INTRODUCTION

The need for a stable and durable source of high-quality protein has become necessary with the population explosion in recent years and aquaculture is considered to be the viable option for reducing the gap between production and human consumption (FAO 2014). Indeed, aquaculture has contributed significantly to the production of seafood since the 1970s however, the aquaculture industry faces a series of challenges (Bentzon-Tilia et al. 2016). As intensive aquaculture is developing, unfavorable impacts and stressful conditions occur more considerably on fish and aquatic system, including larval death, fish disease, and water pollution (Wang et al. 2008a, b).
The success of aquaculture practices depends on a number of correlated factors, including the cultured stock starting from the larvae as well as the aquatic environment. Fish larvae production is often hindered by high mortality rates, which may result from diseases or malnutrition (Conceição and Tandler 2018). Starvation is considered to be the leading cause of large proportion death of up to 99% of newly hatched fish larvae due to the insufficiency of their prey including Artemia, copepods, and rotifers (China and Holzman 2014). In order to improve live prey nourishing efficiency, feed for the zooplankton, e.g. phytoplankton or/ and ciliates, is primarily important (Das et al. 2012; Wikfors 2004). Ciliated protozoa abundance and biomass are highly related to the trophic status of the aquatic ecosystem, which is an integral component of the planktonic food web (Beaver and Crisman 1989).

Bacterioplankton and phytoplankton form the basis of the natural food chain in the aquatic ecosystem and often are grazed by zooplankton including ciliated protozoa as the main sources for its nutritional requirements (Bentzon-Tilia et al. 2016; Evjemo et al. 2003). The capacity of ciliates to graze differs depending on its genera or species as well as the type of bacteria, as some species are more attractive to the grazer than others (Ayo et al. 2001; Gruber et al. 2009). The distinctive characteristics of photosynthetic bacteria (PSB) in feeding prey organisms, bioremediation, probiotics stimulating and controlling disease have made it one of the main eco-friendly practice in aquaculture (Dawood et al. 2018; Wang et al. 2008a; Zhang et al. 2014).

The aims of the present study were (i) to evaluate the efficiency of different methods of ciliate cultivation and (ii) to test PSB as a feed to ciliate.

**MATERIALS AND METHODS**

**Photosynthetic bacteria (PSB) sample collection and isolate enrichment**

Sediment samples were collected from station St. Y in Yamagawa bay, Kagoshima, Japan (Fig. 1 A) from June to July 2016 with a gravity core sampler and the homogenized surface layer at the level of 1 cm depth were used for further analysis.

PSB bacterial strains were enriched by inoculating 0.6 – 0.8g solid sediment or 1 ml liquid sediment into 30 ml Basic I medium under anaerobic conditions as described by Wei et al. (2016). To obtain single bacterial colonies, the enriched cultures were spread on the Basic I double layer agar plates containing 1.5% agar for the bottom layer and 1.2% agar for the upper layer, under 12 hrs light: 12 hrs dark at 30 °C for 3-5 days. To purify the isolates, colonies exhibiting different distinct morphologies were isolated several times by single colony isolation procedure. A pure isolate encoded with PSB was preserved in Basic I media liquid form or agar plates until further analysis.

**PSB identification by 16S rRNA gene analysis**

For PSB identification, 1 ml of liquid bacterial culture was used for DNA extraction using DNeasy Plant Mini (Qiagen, Hilden, Germany). A universal primer set PrSSU.1F and PrSSU.1R was used for PCR amplification of 16S ribosomal RNA genes (16S rDNA) of the extracted DNA (Table 1). The PCR amplification mixture (100 µl) was consisted of 10 µl 10x Ex Taq Buffer (Takara Bio, Otsu, Japan), 4 µl of each dNTP, 5 µl of each primer, 0.5 µl Ex Taq DNA polymerase Hot Start Version (Takara Bio, Otsu, Japan), 5 µl bacterial DNA solution, and 70.5 µl H2O. The thermal cycling was performed by an initial denaturation at 94 °C for 1 min, followed by 25 cycles of denaturation at 94 °C for 30s, annealing at 58 °C for 30s, an extension at 72 °C for 90s, and a final extension at 72 °C was performed for 7 min.

The PCR products were subjected to 1.5 % agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 1 mM ethylene di-amine tetraacetic acid, EDTA) under a constant voltage condition of 100 V using GelMate 2000 electrophoresis system (Toyobo, Osaka, Japan). The electrophoresed gels were stained with Gel Red (GelRed® Nucleic Acid Gel Stain, Biotium, USA) and photographed under a blue trans-illuminator Safe Imager 2.0 (Invitrogen, Life Technologies, Carlsbad, USA). The PCR-amplified 16S rDNA nucleotide sequences were defined using a universal primer set PrSSU.2F, PrSSU.1R, and PrSSU.3R with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, USA) (Table 1).

The obtained sequences were assembled using DNASIS Pro Version 2.7 program (Hitachi Solutions, Tokyo, Japan). To identify the strain, the basic local alignment search tool BLAST program was used to find the closely related sequences in the GenBank DNA database. The partial sequences of 16S rRNA obtained in the present study were submitted to the DNA Data Bank of Japan (DDBJ) database and the accession numbers are shown at the end of the phylogenetic tree branches. Phylogenetic analysis with the neighbor-joining method performed using MEGA-X program (Kumar et al. 2018; Saitou and Nei 1987; Tamura et al. 2004, 2011).

**PSB staining**

PSB was harvested at the end of their logarithmic phase (14 days) by centrifugation at 10,000 rpm for 15 min. The pellets were resuspended in 10 ml buffered saline solution to achieve a density of 1.7±0.2×10⁶ cell
TABLE 1. Primers used in this study.

| Primer   | Method                | Sequence                                      | Reference                  |
|----------|-----------------------|------------------------------------------------|----------------------------|
| PrSSU.1F | PCR                   | 5’-AGAGTTTGATCCTGGCTCAG-3’                    | Wei et al. (2016)          |
| PrSSU.1R | PCR and Sequencing    | 5’-AAAGAAGGTGATCCAGCC-3’                      | Wei et al. (2016)          |
| PrSSU.2F | Sequencing            | 5’-TCCTACGGGAGGCAGCA-3’                       | Sambo et al. (2018)        |
| PrSSU.3R | Sequencing            | 5’-TACCGCGGTCTGGCA-3’                         | Kulakov et al. (2002)      |
| 1427F-GC | Sequencing plus GC clamp | 5’-TCTGTGATGCCTTAGATTTCTGGG-3’ plus GC clamp | Leão et al. (2012)         |
| 1616R    | Sequencing            | 5’-GCGGTGTGTACAAAGGGCAGGG-3’                  | Leão et al. (2012)         |

FIG. 1. Sampling stations map: (A) Yamagawa Bay, south of Kagoshima, Japan with geographical coordinates of the sampling stations, St.Y (31.2042N/130.6310E); (B) Kuwano-ura Bay, Koshiki Island, north west of Kagoshima, Japan at geographical coordinates (31.84633N/129.837E).
ml−1. DAPI staining of the PSB was performed according to Porter and Feig (1980).

In order to obtain the fluorescently-labeled bacteria (FLB), the method of Sherr et al. (1987) was performed by adding 5-(4,6-dichlorotiazin-2-yl) aminofluorescein (DTAF, Fluoresbrite, Polysciences Inc.) at 2µl ml−1 bacterial suspension for 2hrs at 60°C. To get rid of the excess fluorochrome, the FLB bacterial suspensions were centrifuged and resuspended four times.

Ciliated protozoa samples and cultivation

For ciliate experiments, sediment and water samples were collected from established station in Kuwano-ura bay, Koshiki Island, Kagoshima, Japan (Fig. 1 B). Three enrichment mixtures were tested for ciliate cultivation under the same experimental conditions at 23°C, pH 7.2, and continuous lighting with no aeration for 2 weeks. The first mixture (M I) follows the composition used by Mukai et al. (2016), in which 0.1 g anchovy fish meal (iRiKO, Co., Japan) and 0.1 g radish leaves (Brassica rapa) mixed into 5 ml filtered seawater. The second mixture (M II) consists of 0.1 g anchovy fish meal, 0.1 g radish leaves, and 0.1 g sediment mixed into 5 ml autoclaved sea water. In the third mixture (M III), 0.1 g sediment was mixed into 5 ml autoclaved algae media (Daigo’s IMK medium for marine microalgae). During the culture period, sub-samples were fixed with 2% neutralized formaldehyde and the number of ciliates was counted using Sedgwick-Rafter slide on a microscope.

Ciliates community was analyzed using denaturing gradient gel electrophoresis (DGGE) with universal primers set (1427F-GC and 1616R) for 18S rRNA gene (Table 1). The DNA of cultured ciliates was obtained using the Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). Amplification of the 18S ribosomal RNA gene was conducted using a PCR reaction mixture at a volume of 20 µl consisted of 1 µl 10-times diluted DNA, 2 µl 10x Ex Taq Buffer (Takara Bio, Otsu, Japan), 0.8 µl of each dNTP, 1 µl of each primer, 0.1 µl Ex Taq DNA polymerase Hot Start Version (Takara Bio, Otsu, Japan), and 14.1 µl H2O. A touchdown PCR (Don et al. 1991) were optimized with a PCR Thermal Cycler Dice Mini (Takara Bio, Japan) follows at 95° C as an initial denaturation for 5 min, followed by 19 cycles of 95 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min, decreasing the denaturation temperature after cycle 1 by 0.8 °C every cycle followed by 9 cycles of 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min. The final extension was performed using 1 cycle of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 10 min. PCR products were loaded onto 6 %polyacrylamide gel at gradient concentrations (25 – 55 %), 60 V, 60 °C for 16 h using D-Code System (Bio-Rad, Hercules, CA, USA). The gels were stained with SYBR Gold (Invitrogen, Carlsbad, CA, USA), and band presence was manually checked on a blue light trans-illuminator (Invitrogen). To obtain the sequencing data, the DGGE bands were excised from the gels using 1-mL pipette tips and suspended in 100 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid), and then were frozen and thawed to elute the amplified DNA. Sequencing was done using ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, USA) and the closest relatives of the sequenced 18S rRNA genes were determined by the basic local alignment search tool.

Grazing experiment

Two groups of different sized ciliates (greater or less than 30 µm) were used in this study as predators. Before grazing trial, cultured ciliates were harvested by passing cultures through polycarbonate filter (84 µm mesh for big size and 30 µm for small size) to remove larger particulates, then washed twice onto polycarbonate filters (30 µm mesh for big size and 5 µm for small size). Ciliates were kept in sterilized seawater for 6 hrs to clear food vacuoles. Fixed volumes of PSB suspension (0.78 OD660) were placed in 3 polycarbonate bottles (40 ml) contain the same level of sterilized seawater with big ciliate (B= 440±46 individual ml−1), small ciliate (S= 480±59 individual ml−1), or without ciliate as a control group (PSB), respectively.

During the grazing experiment, the reduction in the number of PSB was estimated by a spectrophotometer (HITACHI U-1100, Japan) at 660 nm (Wei et al. 2016), while the ciliates were counted by Sedgwick-Rafter slide under the optical microscope. To confirm ciliates grazing
on PSB, a fluorescently-labeled PSB (FLB) was offered to ciliates in filtered seawater and was examined within 24 hours with epifluorescence microscope.

Data analysis
All experiments were carried out in triplicates to confirm observed results. Statistical analysis (means ± SEM, standard error) was performed with analysis of variance (one way-ANOVA) followed by post-Hoc Duncan’s test using statistical analysis system (SAS) version 8.02 for Windows. Differences in the data were considered to be significant at the level of P ≤ 0.05.

RESULTS

Isolation and identification of PSB Strain
An anoxygenic photosynthetic purple non-sulfur bacterium was isolated from station Y (St. Y) in Yamagawa bay (Fig. 1). Its single bacterial colony was marked after 3-5 days of incubation period on Basic I medium agar plates with a pink color and smooth edges (Fig. 2), while the liquid cultures were obtained after 14 days (1.7±0.2×10^8 cell ml^-1). The microscopic examination of the isolate (named PSB1) showed short rod cell (1-2 µm length) with round ends. Phylogenetic tree including the isolate PSB1 was shown in Fig. 3. The result of phylogenetic analysis showed that closest strain was Rhodobacter sphaeroides (similarity is 100% of 1466bp).

FIG. 3. Photosynthetic bacterial isolate unrooted phylogenetic tree based on the 16S rDNA sequences. Bootstrap values are shown at the branching points. The tree was constructed by the neighbor-joining method. Nucleotide sequence database accession numbers are registered in the DNA Data Bank of Japan (DDBJ) and shown at the end of the tree branches. Bosea eneae which isolated from the sediment of Yamagawa bay was used as an outgroup.

FIG. 4. Growth of the ciliates in different culturing mixtures. Marine seawater or sediment samples were added into the mixtures MI(-----), MII(- - - -) and MIII(----) and cultivated. Error bars, standard deviation; lowercase letters, significant difference between the mixtures (ANOVA with post-hoc Duncan’s test, P≤0.05); not significant differences.

Ciliates cultivation
Figure 4 represents changes in ciliates densities cultured in different media. Ciliates were observed in all flasks and a significant increase in the number of ciliates observed on the third day (112, 167, 340 individual ml^-1) in MI, MII, and MIII, respectively. The peak of ciliates growth was on the seventh day, reaching 1424±54, 1509±61, and 2081±65 individual ml^-1 in MI, MII, and
Mill, respectively. The use of the algae media in Mill mixture produced the highest total number of ciliates.

**Ciliates identification**

The ciliates of “large size” (30 – 84 µm, A-1 and A-2 of Fig. 5) and “small size” (5 – 30 µm, B of Fig. 5) in the enrichment mixtures, whose cell morphology were shown in picture, were analyzed by 18S rDNA PCR-DGGE (results are shown in Fig. 6). Two unique bands were obtained from the large-size ciliates, whose nucleotide sequences were identical to *Euplotes minuta* (100% identity of 162 nucleotides) and *Cyclidium varibonneti* (99.3% identity of 139 nucleotides), respectively. The small-size protozoa provide a single band, and the same nucleotide sequence as the one of *Micromotopion nutans* was obtained with the identity of 87.6% (sequence length is 161 bp).

**Bacteriovorus ciliates grazing**

Figure 7 shows the changes in the abundance of *Rhodobacter sphaeroides* and ciliates during the grazing period. The abundance of *Rhodobacter sphaeroides* in the control group did not alter significantly until 96 hours later, then dropped dramatically and was considered the endpoint of the experiment. Ciliates peak growth rates were between 72 to 96 hrs for big size ciliates (2,193.33±13.34 individual ml⁻¹) and small size ciliates (1846.66±46.67 individual ml⁻¹). A significant reduction in the presence of bacteria was observed at 24 hours from the beginning of the experiment but with no difference between big and small size ciliates. From 36 to 72 hrs, the decrease in the number of bacteria with the larger ciliates was higher than the small size. The decline in the number of *Rhodobacter sphaeroides* as a result of grazing ciliates has been confirmed, using FLB. Microscopic examination of all types of the used ciliates shows clearly the presence of the labeled bacteria (FLB) in their bodies after the feeding trial (Fig. 8).

**DISCUSSION**

Purple non-sulfur photosynthetic bacteria (PNSB) *Rhodobacter sphaeroides* was isolated from St. Y of Yamagawa bay, Kagoshima, Japan. Previous studies indicated that PNSB widely live in diverse environments (Merugu et al. 2014; Okubo et al. 2006; Subudhi et al. 2016). The growth of PNSB in various environments can be attributed to their high ability to utilize a wide range of nutrients (Alloul et al. 2019; Wei et al. 2016) and its ability to survive in difficult environmental conditions (Kosamu and Obst 2009; Yegani et al. 2005). In the present study, *Rhodobacter sphaeroides* developed a rapid growth on Basic I medium as previously recommended by Wei et al. (2016). Also, the rapid growth of *Rhodobacter sp.* has been observed in pure and mixed cultures (Alloul et al. 2019; Kaewsuk et al. 2010).

The importance of ciliates in the aquatic systems and aquaculture has been noted in several studies (Ajeagah Aghaindum and Foto Menbohan 2012; Ayo et al. 2001, 2009; Chen et al. 2012; Gruber et al. 2009; Kar et al. 2017; Mukai et al. 2016; Pfister et al. 2002; Posch et al. 2001). Ciliates can be dramatically boosted using different cultivation media as the results of this study suggest. Ciliates, which are highly nutritious live feeds and preferred resources in terms of its rapid generation, small size, soft bodies, high quality protein and lipid content as well as its ability to adapt to a wide range of environmental conditions, is necessary to maintain the sustainability of aquatic organisms, especially at early stages (Corliss 2002; Das et al. 2012; Kar et al. 2017; Montagnes et al. 2010; Mukai et al. 2016). In the present study, the increased ciliates number three days post cultivation is consistent with the results obtained by Mukai et al. (2016). Also, Das et al. (2012) observed ciliates after 4 – 5 days of the enrichment. The density of cultured ciliates was about 1,424 – 2,081 individual ml⁻¹, which was higher than that obtained by Mukai et al. (2016) (500 – 1,000 individual ml⁻¹) and almost close to Côrtes et al. (2013) (2,500 individual ml⁻¹). These differences can be attributed to different ciliate species or and different culture conditions. The highest number of ciliates resulted from using algae media in the composition of the Mill mixture referring to the importance of algae in supporting the growth of ciliates as previously reported by Pfister et al. (2002).

Ciliates are very abundant phagotrophs that occur in almost all aquatic environments in which they graze on a wide range of algae, bacteria, and other microorganisms.
FIG. 6. Band profiles of the 18S rDNA PCR-DGGE. A, large-size ciliates; B, small-size ciliates. Numbers with dots represent the bands subjected to nucleotide sequencing and homology search analysis. Closest strain of A-1, A-2 and B-1 is *Euplotes minuta*, *Cyclidium varibonneti* and *Micrometopion nutans*, respectively.

FIG. 7. Abundance of the photosynthetic bacterial isolate (---) and ciliates (small size ——, big size ------) in the grazing experiment. Upper panel, bacterial abundances; lower panel, ciliate abundances. Error bars, standard deviation; lowercase letters, significant difference between the mixtures (ANOVA with post-hoc Duncan’s test, $P \leq 0.05$); NS, not significant differences.

FIG. 8. Epifluorescent micrographs of ciliates fed on the fluorescently labeled photosynthetic bacterial isolate. The ciliates and isolate were co-incubated for 24 h and observed under blue excitation after cell fixation with formaldehyde. A-1 and A-2, large-size ciliates; B, small-size ciliates.
as well as boost the microbial biomass remineralization and increase the transfer of nutrients to other organisms (Dziallas et al., 2012). A great section of ciliated protozoa devour on bacteria at rates vary according to bacteria species in terms of cell size, shape, surface properties, motility, and biochemical composition as well as predator size and feeding history (Ayo et al. 2009; Chen et al. 2012; Gruber et al. 2009; Posch et al. 2001; Sakaguchi et al. 2001). In the present study, the ability of PSB (Rhodobacter sphaeroides) to support the growth of ciliated protozoa adds a new feature and importance to these bacteria. In consist with our results, Myung et al. (2006) reported a novel phenomenon of the ingestion of the fluorescently labeled photosynthetic bacterial cells (FLB) by Myrionecta rubra ciliate. Also, Saccà et al. (2009) concluded that photosynthetic bacterial productivity is one of the foundations of protozoan grazing activity, which could channel a large proportion of planktonic production to higher trophic levels. Rhodobacter sphaeroides used in the present study was extensively grazed, reflecting their quality as prey. The size of R. sphaeroides is 1 – 2 µm length which is a perfect size for a large portion of predators (15 – 45 µm size) according to Chen et al. (2012) as the optimum predator: prey size ratio of about 8: 1 proposed by Jönsson (1986). Furthermore, small ciliates exhibited low grazing pressure. In this context, Ayo et al. (2001) pointed out that the size of ciliates is positively associated with the grazing rate as large-size ciliates had a higher grazing rate than small ones. In conclusion, this study indicates the possibility to support the growth of ciliated protozoa using photosynthetic bacteria (R. sphaeroides).

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