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Improvement of DNA Extraction and Electrophoresis Conditions for the PCR-DGGE Analysis of Bacterial Communities Associated to Two Aquaculture Fish Species

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ABSTRACT

Improvement of a denaturing gradient gel electrophoresis (DGGE) method for analyzing bacterial microbiota 16S rDNA in fish is presented. Tilapia and sea bass were collected from different locations in Thailand and France, respectively. The DGGE electrophoresis conditions were optimized which indicated that a decrease in electrophoresis applied voltage and an increase in the running time (80 V for 12 h) provided optimal band resolution. DNA extraction procedure was also optimized. PCR-DGGE analysis patterns of bacterial DNA from tilapia and sea bass showed that there was a unique band pattern for each type of fish and dissimilarity was observed even within the same species. The differences in bacterial community in fish were probably due to different locations of collections.

Keywords: aquaculture, bacteria, ecology, electrophoresis
Abbreviations: DGGE, denaturing gradient gel electrophoresis; **MATAB**, mixed alkyltrimethyl ammonium bromide; **PCR**, polymerase chain reaction; **SDS**, sodium dodecyl sulphate

INTRODUCTION

Aquatic microorganisms not only influence the water quality but are also known to be closely associated with the physiological status of fish and their post-harvest quality. Surveys have been done on the bacterial flora in fish species captured from various geographical locations (Grisez *et al*. 1997; Spanggaard *et al*. 2000; Al-Horbi and Uddin 2003). Microorganisms are found on the outer surfaces (skin and gills) as well as in the intestines of live and newly caught fish. The bacterial flora on tropical freshwater fish are very similar to the flora of temperate species, consisting of *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Lactobacillus*, *Alcaligenes*, *Streptococcus*, *Brevibacterium*, *Escherichia*, *Clostridium*, *Acinetobacter*, *Moraxella* and *Vibrio* which are commonly found on newly-caught fish. *Aeromonas* spp. are ubiquitous inhabitants of aquatic ecosystem such as freshwater; these bacteria have a broad host range and have often been isolated from humans with diarrhoea (Syvokiene and Mickeniene 1999; El-Shafai *et al*. 2004). Some common bacterial flora of water, such as *Pseudomonas fluorescens*, *Aeromonas hydrophila*, *Edwardsiella tarda*, *Vibrio* sp. and *Myxobacteria*, can be found on the body surface or in the intestinal tracts of fish under normal conditions but under environmental stress, they could lead to epizootic diseases (Al-Horbi and Uddin 2003).

The fish microbiota in aquaculture has previously been studied for many purposes. Detection of bacterial communities in fish samples have been mostly done by cultivation-dependent plating techniques and their identification by phenotypical and biochemical characteristics (Grisez *et al*. 1997; Syvokiene and Mickeniene 1999; Spanggaard *et al*. 2000; Al-Horbi and Uddin 2003, 2004; El-Shafai *et al*. 2004). This procedure is time-consuming, and it uses only a limited number of key characteristics. In order to provide better insight into the diversity of bacterial community in fish, there is a need for cultivation-independent techniques enabling the direct information of these populations. In recent years, applied molecular ecology methods have been widely used for analyzing microbial community in various environments and have become a valid complement to traditional techniques. The denaturing gradient gel electrophoresis (DGGE) of 16S rDNA fragments, generated by polymerase chain reaction (PCR-DGGE), is now a popular method for assessing ecology of microbial communities in various environments (Muyzer *et al*. 1993; Ovreas *et al*. 1997; Ampe *et al*. 1999; Dièz *et al*. 2001). DGGE has advantages that provide information on the microorganisms present and the community changes by analyzing DNA bands that migrate separately on DGGE gel, according to the melting point of 16S rDNA fragments (Omar and Ampe 2000; Sekiguchi *et al*. 2002; Ercolino 2004; Watanabe *et al.* 2004).

To our knowledge, there are only four published papers that analyzed microbial communities in fish by DGGE (Spanggaard *et al*. 2000; Huber *et al.* 2004) including two of our team (LeNguyen *et al.* 2007; Maiworé *et al,* 2009). The purpose of the present study was to establish a DGGE method for analyzing dominant bacterial communities in fish with the further objective to create a technique to link bacterial community to the geographical origin of food (fish). To achieve this objective, a DNA extraction method was optimized and voltage (V)-hours (h) combinations of DGGE running conditions were evaluated for use in analysis of fish bacterial communities.

MATERIALS AND METHODS

Fish samples

Tilapias were collected from ponds of three freshwater fish farms in Thailand: Khon Kaen, (450 km Northeast of Bangkok); Nakorn

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Pratom (50 km West of Bangkok) and Ladkrabang (Bangkok). Sea basses were collected from two fish farms in France: Aquanord (Gravelines) and Viviers du Gois (Beauvoir sur Mer), North-West of France. The fish samples were placed immediately on ice and then stored at 20°C.

Improvement of the total DNA extraction

DNA extraction was based on the method of Ampe *et al.* (1999) but modified as follows. The extraction was replicated two times per sample. Around 2 g each of gills, skin and intestine were aseptically removed from each fish specimen and homogenized for 3 min with a vortex with maximum speed after addition of 6 mL sterile peptone water (pH 7.0). The homogenate samples were then centrifuged at 10,000 × *g* at room temperature (25°C) for 15 min. The pellets were suspended into 100 µL of a lysis buffer TE (10 mM Tris-HCl; 1 mM EDTA; pH 8.0) followed by addition of 100 µL of lysozyme (25 mg mL^{-1}) and 50 µL of proteinase K (10 mg mL^{-1}) and the suspension was incubated at 42°C for 20 min. Then, 50 µL of 20% sodium dodecyl sulphate (SDS) was added and the tubes were placed at 42°C for 10 min followed by addition of 1 equivalent volume of the solution in the tube of MATAB (mixed alkyltrimethyl ammonium bromide, Sigma M7635) and the reaction mixture was incubated at 65°C for 10 min. Then the suspension was mixed with 700 µL of phenol-chloroform-isoamyl alcohol (25: 24: 1). After 10 min of centrifugation at 10,000 g, 400 µL of the aqueous upper phase was mixed with 400 µL of chloroform-isoamyl alcohol (24: 1). After another 10 min of centrifugation at 10,000 × *g*, 400 µL of the aqueous upper phase was transferred into a new tube and 700 µL of cold isopropanol was added. To precipitate DNA, tubes were placed at 20°C for 30 min and centrifuged at 10,000 × *g* for 10 min. After elimination of the supernatant, 700 µL of 70% ethanol was added in order to wash the DNA pellets, the tubes were centrifuged at 10,000 g for 10 min. The ethanol was then discarded and the pellets were air-dried for 60 min. The DNA was re-suspended into 100 µL of TE and kept frozen at 20°C.

PCR amplification

The V3 variable region of bacterial 16S rDNA from fish was amplified using primers GC338f (5′-CGCCCGCCGCGCGCGGCG GGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCA GCAG) and 518r (5′-ATTACCGCGGCTGCTGG) (16) as described by Ampe *et al*. (1999). The PCR amplification procedure was performed with a Peltier Thermal Cycler PTC-100 (MJ Research Inc., USA). The final concentrations of the different components were as follows: 0.2 µmol L^{-1} each primer, 200 µmol L^{-1} deoxynucleoside triphosphate dNTPs (dTTP, dATP, dGTP and dCTP), 1.5 mM L^{-1} $MgCl_2$, 1X of reaction *Taq* buffer ($MgCl_2$-free), 0.05 U μl^{-1} *Taq* DNA polymerase (Promega, France).

Template DNA was denatured at 94°C for 5 min. To increase the amplification specificity and to reduce the formation of spurious by-products, a "touchdown" PCR was performed according to the protocol of Muyzer and Smalla (1998). The initial annealing temperature was 10°C above the expected annealing temperature (65°C), and the temperature was decreased by 1°C every second cycle until the touchdown temperature, 55°C was reached; then 10 additional cycles were carried out at 55°C. Primer extension was carried out at 72°C for 3 min. The final extension step was done at 72°C for 10 min. Aliquots (5 µL) of PCR products were analyzed first by conventional electrophoresis in 2.0% (w/v) agarose gel (2.0%) with 1X TAE buffer (40 mM Tris-HCl, 40 mM acetate, 1.0 mM EDTA).

DGGE analysis

DGGE was performed with a DCode system (Bio-Rad, USA) as described previously (Muyzer *et al*. 1999). Samples containing approximately equal amounts of PCR amplicons were loaded into 8% (w/v) polyacrylamide gels (37.5/1, acrylamide/bisacrylamide) in 1X TAE buffer (40 mM Tris-HCl, pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2EDTA at pH 8.0) using a denaturing gradient ranging from 30 to 60% (100% denaturant contains 7 M urea and 40% (v/v) formamide). Electrophoresis was performed at 60°C, initially at 20 V for 10 min and then followed by eight combinations of voltage and time; 200 V for 3.5 h, 200 V for 5 h, 150 V for 6.7 h, 100 V for 10 h, 80 V for 5.5 h, 80V for 12 h, 80 V for 15 h and 80 V for 12.5 h.

After electrophoresis, the gels were stained for 10 min with ethidium bromide (BET: Promega H5041, France, at 50 µg mL^{-1}), de-stained for 20 min in distilled water and the gel was then photographed after UV revelation with a Sony digital camera (Japan).

DGGE fingerprints were analyzed by using Image J Gel analysis software (created by National Institutes of Health, Maryland, USA) and were manually scored by the presence and absence of co-migrating bands, independent of intensity. Pair-wise community similarities were quantified using the Dice similarity coefficient (S_D) (Muyzer *et al*. 1999).

$$S_D = 2\ Nc\ /\ Na + Nb$$

where *Na* represented the number of bands detected in the sample A, *Nb* represented the number of bands detected in the sample B, and *Nc* represented the number of bands common to both samples. Similarity indices were expressed numerically within a range of 0 (completely dissimilar) to 1.0 (perfect similarity). The phylotype richness (S, the number of bands) was calculated. Dendrograms were constructed using the un-weighed pair group method with arithmetic averages (UPGMA) by using DARwin 4.0.290 software (Cirad, Montpellier, France). Binary sequences were generated for individual DGGE lanes by determining the number and position of bands compared to the total number of band positions detected.

RESULTS

Impact of time-voltage combination on DGGE electrophoresis

To determine the effect of time-voltage combination on DGGE electrophoresis, PCR amplicons of sea bass were analyzed by DGGE under various time-voltage combinations. Because 1000 V h^{-1} represented a standard value among many published DGGE protocols (Sigler *et al*. 2004), we have chosen it first as a constant parameter (80 V, 12.5 h) to optimize the time impacts to assess the microbial community structure. Otherwise, decrease and increase of these parameters (voltage and time) were also studied as Sigler *et al*. (2004) did to study bacterial community in old soils. Dice Similarity coefficient (S_D) and phylotype richness (S) were calculated. **Fig. 1** shows the DGGE profiles of 16S rDNA fragments targeting the bacteria obtained from sea bass samples. The variation of time-voltage combinations altered band richness and impacted profile similarity (**Fig. 1**; **Table 1**). It is interesting to note that, decreased electrophoresis voltage and increased electrophoresis time resulted in a higher number of bands. For example, DGGE fingerprints obtained at 80 V for 12 h (G) resulted in a maximum

Table 1 Comparison of similarity indices of DGGE profiles under different combinations of voltage (V) and time (h).

	Combinations of V x h							
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
	1000*	**700**	**1000**	**1000**	**400**	**1200**	**960**	**1000**
Band richness (S)								
	6	4	7	5	3	6	8	8
Dice similarity (S_D)								
A								
B	0.2							
C	0.31	0.18						
D	0.55	0	0.17					
E	0	0.28	0.20	0				
F	0.50	0	0.61	0.55	0			
G	0.43	0	0.67	0.46	0	0.71		
H	0.43	0	0.67	0.46	0	0.71	0.75	

* V × h value

Note: Combination of voltage and time (Vxh): (**A**) 200 V for 5 h; (B) 200V for 3.5 h; (C) 150 V for 6.66 h; (D) 100 V for 10 h; (E) 80 V for 5 h; (F) 80 V for 15 h; (G) 80 V for 12 h; (H) 80 V for 12.5 h.

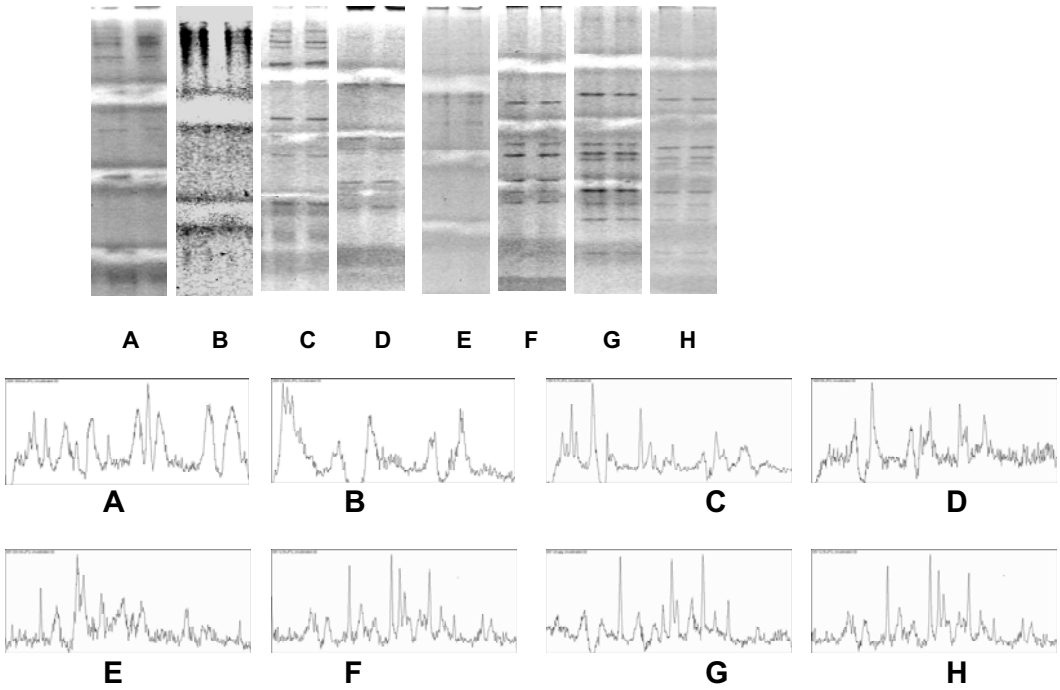

Fig. 1 DGGE fingerprints of 16S rDNA of representative bacteria obtained from sea bass (Viviers du Gois, France) under differing combination of voltage (V) and time (h). (**A**) 200 V for 5 h; (**B**) 200V for 3.5 h; (**C**) 150 V for 6.66 h; (**D**) 100 V for 10 h; (**E**) 80 V for 5 h; (**F**) 80 V for 15 h; (**G**) 80 V for 12 h; (**H**) 80 V for 12.5 h. (**B**) DGGE chromatogram obtained from (**A**) by using ImageJ software.

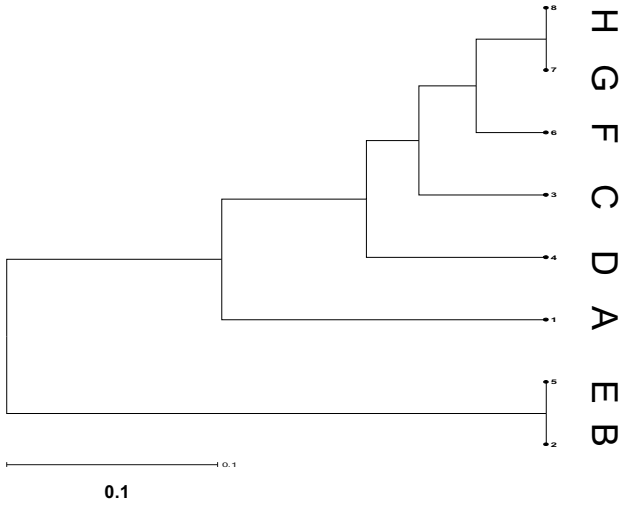

Fig. 2 Dendrogram of PCR-DGGE fingerprints of representative bacteria in sea bass analysed under various time-voltage combinations.

of 8 bands while it resulted in a minimum of 3 bands at 200 V for 3.5 h (B) and at 80 V for 5 h (E). The highest similarities were found upon comparing the combination of volt-time F (80 V, 15 h), G (80 V, 12 h) and H (80 V, 12.5 h) (**Fig. 2**). Altering the electrophoresis time-voltage combinations would theoretically result in minimum and maximum limits of observed bands. Such discrepancy would have a significant effect on band richness and thus the overall community structure assessment.

Analysis of dominant bacteria in fish

Analysis of bacterial community composition by PCR-DGGE was performed on all fish samples (tilapia and sea bass) under electrophoresis condition at 80 V for 12 h, combination that we considered as "standard" to differentiate the DNA bands directly extracted from fish. DGGE band patterns for the 16S rDNA of dominant bacteria from tilapia and sea bass collected from different geographical location

Table 2 Dice similarity coefficients (S_D) comparing the similarities of DGGE fingerprints (**Fig. 3**) from fish samples.

	TN	TL	TK	BS2	BS1	BN2	BN1
TN							
TL	0.2						
TK	0	0					
BS2	0	0	0				
BS1	0.11	0	0	0.4			
BN2	0	0	0	0.21	0.11		
BN1	0	0	0	0.15	0	0.82	

TN: Tilapia from Nakorn Pratom (Thailand); TK: Tilapia from Bangkok (Thailand); BS1, BS2: Sea basses from Aquanord, Graveline (France); BN1, BN2: Sea basses from Viviers du Gois, Beauvoir sur Mer (France)

are shown in **Fig. 3**. DGGE band patterns from all fish samples show some differences. The number of bands per lane varied from 1 to 9 (**Table 2**). Some differences were noted in band position, intensity, and number of bands present in the fish bacterial DGGE patterns throughout the collected locations. Each fish had its own unique profile, indicating variation within fish species but also depending on sampling location as well.

The Dice similarity coefficient (S_D) was used to quantify the similarity of these community fingerprints between each fish samples. There was only moderate similarity (SD = 0.4) between BS1 and BS2. The fingerprints from BN1 and BN2 were highly similar, but not identical community fingerprints (SD = 0.82). There were a complete dissimilarity between tilapia from Thailand (TN, TL, TK) and sea bass (BN1, BN2, BS1, BS2) from France (SD = 0). This pair-wise comparison of community structures suggested a larger community shift in response to different geographical location where the fish samples have been collected than within the fish species.

The number of bands in an individual lane ranged from 1 to 9, corresponding to a total of 24 unique band positions. Cluster analysis was performed with the UPGMA algorithm to study general patterns of community similarity among the different geographic locations where the fish samples were collected (**Fig. 4**). The bacterial communities of Thai tilapia from Nakorn Pratom (TN) were closely related to tilapia from Bangkok (TL) whereas the French sea bass

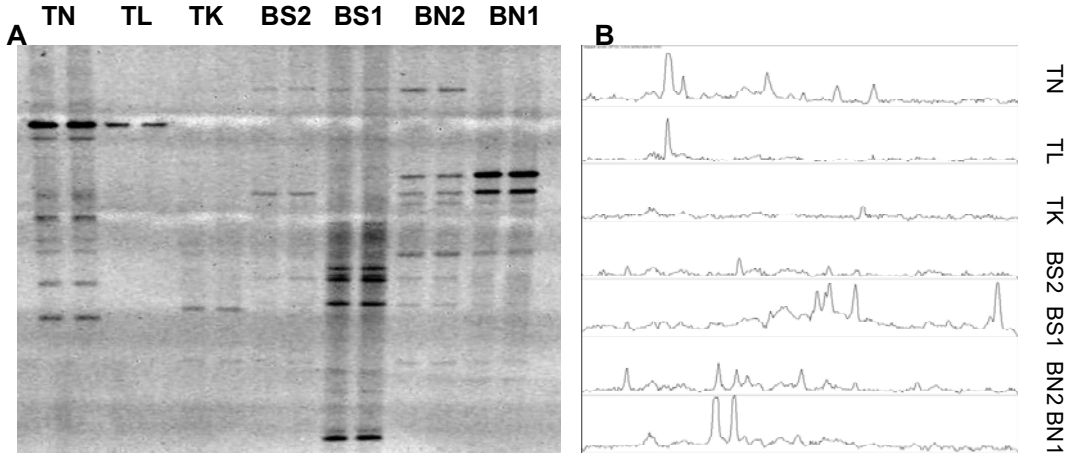

Fig. 3 DGGE analysis of 16S rDNA fragments of the representative bacteria from fish samples collected from Thailand and France. BN1-BN2: sea bass provided by (Viviers du Gois, France; BS1-BS2: sea bass provided by Aquanord, France; TK: tilapia from Khon Kaen, Thailand; TN: tilapia from Nakorn Pratom, Thailand; TL: tilapia from Ladkrabang, Bangkok, Thailand. (**A**) DGGE profiles (negatively converted). (**B**) DGGE chromatogram obtained from (**A**) by using ImageJ software.

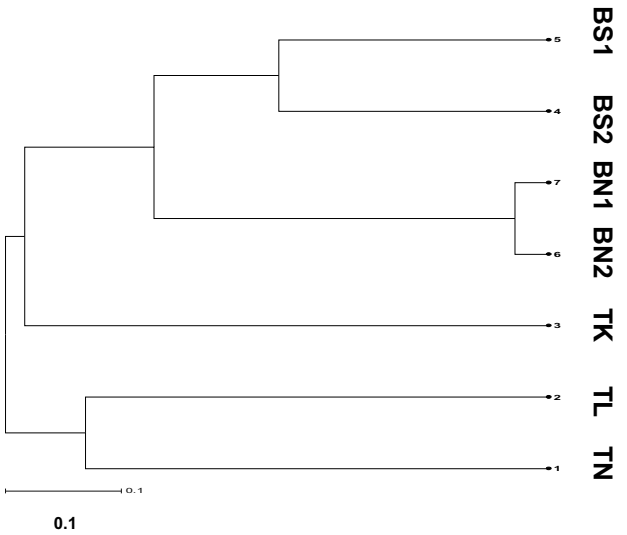

Fig. 4 Dendrogram revealing the relatedness of DGGE fingerprints from fish samples collected from different geographical locations. BN1-BN2: sea bass provided by (Viviers du Gois, France; BS1-BS2: sea bass provided by Aquanord, France; TK: tilapia from Khon Kaen, Thailand; TN: tilapia from Nakorn Pratom, Thailand; TL: tilapia from Ladkrabang, Bangkok, Thailand.

(BS1, BS2, BN1 and BN2) grouped closely according to collected locations. Otherwise, the bacterial communities of tilapia from Khon Kaen (TK) were only distantly related to the sea bass bacterial communities.

DISCUSSION

There are only a few published works that analyzed the bacterial community in fish by PCR-DGGE (Spanggaard *et al*. 2000; Huber *et al*. 2004; LeNguyen *et al*. 2007; Maiworé *et al*. 2009). These authors used a constant time-voltage combination of 200 V for 5.5 h. In this study, we demonstrated the impact of various time-voltage combination of DGGE electrophoresis on assessment of bacterial community in fish. Based on the results, it was apparent that the selection of time (h) – voltage (V) combination seems to had a significant impact on the band pattern generated during DGGE analysis as well as subsequent community structure assessments using all indices (Dice and band richness). At a constant value of time-voltage (1000 V h^{-1} = 80 V for 12.5 h) and when voltage were varied from 80 to 200 V, dissimilarity in band patterns was observed. The highest applied voltage (200 V) greatly impacted the separation and resolution of the bands. Decreasing the applied voltage with increasing running time resulted in optimal resolution of the bands. Furthermore, longer electrophoresis time (80 V for 15 h) caused pattern differences. It might be possible that some internal diffusions of the denaturing gradient occurred following polymerization of acrylamide as well as some 16S rDNA fragments migrated throughout DGGE gels before their melting point, considering that the bottom of DGGE gels are open to the electrophoresis buffer. As demonstrated in this work, changing the electrophoresis conditions could greatly impact subsequent band-based analysis. However, once a DGGE protocol has been established the effect of minor modifications of the protocol (i.e. changing the DGGE gradient or PCR conditions, etc.) should always be tested before drawing any conclusion. We therefore propose two combinations of time-voltage: 200 V for 3.5 h for the DGGE analysis of pure bacterial isolate and 80 V for 12 h for determination of dominant mixed bacterial community in fish samples. In all the cases, it is recommended to add reference samples of DNA from pure bacteria on the two sides of the gel to facilitate the positioning of other bands and to avoid all problems of diffusion or separation due to long time analysis or gradient homogeneity.

The limitation of cultivation methods for studying the bacterial ecological communities has been widely discussed; DGGE genetic fingerprinting techniques could allow comparison of total bacterial communities of different samples through pattern analysis. In this study, bacterial community of fish samples was studied. The results revealed that the bacterial community of fish samples was dependent to the different geographical location from where the fish samples were collected. Apparently, the geographical location of fish samples might have a greater impact on the community profile than fish species. It is important to note that the diversity of bacteria in fish depends also on the environmental and climate conditions, diet and dietary components and physiological status and post harvest conditions of fish. These factors remain to be studied.

This optimized PCR/DGGE method will open new perspectives in the fish processing analysis. It could permit to follow the microbiota ecology of fish during processing and would help to determine the hazard point with more accuracy.

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