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Phylogenetic characterization and in situ detection of bacterial communities associated with seahorses (*Hippocampus guttulatus*) in captivity

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Abstract

Although there are several studies describing bacteria associated with marine fish, the bacterial composition associated with seahorses has not been extensively investigated as these studies have been restricted to the identification of bacterial pathogens. In this study, the phylogenetic affiliation of seahorse-associated bacteria was assessed by 16S rRNA gene sequencing of cloned DNA fragments. Fluorescence *in situ* hybridization (FISH) was used to confirm the presence of the predominant groups indicated by 16S rRNA analysis. Both methods revealed that *Vibrionaceae* was the dominant population in *Artemia* sp. (live prey) and intestinal content of the seahorses, while *Rhodobacteraceae* was dominant in water samples from the aquaculture system and cutaneous mucus of the seahorses. To our knowledge, this is the first time that bacterial communities associated with healthy seahorses in captivity have been described.

Keywords: Seahorses; Captivity conditions; Bacterial community structure; Clone libraries; Fluorescence *in situ* hybridization.

Short title: Bacterial diversity in seahorses

Introduction

Natural populations of many marine species have experienced drastic reductions in number during the last decades, largely due to the effects of overexploitation and inadequate management [19, 31, 34]. Seahorses constitute a group of marine fish, in which the consequences of their exploitation and degradation have been shown [16]. All seahorse species have therefore been listed on Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and included on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species [32]. Knowledge of the biological characteristics of affected species would be of major importance in conservation actions, development of breeding programmes and recovery of wild populations.

Although there are several studies describing bacteria associated with marine fish [12, 28], the bacterial composition associated with seahorses has not been extensively investigated as these studies have been restricted to the identification of bacterial pathogens [2, 8, 53]. Fish are intimately in contact with a complex and dynamic microbial world, and unlike mammals, a large fraction of these microorganisms adhere to and colonize epithelial surfaces. In rare circumstances microorganisms cause disease, either directly, by damaging or traversing epithelial layers, or indirectly, by inducing tissue-damaging inflammatory responses [26]. Because conventional characterization of microorganisms has depended on cultivation-based techniques, our understanding of the microbiota composition has been restricted to those that can be cultured [7]. More recently, however, the development of techniques that allow the genomic analysis of an assemblage of microorganisms, including those species that cannot be cultured by standard techniques, is now providing new ways to study microbial communities in their natural environments. Among them, cloning and sequencing of 16S rRNA genes is

being used to explore the microbial diversity from complex samples (i.e. Elshahed et al. [20]; Hugenholtz et al. [30]; Suzuki et al. [51]). Furthermore, the development of a microscopic tool based on fluorescence *in situ* hybridization (FISH) using rRNA-targeted oligonucleotide probes can be used not only for identification of unculturable species, but also provides a powerful tool for the quantification and visualization of distribution and association of microbial populations [5].

In this study, we determined the bacterial community composition associated to seahorses in captivity using clone libraries of 16S rRNA genes. We also contrasted the composition of these libraries with community structure determined by FISH using oligonucleotide probes to target all relevant identified bacterial groups. It is important to point out that seahorse maintenance requires the provision of live or frozen food, such as *Artemia*, mysid shrimp and copepods [42, 43, 60]. This practice could potentially introduce pathogenic microorganisms into the production system [46]. A better understanding of the bacterial community associated with healthy seahorses is therefore required in order to identify the roles of specific bacterial groups in both diseased and healthy individuals.

Materials and Methods

Captivity conditions and sample collection

Healthy adult seahorses (*Hippocampus guttulatus*) were collected from the coast of Galicia (NW Spain). After the capture, they (n = 6) were held for 1–2 years in experimental units (160 L) at 15–21 ºC and fed exclusively on enriched adult *Artemia* sp. (EG® Type, INVE Aquaculture, Baasrode, Belgium) according to a protocol previously described [43]. Briefly, the seahorses were fed twice daily at 09:30–10:30h and 16:00–18:00h, depending on the photoperiod regime. Food levels were adjusted daily (60–150 *Artemia*/seahorse) according to the season/temperature and to visual

observations of *Artemia* remaining in the experimental units from the previous day. Adult *Artemia* (length > 5.5 mm; 15–25 days old) were enriched with microalgae *Isochrysis galbana* and lyophilized *Spirulina* and Prolon® (INVE Aquaculture). A commercial product ACE® (INVE Aquaculture) was also added to reduce the bacterial load according to the manufacturer's instructions.

Four different types of samples (intestinal content and cutaneous mucus of the seahorses, *Artemia* sp. [1.0 g], and seawater of the aquaculture system [1.0 L]) were collected when seahorses were kept at 17 ºC. All samples were analyzed to compare the community composition of bacterial assemblages as revealed by 16S rRNA cloning and sequencing and FISH.

PCR and cloning

The four samples were centrifuged at 10,000 × *g* for 15 min and genomic DNA was extracted using the phenol-chloroform method [39]. 16S rRNA genes were amplified from total genomic DNA by PCR using general bacterial primers (27F, 5'- AGA GTT TGA TCM TGG CTC AG-3'; 1522R, 5'-AAG GAG GTG ATC CAN CCR CA-3') [24]. In a final volume of 25 µl, reaction mixtures contained 1× PCR buffer, 0.2 mM each dNTP's, 2.0 mM $MgSO_4$, primers (0.2 µM each), 10 ng of template, and 0.025 U of Platinum *Taq* High Fidelity enzyme (Invitrogen). All reactions were conducted in a DNA thermal cycler (GeneAmp PCR System 2700; Applied Biosystems), programmed with an initial 3 min enzyme activation step at 95 ºC, and 30 cycles of 1 min at 95 ºC, 1 min at 55 ºC, and 1.5 min at 72 ºC. A reconditioning step was conducted in order to reduce heteroduplex formation during PCR [56]. Briefly, 2.5 µl of PCR product from the first 30-cycle PCR was mixed with 22.5 µl of the same PCR cocktail used for the original PCR and the reactions were run after 3 min enzyme activation at 94 ºC followed by 3 cycles as above, and one hold at 72 ºC for 10 min. The amplified

products were purified and concentrated with the UltraClean purification kit (Mo Bio, Inc.), ligated into a TOPO TA cloning vector (Invitrogen, San Diego, CA), and electrotransformed into competent *Escherichia coli* cells according to the manufacturer's instructions. The transformed cells were spread onto Luria-Bertani plates containing 50 μg ml^{-1} kanamycin and treated with 40 mg ml^{-1} X-gal. Plates were incubated overnight at 37 °C. White colonies were picked into 96-well microtiter plates containing selective Luria-Bertani media and incubated for 18h at 37 °C. Sterile glycerol (15% final concentration) was added to each well, and the clones were stored at –80 °C until further processing. For each library, six replicates were pooled prior to cloning in order to minimise PCR bias and sample variation. Library clones were screened by *Hae*III RFLP analysis of 16S rRNA sequences as described previously [52].

Sequencing and phylogenetic analysis

All clones that had unique RFLP patterns were sequenced by the dideoxynucleotide-termination reaction using the Big Dye v3.1 kit (Applied Biosystems). Clone sequences were checked for chimeras by CHIMERA_CHECK [15]. Sequences from this study and reference sequences, as determined by BLAST [3] and Eztaxon [14] analyses, were subsequently aligned using the Fast Aligner algorithm in the ARB package [36]. All alignments were then visually verified and adjusted by hand according to the *E. coli* SSU rRNA secondary structure. Sequences from randomly chosen clones were compared and high similarity (equal to or greater than 98.5%) was observed. Therefore, sequences showing more than 98.5% similarity were considered to belong to the same operational taxonomic unit. Neighbor-joining trees incorporating a Jukes-Cantor distance correction were created from the alignments using the ARB software package [36]. Clone library coverage was calculated according to Singleton et al. [50], and

differences between clone libraries were statistically compared using ∫-LIBSHUFF [48]. ∫-LIBSHUFF uses distance matrices (in this case, Jukes-Cantor matrices) to estimate the coverage values for the clone libraries being compared over a range of taxonomic levels, enabling detection of significantly different microbial communities defined by nonoverlapping coverage. Randomizations (10,000) were run to determine the significance (P = 0.05) of overlapping coverage after correction for multiple pairwise comparisons using the Bonferroni method.

FISH

Freshly sampled portions from the four original samples (intestinal content and cutaneous mucus of the seahorses, *Artemia* sp., and seawater of the aquaculture system) were centrifuged and fixed with 4% (wt/vol) paraformaldehyde for 3 h as described by Manz et al. [38]. Fixed samples were transferred to slides (Marienfeld Laboratory Glassware, Germany) and air-dried overnight before dehydration by sequential washes in 50, 80, and 100% (vol/vol) ethanol for 3 min each. The oligonucleotide probes were synthesized and directly labelled with Cy3 and Cy5 or with FluosPrime (5,6-carboxyfluorescein-*N*-hydroxysuccinimide ester), all of which were purchased from Thermo Electron GmbH (Ulm, Germany). Sequences and optimal hybridization conditions for probes used are listed in Table 1. Samples were then mounted with Citifluor AF1 (Citiflour Ltd, UK) prior to microscopic observation, and image analysis was conducted on a Zeiss LSM 510 confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany) with the Zeiss Image Browser software. Different controls were employed to ensure the reliability of the obtained probe signals, such as the nonEUB338 probe, DAPI, hybridization without probes, and appropriate reference strains as positive as well as negative controls as described by Lee et al. [35]. Means were calculated from 10 randomly chosen fields on each filter section, corresponding from 600 to 800 DAPI-

stained cells. Concerning statistical analysis, we used nonparametric methods such as the Mann-Whitney U test.

Results

Bacterial diversity indicated by 16S rRNA gene analysis

PCR amplification of 16S rRNA genes from total DNA isolated from all four samples (intestinal content and cutaneous mucus of the seahorses, *Artemia* sp., and seawater of the aquaculture system) using 16S rRNA primers specific for bacteria yielded a band of the expected size of 1.5 kb. Combined PCR products were cloned into the vector pCR2.1-TOPO, yielding approximately 200 independent clones in each library. Ninety-eight of these clones from each library were analyzed and grouped by amplified ribosomal DNA restriction analysis. In total, 108 independent clones were sequenced and subjected to phylogenetic analysis. The statistical comparison showed significant differences in the composition of libraries between the cutaneous mucus and water samples (P = 0.001). Similar results were obtained when the intestinal content and *Artemia* sp. samples were compared with cutaneous mucus (P = 0.001) or water samples (P = 0.001), but not between *Artemia* sp. and intestinal contents (P = 1.07).

Clone libraries of 16S rRNA genes amplified from *Artemia* sp. and intestinal contents showed a high number of sequences affiliated to the class *Gammaproteobacteria*, ranging from 75.4 to 79.4 % of the clones analyzed (Figure 1). The predominant sequences were affiliated with the family *Vibrionaceae* and comprised from 44.2 to 49.7 % of the total abundance. Clones belonging to *Actinobacteria* (3.6–7.7 %), *Alphaproteobacteria* (5.5–7.2 %), *Bacteroidetes* (4.4–7.2 %) and *Betaproteobacteria* (2.2–6.0 %) were also found in both samples.

The *Alphaproteobacteria* group was found to be the dominant bacterial population in cutaneous mucus and water samples. The abundance of *Alphaproteobacteria*,

particularly represented by sequences of the family *Rhodobacteraceae*, in the clone libraries of both samples ranged from 53.7 to 61.3 % (Figure 2). Clones belonging to the *Gammaproteobacteria* group were also found (16.8–24.1%), followed by *Bacteroidetes* (9.6–11.0 %), *Actinobacteria* (6.0–7.2 %) and *Betaproteobacteria* (4.8–4.9 %).

FISH analysis

Most of the bacteria visualized with DAPI staining (92.8 ± 0.4) were detectable with the EUB338 probe specific for *Bacteria*. The background signal of samples, observed with the probe NON338, was negligible (< 0.1%).

FISH with several group-specific probes confirmed the general community structure indicated by 16S rRNA gene analysis of the bacterial clone libraries. Counts made with the GAM42a probe revealed that the *Gammaproteobacteria* was dominant in *Artemia* sp. (54.7 %) and intestinal content of *H. guttulatus* (64.2 %), especially members of the family *Vibrionaceae* as indicated by hybridization with the GV probe (Table 2). The *Betaproteobacteria* represented the second major phylogenetic group in *Artemia* sp. and intestinal content samples, while other groups such as *Alphaproteobacteria*, *Actinobacteria* and *Bacteroidetes* were less abundant (Table 2).

Quantification by FISH using the ALF968 probe, which targets most of the *Alphaproteobacteria*, showed that this group was prevalent in the seawater samples and cutaneous mucus, ranging from 56.8 to 59.7 %, respectively (Table 3). Members of other phylogenetic groups such as *Betaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria* and *Bacteroidetes* were also detected in the water samples and cutaneous mucus but in lower proportions (Table 2).

Discussion

The genus *Vibrio* is one of the most common and widely distributed groups of marine bacteria [40, 54, 55]. Although *Artemia* sp. cysts have been found to contain few bacteria [17], the bacterial load, particularly *Vibrionaceae* levels, may thus drastically increase in *Artemia* sp. nauplii if *Vibrionaceae* species are present in the surrounding water or/and in the enrichment medium. This suggests that *Vibrionaceae* species were present in the seawater and thus colonize *Artemia* sp. after hatching. In the present study, the 16S rRNA gene clone libraries from *Artemia* sp. and intestinal contents of *H. guttulatus* showed a high number of sequences affiliated to *Vibrionaceae* (Fig. 1). Statistical comparison did not reveal significant differences between the clone libraries from these sources. These observations indicate thus that *Artemia* sp. may have a strong influence on the bacterial community structure in the intestinal content of *H. guttulatus*. The most abundant clones found in both sources were closely related to the species *Vibrio splendidus* and *Vibrio cyclitrophicus*. The FISH analysis also revealed that *Vibrionaceae* were the dominant bacterial population in *Artemia* sp. and intestinal content, ranging from 45.8 to 48.5 %, respectively (Table 2). *Vibrio splendidus* and *V. cyclitrophicus* are ubiquitous microorganisms in seawater and have been frequently isolated from marine fish and invertebrates [10, 54]. However, some studies have suggested that these species are pathogenic to several marine organisms [18, 27, 40, 45, 57].

The 16S rRNA gene clone libraries from the seawater samples and cutaneous mucus showed a high number of sequences affiliated to *Rhodobacteraceae* (Fig. 2). However, statistical comparison showed significant differences in the composition of libraries between the two samples. The most abundant clones in the water sample were closely related to the genera *Antarctobacter*, *Nereida* and *Phaeobacter*, while the genera *Phaeobacter* and *Ruegeria* were dominant in the cutaneous mucus. Microscopic

enumeration of bacteria hybridized with specific probes also revealed a community dominated by *Rhodobacteraceae* (GRb probe) in both samples, ranging from 51.0 to 52.1 % (Table 2). Members of the family *Rhodobacteraceae*, particularly the genus *Roseobacter*, are ubiquitous in marine environments and have been found to produce antibacterial compounds against several species, including members of the family *Vibrionaceae* [9, 11, 58]. These properties could explain their dominance as they may compete with other microorganims for space and essential nutrients in the culture system.

Several studies have found a close correspondence between clone library composition and in situ community composition determined by FISH [13, 49]. However, our study suggests that clone libraries of 16S rRNA genes amplified using general bacterial primers overestimates the relative abundance of *Gammaproteobacteria* (clone libraries, 16.8-79.4%; FISH, 5.2-64.2%) and underestimates the *Betaproteobacteria* group (clone libraries, 2.2-6.0%; FISH, 13.3-22.1%), when we compare with the results obtained by FISH. Factors such as cloning biases [1], variation in genome size [21, 23], rRNA gene copy number [33] and ribosome content [22], could have resulted in larger differences than we observed between clone libraries and the community structure determined by FISH. For example, a computer search using the Ribosomal RNA Operon Copy Number Database [33] revealed that the 222 members of the *Gammaproteobacteria* group that have been examined average 5.75 copies of the rRNA operon, while the 60 *Betaproteobacteria* have only 3.90 copies on average. Based on these data, we would expect the *Betaproteobacteria* group to be underrepresented in clone libraries.

In summary, molecular analysis of bacterial community composition detected by both methods (clone libraries and FISH) in *Artemia* sp. and intestinal content of seahorses showed that the dominant population appeared to be *Vibrionaceae*. Because this family

has been frequently associated with disease in several aquatic organisms, the information obtained here on their abundance is significant. The dominance of *Rhodobacteraceae* in the cutaneous mucus and water samples analyzed in this study is also important since several species of this family have exhibited potential probiotic properties [29, 44]. Therefore, further studies may benefit from these molecular approaches that determine which phylogenetic groups of bacteria dominate in the associated microbiota, as specific groups may play substantial roles in normal and perturbed host physiological states.

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References

[1] S.G. Acinas, R.S. Sarma-Rupavtarm, V. Klepac-Ceraj, M.F. Polz, PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same simple. Appl. Environ. Microbiol. 71 (2005) 8966–8969.

[2] E. Alcaide, C. Gil-Sanz, E. Sanjuan, D. Esteve, C. Amaro, L. Silveira, *Vibrio harveyi* causes disease in seahorse, *Hippocampus* sp. J. Fish Dis. 24 (2001) 311–313.

[3] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool. J. Mol. Biol. 215 (1990) 403–410.

[4] R. Amann, B.M. Fuchs, Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. Nat. Rev. Microbiol. 6 (2008) 339–348.

[5] R. Amann, B.M. Fuchs, S. Behrens, The identification of microorganisms by fluorescence in situ hybridisation. Curr. Opin. Biotechnol. 12 (2001) 231–236.

[6] R.I. Amann, B.J. Binder, R.J. Olson, S.W. Chisholm, R. Devereux, D.A. Stahl, Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56 (1990) 1919–1925.

[7] B. Austin, The bacterial microflora of fish. Scientific World J. 6 (2006) 931–945.

[8] J.L. Balcázar, A. Gallo-Bueno, M. Planas, J. Pintado, Isolation of *Vibrio alginolyticus* and *Vibrio splendidus* from cautive-bred seahorses with disease symptoms. Antonie van Leeuwenhoek. in press.

[9] J.L. Balcázar, T. Rojas-Luna, D.P. Cunningham, Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus*. J. Invertebr. Pathol. 96 (2007) 147–150.

[10] R. Beaz-Hidalgo, I. Cleenwerck, S. Balboa, M. De Wachter, F.L. Thompson, J. Swings, P. De Vos, J.L. Romalde, Diversity of vibrios associated with reared clams in Galicia (NW Spain). Syst. Appl. Microbiol. 31 (2008) 215–222.

[11] J.B. Bruhn, L. Gram, R. Belas, Production of antibacterial compounds and biofilm formation by *Roseobacter* species are influenced by culture conditions. Appl. Environ. Microbiol. 73 (2007) 442–450.

[12] M.M. Cahill, Bacterial flora of fishes: a review. Microb. Ecol. 19 (1990) 21–41.

[13] S.C. Cary, M.T. Cottrell, J.L. Stein, F. Camacho, D. Desbruyeres, Molecular identification and localization of filamentous symbiotic bacteria associated with the hydrothermal vent annelid *Alvinella pompejana*. Appl. Environ. Microbiol. 63 (1997) 1124–1130.

[14] J. Chun, J.H. Lee, Y. Jung, M. Kim, S. Kim, B.K. Kim, Y.W. Lim, Eztaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. Int. J. Syst. Evol. Microbiol. 57 (2007) 2259–2261.

[15] J.R. Cole, B. Chai, R.J. Farris, Q. Wang, S.A. Kulam, D.M. McGarrell, G.M. Garrity, J.M. Tiedje, The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res. 33 (2005) D294–D296.

[16] J.M.R. Curtis, A.C.J. Vincent, Use of population viability analysis to evaluate CITES trade-management options for threatened marine fishes. Conserv. Biol. 22 (2008) 1225–1232.

[17] M. Dehasque, L. Verdonck, P. Sorgeloos, J. Swings, P. Léger, K. Kersters, Determination of the bacterial contamination in live food production systems in marine fish hatcheries in southern Europe, In: P. Lavens, P. Sorgeloos, E. Jaspers, F. Ollevier (Eds.), LARVI'91–Fish and Crustacean Larviculture Symposium. European Aquaculture Society, Ghent, 1991, pp. 339–402.

[18] H. Deng, C. He, Z. Zhou, C. Liu, K. Tan, N. Wang, B. Jiang, X. Gao, W. Liu, Isolation and pathogenicity of pathogens from skin ulceration disease and viscera

ejection syndrome of the sea cucumber *Apostichopus japonicus*. Aquaculture 287 (2009) 18–27.

[19] N.K. Dulvy, J.R. Ellis, N.G. Goodwin, A. Grant, J.D. Reynolds, S. Jennings, Methods of assessing extinction risk in marine fishes. Fish Fish. 5 (2004) 255–276.

[20] M.S. Elshahed, J.M. Senko, F.Z. Najar, S.M. Kenton, B.A. Roe, T.A. Dewers, J.R. Spear, L.R. Krumholz, Bacterial diversity and sulfur cycling in a mesophilic sulfide-rich spring. Appl. Environ. Microbiol. 69 (2003) 5609–5621.

[21] V. Farrelly, F.A. Rainey, E. Stackebrandt, Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl. Environ. Microbiol. 61 (1995) 2798–2801.

[22] F. Fegatella, J. Lim, S. Kjelleberg, R. Cavicchioli, Implications of rRNA operon copy number and ribosome content in the marine oligotrophic ultramicrobacterium *Sphingomonas* sp. strain RB2256. Appl. Environ. Microbiol. 64 (1998) 4433–4438.

[23] G.B. Fogel, C.R. Collins, J. Li, C.F. Brunk, Prokaryotic genome size and SSU rDNA copy number: Estimation of microbial relative abundance from a mixed population. Microb. Ecol. 38 (1999) 93–113.

[24] S.J. Giovannoni, The polymerase chain reaction. In: E. Stackebrandt, M. Goodfellow (Eds.), Nucleic acid techniques in bacterial systematic, John Wiley & Sons, New York, 1991, pp. 177–203.

[25] L. Giuliano, M. De Domenico, E. De Domenico, M.G. Höfle, M.M. Yakimov, Identification of culturable oligotrophic bacteria within naturally occurring bacterioplankton communities of the Ligurian Sea by 16S rRNA sequencing and probing. Microb. Ecol. 37 (1999) 77–85.

[26] G.D. Gómez, J.L. Balcázar, A review on the interactions between gut microbiota and innate immunity of fish. FEMS Immunol. Med. Microbiol. 52 (2008) 145–154.

[27] J. Gómez-León, L. Villamil, M.L. Lemos, B. Novoa, Isolation of *Vibrio alginolyticus* and *Vibrio splendidus* from aquacultured carpet shell clam (*Ruditapes decussatus*) larvae associated with mass mortalities. Appl. Environ. Microbiol. 71 (2005) 98–104.

[28] G.H. Hansen, J.A. Olafsen, Bacterial interactions in early life stages of marine cold water fish. Microb. Ecol. 38 (1999) 1–26.

[29] M. Hjelm, Ø. Bergh, A. Riaza, J. Nielsen, J. Melchiorsen, S. Jensen, H. Duncan, P. Ahrens, H. Birkbeck, L. Gram, Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (*Scophthalmus maximus*) rearing units. Syst. Appl. Microbiol. 27 (2004) 360–371.

[30] P. Hugenholtz, B.M. Goebel, N.R. Pace, Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180 (1998) 4765–4774.

[31] J.A. Hutchings, J.D. Reynolds, Marine fish population collapses: consequences for recovery and extinction risk. Bioscience. 54 (2004) 297–309.

[32] IUCN Red list of Threatened Species, 2006. Available at www.redlist.org.

[33] J.A. Klappenbach, P.R. Saxman, J.R. Cole, T.M. Schmidt, rrndb: the ribosomal RNA operon copy number database. Nucleic Acids Res. 29 (2001) 181–184.

[34] W.S. Lakra, V. Mohindra, K.K. Lal, Fish genetics and conservation research in India: status and perspectives. Fish Physiol. Biochem. 33 (2007) 475–487.

[35] N. Lee, P.H. Nielsen, H. Aspegren, M. Henze, K.H. Schleifer, J. la Cour Jansen, Long-term population dynamics and in situ physiology in activated sludge

systems with enhanced biological phosphorus removal operated with and without nitrogen removal. Syst. Appl. Microbiol. 26 (2003) 211–227.

[36] W. Ludwig, O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A.W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, K.H. Schleifer, ARB: a software environment for sequence data. Nucleic Acids Res. 32 (2004) 1363-1371.

[37] W. Manz, R. Amann, W. Ludwig, M. Vancanneyt, K.H. Schleifer, Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *Cytophaga-Flavobacter-Bacteroides* in the natural environment. Microbiology. 142 (1996) 1097–1106.

[38] W. Manz, R. Amann, W. Ludwig, M. Wagner, K.H. Schleifer, Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. Syst. Appl. Microbiol. 15 (1992) 593–600.

[39] R. Massana, A.E. Murray, C.M. Preston, E.F. DeLong, Vertical distribution and phylogenetic characterization of marine planktonic Archaea in the Santa Barbara channel. Appl. Environ. Microbiol. 63 (1997) 50–56.

[40] K.H. Nealson, B. Wimpee, C. Wimpee, Identification of *Vibrio splendidus* as a member of the planktonic luminous bacteria from the Persian Gulf and Kuwait Region with *luxA* probes. Appl. Environ. Microbiol. 59 (1993) 2684–2689.

[41] A. Neef, R. Witzenberger, P. Kämpfer, Detection of sphingomonads and *in situ* identification in activated sludge using 16S rRNA-targeted oligonucleotide probes. J. Ind. Microbiol. Biotechnol. 23 (1999) 261–267.

[42] M.F. Payne, R.J. Rippingale, Evaluation of diets for culture of the calanoid copepod *Gladioferens imparipes*. Aquaculture. 187 (2000) 85–96.

[43] M. Planas, A. Chamorro, P. Quintas, A. Vilar, Establishment and maintenance of threatened long-snouted seahorse, *Hippocampus guttulatus*, broodstock in captivity. Aquaculture. 283 (2008) 19–28.

[44] M. Planas, M. Pérez-Lorenzo, M. Hjelm, L. Gram, I.U. Fiksdal, Ø. Bergh, J. Pintado, Probiotic effect in vivo of *Roseobacter* strain 27-4 against *Vibrio* (*Listonella*) *anguillarum* infections in turbot (*Scophthalmus maximus* L.) larvae. Aquaculture. 255 (2006) 323–333.

[45] H.I. Reid, J.W. Treasurer, B. Adam, T.H. Birkbeck, Analysis of bacterial populations in the gut of developing cod larvae and identification of *Vibrio logei*, *Vibrio anguillarum* and *Vibrio splendidus* as pathogens of cod larvae. Aquaculture 288 (2009) 36–43.

[46] E. Ringø, T.H. Birkbeck, Intestinal microflora of fish larvae and fry. Aquacult. Res. 30 (1999) 73–93.

[47] C. Roller, M. Wagner, R. Amann, W. Ludwig, K.H. Schleifer, *In situ* probing of Gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides. Microbiology. 140 (1994) 2849–2858.

[48] P.D. Schloss, B.R. Larget, J. Handelsman, Integration of microbial ecology and statistics: a test to compare gene libraries. Appl. Environ. Microbiol. 70 (2004) 5485–5492.

[49] A. Schramm, D. de Beer, M. Wagner, R. Amann, Identification and activities in situ of *Nitrosospira* and *Nitrospira* spp. as dominant populations in a nitrifying fluidized bed reactor. Appl. Environ. Microbiol. 64 (1998) 3480–3485.

[50] D.R. Singleton, M.A. Furlong, S.L. Rathbun, W.B. Whitman, Quantitative comparisons of 16S rRNA gene sequences libraries from environmental samples. Appl. Environ. Microbiol. 67 (2001) 4374–4376.

[51] M.T. Suzuki, L.T. Taylor, E.F. DeLong, Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays. Appl. Environ. Microbiol. 66 (2000) 4605–4614.

[52] M.T. Suzuki, M.S. Rappé, Z.W. Haimberger, H. Winfield, N. Adair, J. Ströbel, S.J. Giovannoni, Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. Appl. Environ. Microbiol. 63 (1997) 983–989.

[53] E.A. Tendencia, The first report of *Vibrio harveyi* infection in the sea horse *Hippocampus kuda* Bleekers 1852 in the Philippines. Aquacult. Res. 35 (2004) 1292–1294.

[54] F.L. Thompson, T. Iida, J. Swings, Biodiversity of vibrios. Microbiol. Mol. Biol. Rev. 68 (2004) 403–431.

[55] J.R. Thompson, S. Pacocha, C. Pharino, V. Klepac-Ceraj, D.E. Hunt, J. Benoit, R. Sarma-Rupavtarm, D.L. Distel, M.F. Polz, Genotypic diversity within a natural coastal bacterioplankton population. Science 307 (2005) 1311–1313.

[56] J.R. Thompson, L.A. Marcelino, M.F. Polz, Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by reconditioning PCR. Nucleic Acids Res. 30 (2002) 2083–2088.

[57] R. Thomson, H.L. Macpherson, A. Riaza, T.H. Birkbeck, *Vibrio splendidus* biotype 1 as a cause of mortalities in hatchery-reared larval turbot *Scophthalmus maximus* (L.). J. Appl. Microbiol. 99 (2005) 243–250.

[58] I. Wagner-Dobler, H. Biebl, Environmental biology of the marine *Roseobacter* lineage. Annu. Rev. Microbiol. 60 (2006) 255–280.

[59] G. Wallner, R. Amann, W. Beisker, Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry. 14 (1993) 136–143.

[60] C.M.C. Woods, F. Valentino, Frozen mysids as an alternative to live *Artemia* in culturing seahorses *Hippocampus abdominalis*. Aquacult. Res. 34 (2003) 757–763.

Table 1. Sequences of oligonucleotide probes used for FISH

Table 2. Quantification of various phylogenetic groups by FISH

Figure 1. Phylogenetic trees based on comparative analysis of 16S rRNA genes (approximately 1400 bp) from selected clones affiliated to the *Betaproteobacteria* and *Gammaproteobacteria.* Clones from the intestinal content, cutaneous mucus, *Artemia* sp. and seawater were designated as IC, CM, ART or W. The number of clones in the corresponding RFLP group is shown in square brackets. Selected sequences from the phylum of *Thermotogae* were used to root the trees. The scale bar represents nucleotide substitutions. The nucleotide sequences derived from this study have been deposited in the GenBank/EMBL/DDBJ databases under accession numbers FM878642-FM878657, FM882248, FM958451-FM958470, FN582312-FN582328.

Figure 1. Phylogenetic trees based on comparative analysis of 16S rRNA genes (approximately 1400 bp) from selected clones affiliated to the *Alphaproteobacteria*, *Actinobacteria* and *Bacteroidetes.* Clones from the intestinal content, cutaneous mucus, *Artemia* sp. and seawater were designated as IC, CM, ART or W. The number of clones in the corresponding RFLP group is shown in square brackets. Selected sequences from the phylum of *Thermotogae* were used to root the trees. The scale bar represents nucleotide substitutions. The nucleotide sequences derived from this study have been deposited in the GenBank/EMBL/DDBJ databases under accession numbers FM878642-FM878657, FM882248, FM958451-FM958470, FN582312-FN582328.

Table 1

Probe	Sequence (5'–3')	rRNA target site (position)	Specificity	Group coverage (%)	% FA[c]	Reference
EUB338	GCTGCCTCCCGTAGGAGT	16S (338-355)	Domain *Bacteria*	92[a]	35	Amann *et al*. [6]
NON338	ACTCCTACGGGAGGCAGC	16S (338-355)	Complementary to EUB338	< 0.01[a]	0	Wallner *et al*. [59]
HGC69a	TATAGTTACCACCGCCGT	23S (1901-1918)	*Actinobacteria*	93[b]	25	Roller *et al*. [47]
ALF968	GGTAAGGTTCTGCGCGTT	16S (968-986)	*Alphaproteobacteria*	79[a]	35	Neef *et al*. [41]
BET42a	GCCTTCCCACTTCGTTT	23S (1027-1043)	*Betaproteobacteria*	86[b]	35	Manz *et al*. [38]
GAM42a	GCCTTCCCACATCGTTT	23S (1027-1043)	*Gammaproteobacteria*	76[b]	35	Manz *et al*. [38]
CF319a	TGGTCCGTGTCTCAGTAC	16S (319-336)	*Bacteroidetes*	43[a]	35	Manz *et al*. [37]
GV	AGGCCACAACCTCCAAGTAG	16S (841-822)	*Vibrio*	84[a]	30	Giuliano *et al*. [25]
GRb	GTCAGTATCGAGCCAGTGAG	16S (645-626)	*Rhodobacteraceae*	68[a]	30	Giuliano *et al*. [25]

[a] The probe specificity was carried out using the PROBE MATCH tool of the Ribosomal Database Project.

[b] Data obtained from Amman and Fuchs [4].

[c] Values represent percent formamide in the hybridization buffer.

Table 2

Group	Fraction (%) of total cells (mean ± SD) detected with probe[a]			
	Intestinal content	*Artemia*	Cutaneous mucus	Water
Actinobacteria	5.81 ± 1.10	2.36 ± 1.41	6.40 ± 0.59	4.61 ± 1.83
Alphaproteobacteria	7.40 ± 1.26	7.83 ± 4.25	59.72 ± 2.86	56.82 ± 7.60
Betaproteobacteria	13.28 ± 0.51	22.14 ± 7.81	16.89 ± 1.56	14.45 ± 3.95
Gammaproteobacteria	64.23 ± 3.79	54.72 ± 10.88	5.16 ± 1.29	8.04 ± 2.90
Bacteroidetes	2.07 ± 0.46	5.80 ± 3.57	5.11 ± 1.03	8.37 ± 1.87
Rhodobacteraceae	6.71 ± 0.94	7.45 ± 4.81	52.12 ± 9.23	50.98 ± 10.49
Vibrionaceae	48.46 ± 5.40	45.75 ± 12.88	4.39 ± 1.25	6.08 ± 2.02
Bacteria	92.79 ± 7.05	92.85 ± 6.31	93.28 ± 7.23	92.29 ± 7.66

[a]Percent detection compared to DAPI. Numbers were corrected by subtracting NON338 counts.

Figure 1

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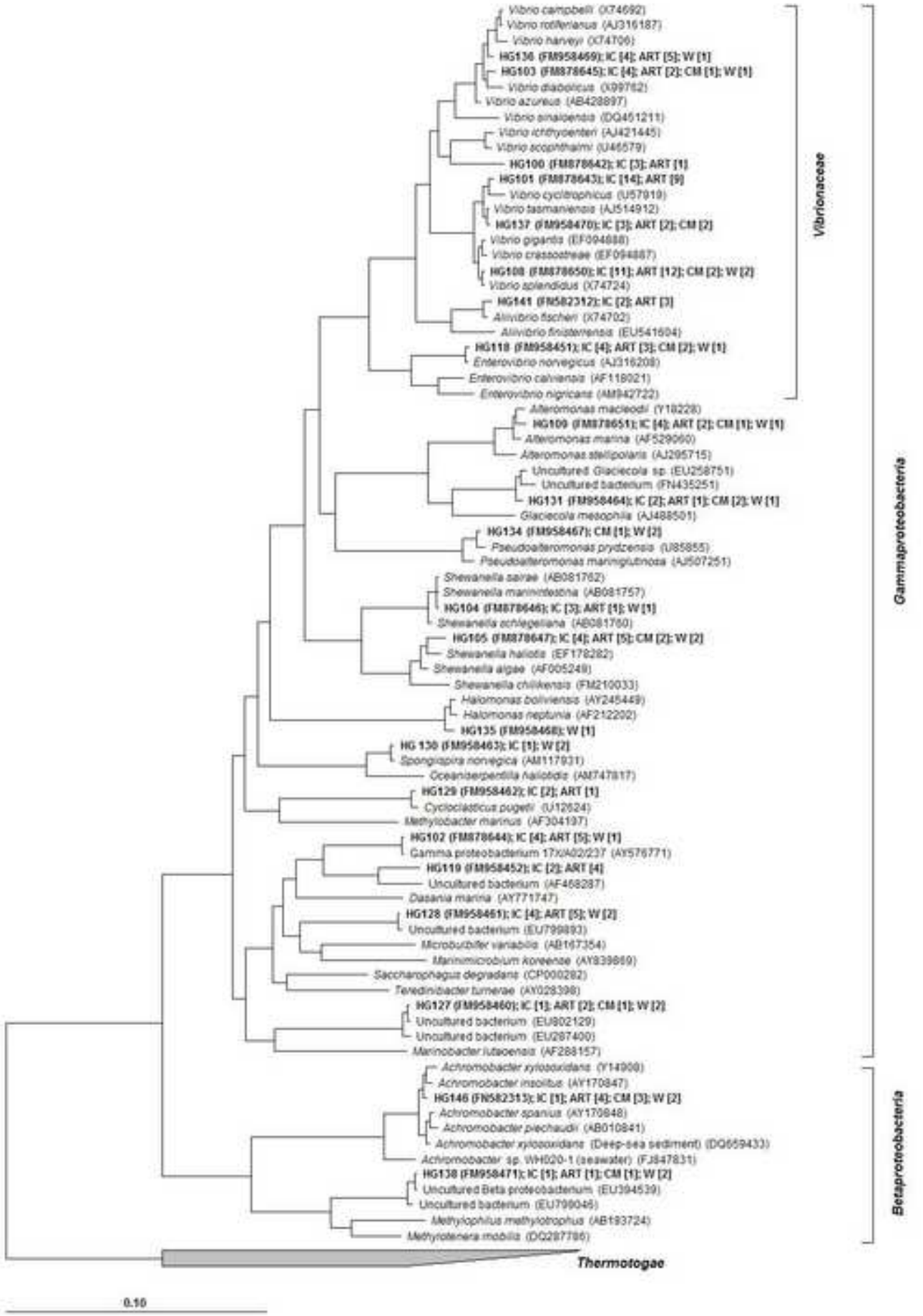

Figure 2

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