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Full Length Research Paper

Comparison of bacterial communities of tilapia fish from Cameroon and Vietnam using PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis)

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Fishes in general and tilapia in particular are traded all over the world. However, it is difficult to find out their exact geographical location. One of the techniques used in the traceability of fish and its by-products consist in analysing in a global way the whole viable and non viable bacterial communities. For this purpose, the molecular technique employing the bacterial 16S DNA banding profiles generated by PCR-DGGE (polymerase chain reaction-Denaturing gradient gel electrophoresis) was used to evaluate the differences between the bacterial profiles of fishes from Vietnam (An Giang, south province) and those of Cameroon (Yagoua, Maga, Lagdo). The different PCR-DGGE 16S rDNA banding profiles obtained were analysed and results showed that there were specific bands for each geographical location though some bands common to Cameroon and Vietnam were observed. This method could be used as a rapid analytical traceability tool for fish products and could be considered as a provider of a unique biological bar code.

Key words: Traceability, PCR-DGGE, bacterial community.

INTRODUCTION

The fishery sector is more or less unstructured because few information are archived and transmitted. Generally, eviscerated fishes and those from which the heads have been removed could be transferred from one box to another, without any one realizing that a transaction has been done. Only administrative documents remain to certify the fish origin. When these papers are lost or when there is sanitary problem linked to fish, it is necessary to determine its geographical origin. This operation remains fastidious and not necessarily reliable. Moreover the European regulation 178/2002 applicable since the 1st January 2005 imposes traceability and labelling to imported products in Europe. Traceability is the capacity to find the history, use or origin of a food (activity, process and product, etc.) by registered method (ISO 8402, 1994).

Practically, it is difficult to determine exactly the geographical origin of a food product. Among the methods used for this purpose, there is one that allows analysing in a global way the whole viable and non viable bacterial communities in fish samples. In fact, aquatic micro-organisms are known to be closely associated with the physiological status of fish (Horsley, 1973; Shewan, 1977). The water composition, temperature and the weather conditions can influence the bacterial communities of fish. De Sousa and Silva-Souza (2001) with their research on the bacterial communities of the Congonhas River in Brazil showed that there was direct relationship between the bacterial community of the river and the fish commensal bacteria. To trace or find out a product source or origin could mean analysing the whole commensal bacterial community in this product. These microbial

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communities characterised in fishes of a given region could be used to establish the fish geographical origin (Montet et al., 2004).

Many methods are used to isolate micro-organisms in food. These methods include the culture-dependant and culture-independent methods. The first are classical methods based on microbial isolation on Petri dishes, the second methods are genetic methods that provides a bacterial profile representing the genetic diversity of a specific environment (Giovanni et al., 1990). Recently, a variety of new methods have been developed to directly characterize the micro-organisms in particular habitats without the need for enrichment or isolation (Head et al., 1998). Typically these strategies examine the total microbial DNA (or RNA) derive from mixed microbial populations to identify individual constituents (Hugenholtz and Pace, 1996). This approach eliminates the necessity for strain isolation, thereby negating the potential biases inherent to microbial enrichment. Moreover, studies which have employed such direct analysis have repeatedly demonstrated a tremendous variance between cultivated and naturally occurring species, there by dramatically altering perception on the true microbial diversity present in various habitats (Hugenholtz et al., 1998).

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) technique is one of the genetic techniques and it combines two stapes: one of amplification by polymerase chain reaction (PCR) and the second of electrophoresis on acrylamide gel. When running PCR, only the variable region of bacterial 16SrDNA from fish is amplified (Leesing, 2005). Separation of PCR products in DGGE (denaturing gradient gel electrophoresis) is based on the decrease of the electrophoretic mobility of partially melted double stranded DNA molecules with different sequences. These will have different melting behaviour and will stop migrating at different position in gel (Leesing, 2005; Muyzer et al., 1993). The main advantage of this technique is that it permits the analysis in the same time of cultivable and non-cultivable aerobic and anaerobic bacteria (Yang et al., 2001).

In this study, PCR-DGGE of 16S rDNA was used to study the natural distribution of bacteria in Cameroonian fishes and those coming from Vietnam in order to compare their banding profiles. The biological bar code of the fishes could be obtained at the end of this study in order to differentiate them in international trade.

MATERIALS AND METHODS

Fish sampling

The fish samples used in this study were tilapia and they were collected both in Cameroon and Vietnam. In Cameroon (Figure 1a), the fishes were collected in three lakes situated in the three regions: Far north (Yagoua), North (Lagdo) and Adamawa (Tibati). In Vietnam (Figure 1b), the fishes were collected from a pound in Ah Giang in the North province. All the samples were collected in the month of August 2006 within the rainy season. On each site, four fishes were collected and transferred in sterile storage bags. The samples were maintained on ice and transported to the laboratory. The skin, gills and intestines were then aseptically removed from each fish and put in separate sealed plastic bags and then kept frozen at -20°C until the DNA was extracted.

Total DNA extraction

The DNA extraction was based on the method of Ampe et al. (1999) and Leesing (2005) but modified and optimised. For all the samples, 2 g of the mixture gills + skin + intestine were homogenized for 3 min with vortex after addition of sterile peptone water. Four tubes of 1.5 ml containing the resulting suspension were then centrifuged at 10000 g (Biofugopico Heraeus, Germany) at 4°C. To the pallet obtained, 100 µl of buffer TE (10 mM Tris-HCl pH 8, 1 mM EDTA; pH 8, Promega, France), 100 µl of lysozyme solution (25 mg/ml, Eurobio, France) and 50 µl of proteinase K solution (20 mg/ml, Eurobio, France) were added. Samples were mixed in vortex for 1 min and incubated at 42°C for 20 min. 50 µl of 20% sodium dodecyl sulphate (SDS) was added and the mixture was incubated at 42°C for 10 min. The SDS is an anionic detergent which completes the proteinase K action. 400 µl of mixed alkyl trimethyl ammonium bromide (MATAB) 2% was added to hydrolysis products (65°C/10 min). After cellular lyses, a volume of phenol/chloroform/isoamyl alcohol (25/24/1, Carlo Erba, France) was added and the mixture was centrifuged for 10 min to allow the DNA extraction. A volume of chloroform/isoamyl alcohol (24/1) mixture was added and centrifuged in order to purify the DNA and to remove the remaining phenol. Proteins and the remaining polysaccharides in the aqueous phase were recovered at the interface with the organic phase after centrifugation at 10000 g for 10 min. The total DNA was precipitated with isopropanol (- 20°C) followed by centrifugation. A volume of 70% ethanol was added to remove water around the DNA molecule. The DNA obtained was suspended in 50 µl of tris-EDTA and stored at -20°C.

DNA amplification by polymerase chain reaction (PCR)

The 16S rDNA were specifically amplified by PCR using bacterial couple of primers described by Muyzer et al. (1993): Gc338f (5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG, Sigma, France) and 518r (5'ATTACCGCGGCTGCTGG, Sigma, France) (Ovreas et al., 1997; Ampe et al., 1999; Leesing, 2005). Amplification of the V3 variable region of bacterial communities' 16S rDNA of fishes was realised using a GC clamp of 40 nucleotides. This clamp was added to forward primer 5' of 338f in order to insure that DNA fragment will partially remain double- stranded (Sheffield et al., 1989). Each mixture (final volume of 50 µl) contained about 100 ng of template DNA, all the primers at 0.2 µM, all the deoxyribonucleotide triphosphate (dNTPs) at 200 µM, 1.5 mM $MgCl_2$ 5 µl of 10X of taq buffer $MgCl_2$ free (Promega, France) and 5 U of Tag polymerase (Promega, France).

During PCR reaction, non-specific hybridizations could be created; these hybridizations are due to complementary micro-sequences. To improve the specificity of the reaction, a ''Touchdown'' PCR was performed according to the protocol of Diez et al. (2001). An initial denaturation at 94°C for 1 min and 10 touchdown cycles of denaturation at 94°C for 1 min, then annealing at 65°C (with an increasing temperature 1°C per cycle) for 1 min. and extension at 72°C for 3 min followed by 20 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min.

PCR products (5 µl) were then analysed by conventional electrophoresis in 2% (w/v) agarose gel with TAE buffer 1X (40 mM Tris-HCl pH 7.4, 20 mM Sodium Acetate, 1.0 mM Na_2-EDTA). 8 µl of

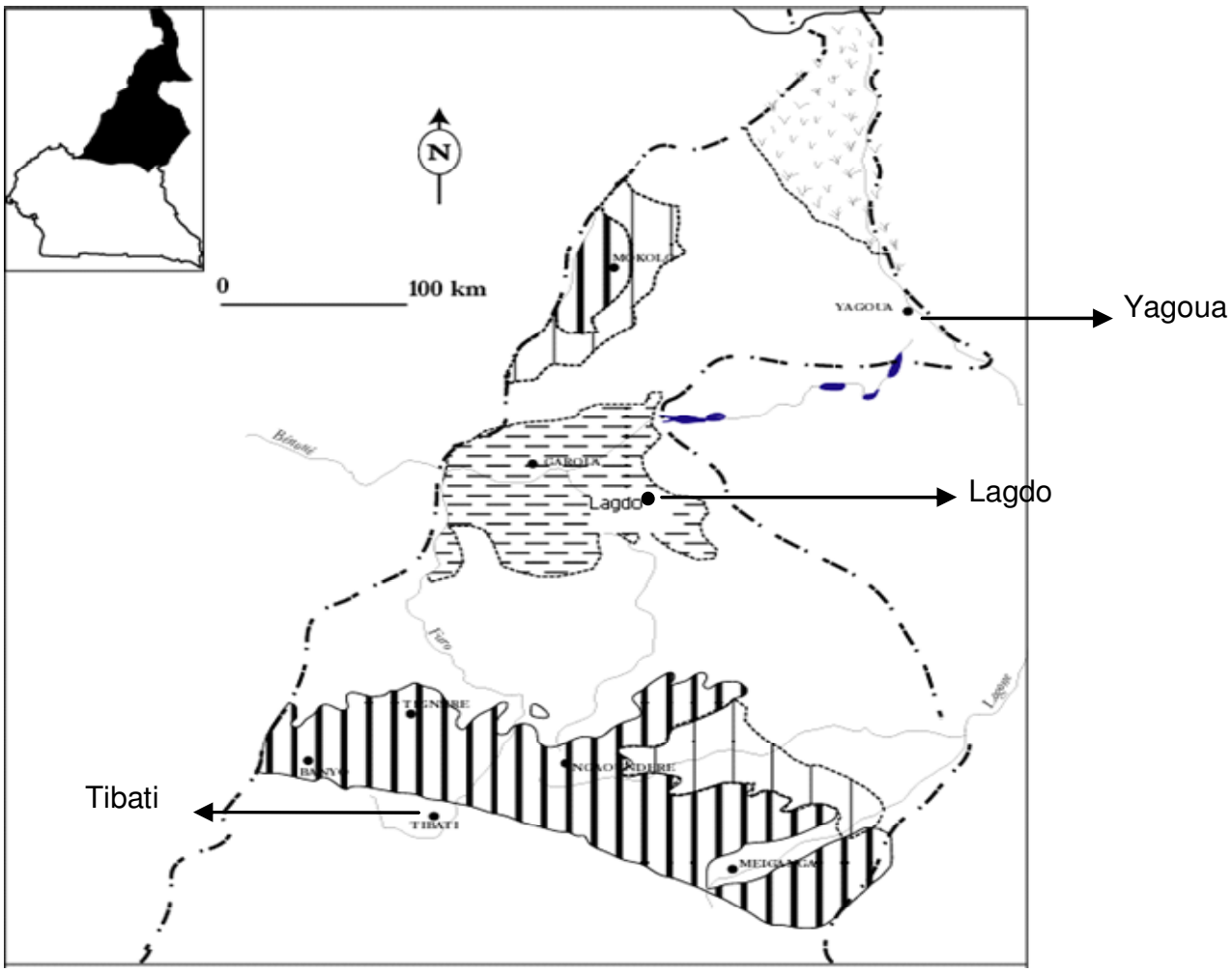

Figure 1a. The expansion of the northern part of Cameroon with three different sampling locations: Tibati, Lagdo and Yagoua.

products and 2 µl of stain (Bromophenol blue) were loaded in wells. The migration lasted for 60 min at 100 volts and was quantified with DNA mass leader of 100 pb (Promega G2101 France). At the end of migration, the agarose gel was immersed in 50 µg/ml of ethydium bromide (EB: Promega H5041, France) for 10 to 15 min (Ampe et al., 1999), rinsed with distilled water for 15 to 30 min. and then, observed on UV transilluminator using Gel Smart 7.3 system (Clara Vision , Les Ulis, France).

DGGE electrophoresis

Polymerase chain reaction (PCR) products were analyzed by denaturing gradient gel electrophoresis (DGGE) using the DCodeTM detection system (Bio-Rad laboratory, Hercules, USA). The method was first described by Muyzer et al. (1993) and improved by Leesing (2005). Samples containing approximately equal quantities of amplicons were loaded into 8% w/w polyacrylamide gel (Acrylamide*/N, N'*- metylene bisacrylamide, 37/5/1, Promega, France) with a denaturing gradient urea formamide spreading 30 to 60% (100% corresponding to 7 M of urea and 40%v/v, formamide, Promega, France). Volumes of PCR products used were 20 µl. Electrophoresis was performed at 60°C in TAE Buffer (2 M sodium-acetate, 0.05 M EDTA, pH 8.3) at 20 V for 10 min and then 80 V for 12 h. After electrophoresis, the gels were stained with ethidium bromide (50 mg/l) for 30 min and rinsed in distilled water for 20 min. The gels were photographed on a UV transilluminator using Gel Smart 7.3 System (Clara Vision, Les Ulis, France).

Gels analysis

Individual lanes of the gel images were straightened and aligned using ImageQuant TL software vision 2003 (Amesham Biosciences, USA). Gels were analyzed by the software picture as for TL (Picture Analysis Software, 2003) that permits to situate automatically bands on gels and generate their corresponding Rf (fronts of migration). Banding patterns were standardised with two reference patterns of *Escherichia coli* and *Lactobacillus plantarum* DNA amplicons which were included in all the gels. The generated bands represent different species of bacteria in a population. On the gel, an individual discrete band refers to a unique phylotype (Muyzer et al., 1996). The DGGE gels were manually scored by presence (1) or absence (0) of band independent of the intensity. Patterns were compared using the dice similarity index (S_D) calculated according to the following formula (Heyndrickx et al., 1996):

$$S_D = 2Nab / (Na+Nb)$$

Where, Nab = number of bands common to sample A and B; Na

Figure1b. Sampling locations in Ha Giang (North province in Vietnam).

and Nb = number of bands detected in sample A and B, respectively.

Similarities index were expressed within a range of 0 (completely dissimilar) to 1 (perfectly similar). Dendogram was constructed using statgraphics plus version 5 software (Sigma plus, France). Similarities in community structure were determined using the cluster analysis with Euclidian distance measure.

RESULTS

Analyses were carried on tilapia fish from Cameroon and those of Vietnam. In the two countries, the fish samples were collected during the rainy season. A direct DNA extraction was carried on the mixture of fish organs of each site. After the DNA amplification, electrophoresis was done on agarose gel to ensure that there was sufficient amplicons. The bands obtained from the four replicates samples were situated between 298 and 220 bp of the molecular leader, which is in agreement with the awaited size of polymerase chain reaction products.

The DGGE patterns of four replicates for each location revealed the presence of 8-11 visible and distinct bands of bacteria in fish (Figure 2). Theoretically, a band corresponds to a bacterial species. This gel showed also

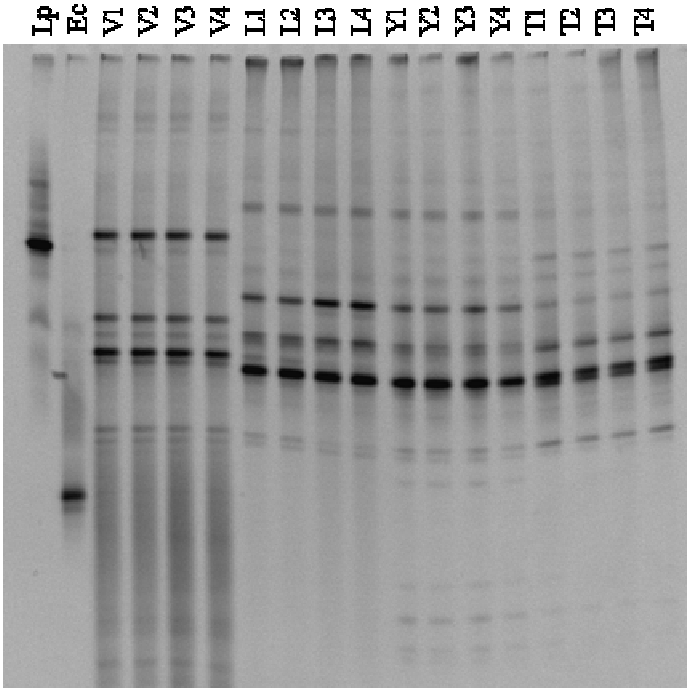

Figure 2. PCR-DGGE of 16S rDNA banding profiles of samples collected from Vietnam and Cameroon. V1 - V4 samples from Vietnam; Y1 - Y4: Samples from Yagoua T1 - T4: samples from Tibati; L1 - L4: samples from Lagdo.

Table 1. Index of similarity (%) between PCR-DGGE profiles of the molecular markers according to the sampling regions.

	V1	V2	V3	V4	L1	L2	L3	L4	Y1	Y2	Y3	Y4	T1	T2	T3	T4
V1	100															
V2	100	100														
V3	100	100	100													
V4	100	100	100	100												
L1	53	53	52,6	53	100											
L2	53	53	52,6	53	100	100										
L3	56	56	55,6	56	82	82	100									
L4	56	56	55,6	56	82	82	100	100								
Y1	48	48	55,6	56	60	60	55	55	100							
Y2	48	48	55,6	56	60	60	55	55	100	100						
Y3	48	48	47,6	48	55	55	63	63	100	100	100					
Y4	48	48	47,6	48	55	55	63	63	100	100	100	100				
T1	42	42	42,1	42	67	67	59	59	70	70	74	74	100			
T2	42	42	42,1	42	67	67	59	59	70	70	74	74	100	100		
T3	47	47	47,1	47	63	63	67	67	67	67	67	67	88	87,5	100	
T4	47	47	47,1	47	63	63	67	67	67	67	67	67	88	87,5	100	100

that among the sixteen samples analysed, there were three intensive common bands (Table 1) for all the fishes analysed at the Rf of 0.48, 0.53 and 0.61, respectively. The statistical analysis of the DGGE gel patterns for the four replicates of fish samples from four different locations (An Giang Province (Vietnam), Yagoua, Lagdo and Tibati (Cameroon)) showed the community similarity among the different geographical locations where the fish

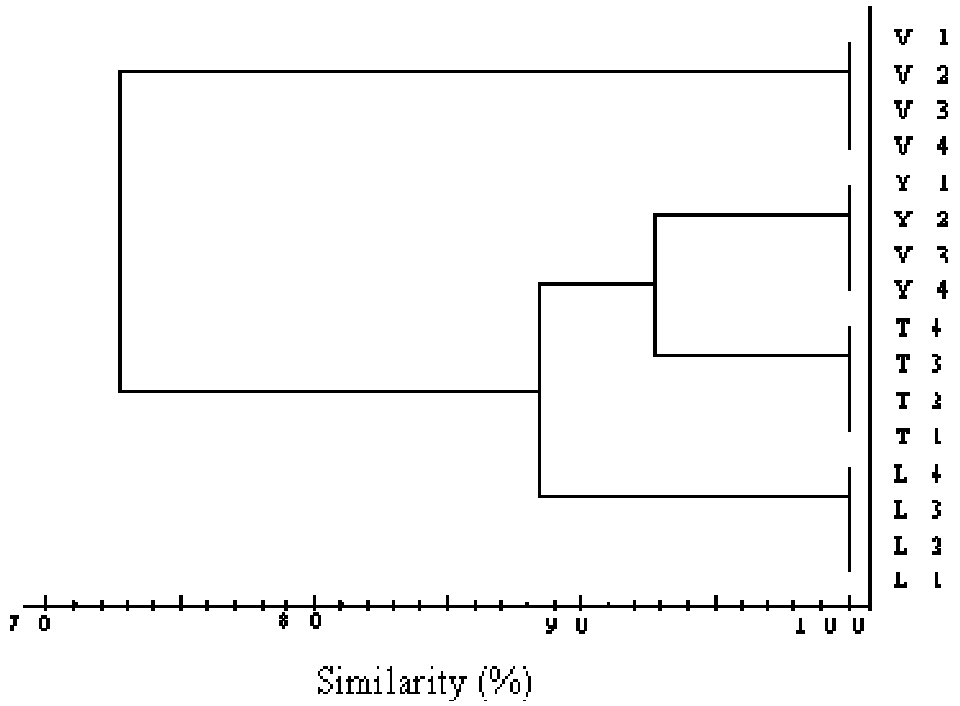

Figure 3. Cluster analysis of 16S rDNA banding profile for fish bacterial communities Cameroon and Vietnam. Y1 - Y4 = Samples from Yagoua; T1 - T4 = samples from Tibati; L1 - L4 = samples from Lagdo.

samples were collected (Figure 2). At 88% similarities level, two main clusters were observed. The first cluster included the samples of Vietnam (V1-V4) while the second cluster comprised the samples from Cameroon. There was also the formation of two secondary clusters at 92% similarities level. The samples from Lagdo formed the first cluster while the samples from Tibati and Yagoua formed the second cluster. The pattern obtained for the bacterial community for four replicates of the same site was totally (100%) similar. The cluster analysis of 16S rDNA banding profile for fish bacterial communities from Cameroon and Vietnam are shown in Figure 3.

DISCUSSION

Tilapia is a very popular commodity around the world and its consumption has increased over the last decades in many countries. In the world, its production exploded and passed from 400000 tons in 1990 to 1 800000 tons in 2004 (Lazard, 2007). Some of the reasons for the popularity are the relatively low cost of production and the high nutritional value. Since the recent major food crises in Europe (Bovine Spongiform Encephalopathy, Salmonella and avian influenza), the issues surrounding food safety and security continue to be topics of concern throughout the supply chain and regulations across Europe continue to be tightened in order to provide a greater degree of assurance in quality and safety. One of the great concerns of the customers is the traceability of the products. Although the determination of geographical origin is one demand of the traceability of import-export products, there are no existing scientific methods which permits the origin of food to be followed or determined precisely (Le Nguyen et al., 2008). The main objective of the present study was to identify and validate some pertinent biological markers which come from the environment of the fish to assure traceability of tilapia from Cameroon and Vietnam during international trade. Aquatic micro-organisms are known to be closely associated with the physiological status of fish. Micro-organisms are found on the entire external surface (skin and gills) and in intestines of a living or freshly collected fish (Shewan, 1977; Liston, 1980). The micro flora of gastro-intestinal tract is important for fish and for environment (Billard, 1995). The skin is in direct contact with water and the gills that filter the air from water that goes to the lung of fish is a good accumulator of the environmental bacteria. The intestine contains also high amount of bacteria. Numerous studies of the microbiota in fish captured from various geographical locations have been done (Grisez et al., 1997; Spanggaard et al., 2000; Al Harbi and Uddin, 2003; Leesing, 2005). The results obtained by these authors clearly demonstrated that a link exists between the bacterial communities of the river and the fish.

PCR-DGGE method was used to analyze the bacteria in fish in order to create a technique to link bacterial com-

munities to the geographical origin and avoid the individual analysis of each bacterial strain. The PCR-DGGE analysis of mixed bacterial populations is widely used in environmental microbiology (Van der Gucht et al., 2005). The amplification of variable regions of the 16S rDNA followed by DGGE analysis led to fingerprints of the microbial community characterized by a correspondence between bands and microbial species (Mauriello et al., 2003). This technique has been recently applied to food ecosystems where the microbial community was identified from PCR-DGGE profiles after a direct DNA extraction from food samples (Ampe et al., 1999; Dewettinck et al., 2001; Mauriello et al., 2003; Ercolini et al., 2004).

In the present study, PCR-DGGE fingerprints were obtained after a direct DNA extraction from fishes. Statistical analysis of PCR-DGGE profiling showed significant differences in migration patterns on the DGGE gel. Thus the bacterial profile is specific for every geographical location (nowhere a similarity of 100% was found between two geographical locations) although there were some similarities. The variations may also be due to the water supply which can be affected by the pollution from urban life. However, the four replicates for each sampling location had statistically similar DGGE patterns throughout the study.

The analysis of the DGGE patterns showed that higher similarity exist between the DGGE patterns of fishes from Cameroon although these three geographical locations were distanced with about 300 km from each other. There was a higher similarity between the samples of the same country (Cameroon) than the samples from different countries (Cameroon and Vietnam).

Conclusion

The PCR-DGGE of fish bacterial community showed that DGGE profiles are specific for a given geographical region. The bacterial communities in fishes collected from Vietnam, revealed by PCR-DGGE approach, were different from those of Cameroon although there were some common bands to the two countries. Thus, PCR-DGGE is a quicker technique (less than 24 h) which has good potential in differentiating fishes and also in ascertaining the geographical origin of fishes. So it can play an important role in the quality control of fishes during commercial trade.

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