). Phylogenetic tree confirmed the close relationship of these strains at the same clade (Figure 2). Some variations in the alignment of representing 16S RNA nucleotides showed a close relationship (Figure 3). The phenomenon of very close relationship (99%) within genus *Bacillus* was reported previously regarding the proposal of *B. tequilensis* as a novel species separated genetically from *B. subtilis.* Pulsed Field Gel Electrophoresis (PFGE) of type strain 10b^T revealed the restriction profile that distinguishes this strain from *B. subtilis.* DNA–DNA relatedness of strain 10b^T that was less than 70% with *B. subtilis* and other closely related species strongly support the species separation to novel species, *B. tequilensis* (Gatson et al. 2006).

Originally, *B. tequilensis* type strain 10b^T (=ATCC BAA-819^T =NCTC 13306^T) was isolated from 2000-year-old shaft-tomb at a site called Huitzilapa, near the city of Tequila in the Mexican state of Jalisco (Gatson et al. 2006). Our finding of the isolates that have the highest similarity to *B. tequilensis* in fish is not surprising, since this species may distribute in aquatic environment. Current publication reported a bacterium identified as *B. tequilensis* was successfully isolated from tilapia pond soil with the capacity of degrading unionized ammonia-nitrogen (Reyes et al. 2019). However, the presence of *Bacillus* in the gut of *N. sumatranus* was firstly documented in this study. *B. subtilis* is suggested to have broad distribution among various freshwater fish. Eight species in genera *Bacillus* have been found in the gut of guppy fish (*Poecilia reticulata*) including *B. cereus, B. subtilis, B. amyloliquefaciens, B. licheniformis, B. altitudinis, B. megaterium, B. marisflavi,* and *B. anthracis* (Kayath et al. 2019). The functionality of *B. subtilis* in aquaculture have been widely investigated. *B. subtilis* ZFB19 isolated from the gut of healthy grass carp showed antagonistic activity against *Edwardsiella piscicida* which was resistant to various antibiotics (Ren et al. 2019). Other studies also demonstrated the probiotic properties of *B. subtilis* in various aquatic animals, including grouper *Epinephelus coioides* (Liu et al. 2012) and white shrimp *Litopenaeus vannamei* (Zokaeifar et al. 2012). *B. subtilis* is suggested to be involved in controlling the fish pathogens by quorum sensing (QS) inhibition. Communication amongst the pathogenic Gram-negative bacteria by synthesizing, secreting, and responding to an autoinducer of QS systems−N-acyl-homoserine lactones (AHLs) were disturbed by AHL-lactonase, an enzyme catalyzing the degradation of the AHL. Previously, the expression of *aii*A gene that encodes AHL-lactonase was reported in *B. subtilis* BS-1. The entire coding region of the gene of this species was successfully obtained by using the primer set AiiA1/AiiA2 in the PCR amplification (Pan et al. 2008). Using the same primer set, the *aii*A gene was also recognized in other indigenous *Bacillus* isolated from *Clarias gariepinus* of Indonesia (Novita et al. 2015).

In this study, we reported the presence of one isolate related to *L. pentosus* (100% similarity). In general, *L. pentosus* is similar to *L. plantarum*, however, DNA-DNA hybridization demonstrated 36% to 85%. In addition, *L. pentosus* could be distinguished from *L. plantarum* in producing acid from D-xylose and glycerol. The type strain of *L. pentosus* NCDO 363^T (=ATCC 8041^T) was originated from corn silage (Zanoni et al. 1987). Although genera *Lactobacillus* in the gut of freshwater fish has been previously reported (Romero et al. 2014), the presence of *Lactobacillus pentosus* in the gut of *N. sumatranus* was firstly reported in this study. Previously, *L. pentosus* PL11 has been isolated from gut of Japanese eels (*Anguilla japonica*). In addition, *L. pentosus* PL11 has been also characterized and evaluated for probiotic properties of production of digestive enzymes, bile and acid tolerance, adhesion to intestinal mucus, and antibacterial activity to inhibit fish pathogen (Lee et al. 2015). Specifically, *L. pentosus* PL11 demonstrated the immunomodulation of the inflammatory responses in fish owing to infection by pathogenic *Edwardsiella tarda* (Birhanu et al. 2016). Considering the species relativeness to previously published *L. pentosus*, the isolate MT6 is promising to be explored as probiotic agent in further research.

The 16S rRNA nucleotide of isolate DT8 was similar to *Klebsiella pneumoniae* (99.79%). *K. pneumoniae* has been recognized as a cause of antibiotic-resistant bacterial infections in immunocompromised individuals. The type strain of this bacterium carried 5.54 Mb genome, in which several genes involved in the lactam resistance and toxin/antitoxin roles (Daligault et al. 2014). In aquatic environments, *K. pneumoniae* has previously been reported as an opportunistic pathogen that causes fish infections. This species is aquatic-borne as observed in the tropical estuaries (Barati et al. 2016). In addition, *K. pneumoniae* was isolated in the infected farmed Indian major carp *Labeo rohita* (Das et al. 2018). We did not observe specifically the potential pathogenicity of isolate DT8 in this study, however, the isolate may be useful as indicator micro-organisms for screening of antipathogenic biological agents.

Host ecology and environment is widely accepted to influence microbiota in the fish's gut (Wong and Rawls 2012). The manipulation of the fish microbiota may represent a new possibility in the prevention or management of pathological and physiological disorders (Thakur et al. 2014). Altogether, this study reflects that bacterial isolates from *N. sumatranus* are useful for further investigation of functional properties and bioprospecting to aquaculture.

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