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Analysis on the composition of culturable bacterial communities in several tissues of puffer fish *Takifugu obscurus*

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Abstract: Using 16S rDNA PCR denaturing gradient gel electrophoresis (PCR-DGGE) technology, we investigated the composition of culturable bacterial communities in the skin, intestines, and TTX-accumulating tissues (ovary, liver) of the obscure striped puffer fish *Takifugu obscurus* fed natural or artificial diets. A total of 45 species of different culturable bacteria were isolated and classified according to phylogenetic analysis. Of these species, those belonging to the class γ-Proteobacteria dominated the microbial community in the puffer fish, while others belonged to the group of low G + C Gram positive bacteria, and the group of high G + C Gram positive bacteria. Large differences in the bacterial assemblage isolated from the intestines, TTX-producing organs and skins of fish fed natural and artificial diets were observed. No matter fed with natural or artificial diets, the tissue of intestine, TTX-accumulating organ, or skin, was found to contain bacteria from TTX-producing genera reported to-date. Puffer fish restricted to artificial diets from birth still had large assemblages of TTX-producing bacteria, indicating that food chain accumulation of TTX contributes little to the toxicity of these fish.

Key words: *Takifugu obscurus*; 16S rDNA; denaturing gradient gel electrophoresis (DGGE); bacterial composition; TTX-producing bacteria

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The origin of tetrodotoxin (TTX) has become highly controversial for a long period of time. Since TTX was isolated from such a diversity of animal species, which were totally unrelated to each other phylogenically [1], it had been postulated that puffer fish may have absorbed and accumulation tetrodotoxin through the food chain [2]. However, as several TTX-producing bacteria had been isolated from sea sediments [3], some researchers started to doubt the point of view. What's more the indigenous bacterial microflora in intestine, TTX-accumulation organs (ovary and liver), or skin, are not clear. The indigenous microflora of fish, particularly the microbial ecology of the digestive tract, has been investigated by culture-dependent methods [4] and identification of the fish microflora has typically relied on phenotypic and biochemical key characteristics [5]. Recently, molecular method of using 16S rDNA was used successfully to characterize the microflora in farmed and wild salmon [6], and in zebrafish larvae [7]. To investigate the TTX-producing bacterial composition and its different impaction from the different diets, The PCA and TCBS mediums, which generally

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were used as selection medium for screening TTX-producing bacteria [8], were used to culture the bacteria from the intestine, TTX-accumulation organs, and skin of puffer fish. A big variety of microbial community composition in these organs existed between two groups of puffer fish with natural or artificial diets fed. However, the puffer fish restricted to artificial diets from birth still had large assemblages of TTX-producing bacteria in this research, indicating that food chain accumulation of TTX contributes little to the toxicity of these fish.

1 Materials and methods

1.1 Sampling of puffer fish

Three puffer fish, weighing an average of 0.493 kg, were fed with natural fish and shrimp (group N), while three other artificially-bred puffer fish, weighing an average of 0.404 kg, were fed with artificial food (group C) from birth. All fish were collected from Qidong farm, Jiangsu province, China. All living fishes were transported to the laboratory in Shanghai Fisheries University, Shanghai, China. The skin, intestine, liver and ovary of each fish were sampled aseptically, then the contents of the same tissues of three fishes in each group were pooled together to provide a representative averaged sample.

1.2 DNA extraction from cultured bacterial

After washing with 0.8% NaCl solution several times, the bacterial cells from the intestine were collected as pellets by centrifugation. Another procedure was used for collecting bacterial cells from liver, ovary and skin. The tissue was crushed first with a mortar in 0.8% NaCl solution, then, the liquid was taken off to a new tube. The bacterial cells were harvested by centrifugation.

The bacterial cells were diluted by 0.8% NaCl solution with proportion to 1∶10, 1∶100, 1∶1000. 100 μL of the diluent, was aseptically spread on PCA and TCBS plates, respectively[8]. The plates were incubated in darkness at 23 ℃ for 3 days. Finally, the colonies on the plates were collected for further bacterial DNA extraction. Total bacterial genomic DNA was extracted as the previously described [9].

1.3 Nest- PCR of 16S rDNA

Nested amplification of 16S rDNA was more effective than direct amplification in analysis of bacterial diversity based on previous research [10]. In this research, we used a pair of bacterial universal primers [9] first to amplify the full-length sequence of 16S rDNA from the DNA extracted from tissue bacteria, and the product was used as the template in the next amplification for the variable V3 region of 16S rDNA [11].

1.4 DGGE analysis of PCR products and sequencing of 16S rDNA

DGGE was performed with a D-code System (Bio-Rad, Germany) to separate the above PCR products. The DNA bands on the DGGE gel could show the bacterial diversity. The details were previously described by Yang G. *et al.* [12]

1.5 Phylogenetic Analysis of 16S rDNA Sequences

The V3 region of the 16S rDNA corresponding to the *E. coli* 16S rDNA position was determined. Close relatives and phylogenetic affiliation of the obtained sequences were determined by using the BLASTN search program at the NCBI web site. Only the two most similar sequences in GenBank were selected for phylogenetic tree construction. Multiple alignment and calculation of the distance matrix were conducted using the MEGA 3.0 package[13]. A phylogenetic tree was constructed using neighbor-joining analysis with 1000 replicates of bootstrap analysis.

2 Results

2.1 The pattern of bands distribution of 16S rDNA on DGGE gel

A total of 45 bands were excised from the DGGE gel and named P1 to P19(Figure 1A), and T1 to T26(Figure 1B). Based on the distribution patterns of the 16S rDNA DGGE bands, differences existed in the bacterial community found between the two groups fish fed different diets. In skin, Group N(fishes fed with natural diet) had three specific bands(P10, T11, T25) out of a total of seven bands, and Group C(fishes fed with artificial diet) had eight specific bands(P1, P2, P3, P5, T3, T18, T19 and T23) out of a total of 12 bands. In TTX - accumulating organs(ovary and liver), a total of 20 bands representing culturable bacteria from Group N and a total of 17 bands from Group C were obtained. Of these, 13 bands(P1, P2, P8, P15, P16, T1, T3, T7, T14, T15, T17, T20 and T21) were specific to Group N, while 10 bands(P5, P6, P7, P11, P14, T2, T6, T9, T13 and T16) were specific to Group C. The biggest difference in the bacterial assemblage between the two diet groups was found in the intestines. 15 bands were found in Group N, and 14 bands were observed in group C, while only four bands, P1, P5, P8 and T12, were shared by both groups.

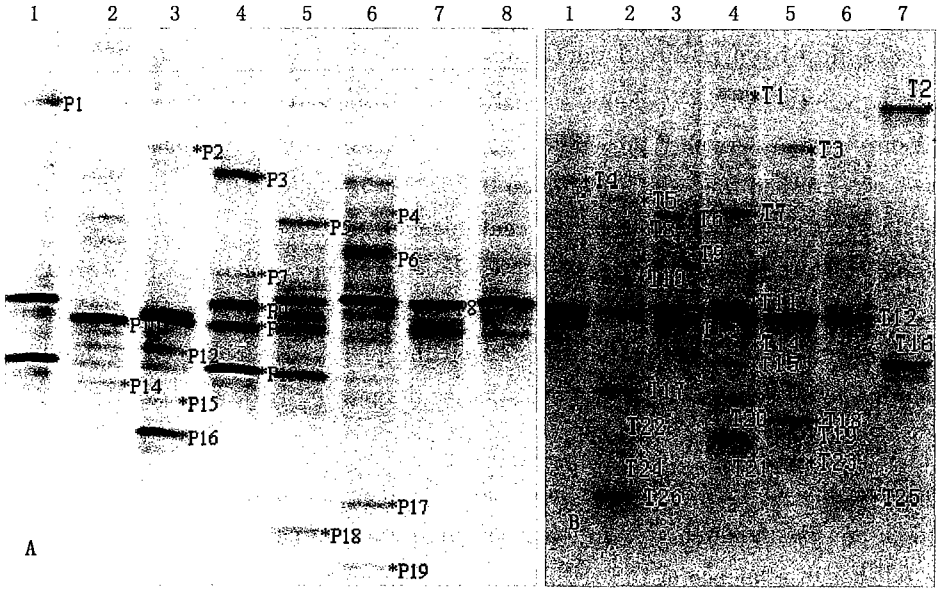

Fig. 1 DGGE patterns of 16S rDNA V3 region fragments obtained after enzymatic amplification and DNA from bacteria cultured on PCA medium (A) or TCBS medium (B).

A. Lane 1. Liver of Group N, Lane 2 Liver of group C, Lane 3 Ovary of Group N, Lane 4 Ovary of Group C, Lane 5 Intestines of Group N, Lane 6. Intestines of Group C, Lane 7. Skin of Group N, Lane 8. Skin of Group C. B. Lane 1. Intestines of Group C, Lane 2. Intestines of Group N, Lane 3 Ovary of Group C, Lane 4 Ovary of Group N, Lane 5. Skin of Group C, Lane 6. Skin of Group N, Lane 7. Liver of Group C. No bacteria from liver of puffer fish fed with natural diet cultured on the TCBS medium. * shows bands cut. P. refer to the bands from PCA medium bacteria. T. refers to the bands from TCBS medium bacteria.

2.2 Phyologenetic analysis of bacterial diversity

A total 45 sequences were obtained from amplification with the 16S rDNA V3 primer pair. GenBank accession number of each sequence showed in the Table 2. The lengths of these sequences were from 169 bp to 195 bp. These sequences were used as queries for BLASTN searches. The two 16S rDNA sequences of identified bacteria with the highest sequence similarities to the query were obtained from the GenBank database and used along with the 45 sequences for phylogenetic analysis. Most bacteria from the various tissues of *T.*

obscurus were clusted into γ-Proteobacteria, rather small fraction of bacteria were affiliated to High G + C Gram positive, or Low G + C Gram positive bacteria (Figures 2,3,4).

Tab. 1 Phylogenetic affiliations of 16S rDNA sequences obtained in this research

Band	accession numbers	Length /bp	Phylogenetic affiliation	closely related species and their accession number of 16S rDNA	Similarity (%)	Source
P1	DQ460734	195	γ-proteobacteria	*Shewanella colwelliana*, AY653177	98	sea water of the Yellow Sea in Korea
P2	DQ460735	194	γ-proteobacteria	Alteromonadaceae bacterium P3, AY902206	98	marine bacterial biofilms
P3	DQ460736	194	γ-proteobacteria	*Pseudomonas* sp. HD-2000/16, AF288724	100	environmental samples
P4	DQ460738	194	γ-proteobacteria	*Vibrio harveyi* ACMM 645, AY264934	98	identification of Vibrio harveyi isolates
P5	DQ460739	194	γ-proteobacteria	*Moraxella* sp. AG-24, X86615	99	a deep subsurface environment
P6	DQ460740	194	γ-proteobacteria	*Vibrio parahaemolyticus* MP-2, AY911391	100	Hong Kong
P7	DQ460741	194	γ-proteobacteria	*Shewanella affinis* KMM 3586, AF500080	99	the North-West Pacific Ocean
P8	DQ460743	194	γ-proteobacteria	*Pseudoalteromonas* sp. SM9913, AY305857	99	adeep-sea psychrotrophic bacterial strain
P9	DQ460744	194	γ-proteobacteria	*Shewanella putrefaciens* B3, AJ491825	100	biofilm and planktonic communities
P10	DQ460745	194	γ-proteobacteria	*Vibrio* sp. M12 -2C, AY730244	99	Mono Lake, California
P11	DQ460746	193	γ-proteobacteria	*Shewanella* sp. NB251, AJ866961	98	a tidal flat sediment
P12	DQ460747	193	γ-proteobacteria	*Listonella anguillarum* S010623-1, AY963632	98	turbot Scophthalmus maximus L.
P13	DQ460748	194	γ-proteobacteria	*Shewanella affinis* KMM 3586, AF500080	100	the North-West Pacific Ocean
P14	DQ460749	194	γ-proteobacteria	*Shewanella* sp. LMG 23025, AJ967028	99	Mediterranean fish
P15	DQ460750	195	Low G + C Gram Positive Bacteria	*Bacillus* sp. 'Mali 10', AY211104	100	Desert Dust Events in Mali, West Africa
P16	DQ460752	195	Low G + C Gram Positive Bacteria	*Bacillus marisflavi* TF-11, AF483624	99	sea water of a tidal flat of the Yellow Sea in Korea
P17	DQ460754	194	γ-proteobacteria	*Vibrio* sp. Ms 121, AY270185	100	sub-tropical regions
P18	DQ460755	174	high G + C Gram Positive Bacteria	*Arthrobacter* sp. GWS-BW-H45M, AY370618	100	the German Wadden Sea
P19	DQ460757	194	γ-proteobacteria	*Vibrio* sp. Clone 1C3, AI627988	100	Sulfide-oxidizing biofilms
T1	DQ460758	193	γ-proteobacteria	*Aeromonas* sp. D6, DQ103510	98	the marine ecosystem subantartic
T2	DQ460759	194	Low G + C Gram Positive Bacteria	*Staphylococcus equorum* NRL 99/760, DQ006839	99	human clinical specimens
T3	DQ460761	194	γ-proteobacteria	*Pseudomonas fluorescens*, DQ207731	100	environmental sample
T4	DQ460764	194	γ-proteobacteria	*Shewanella putrefaciens*, AY321590	100	the deep subsurface
T5	DQ460765	194	γ-proteobacteria	*Vibrio ordalii* NCMB2168, AY628646	99	salmo salar reared at pen-site on the marine coasts of southern chile
T6	DQ460766	194	γ-proteobacteria	*Vibrio aestuarianus* 03/015, AJ845022	100	summer mortality of oyster (Crassostrea gigas)
T7	DQ460767	195	Low G + C Gram Positive Bacteria	*Enterococcus aquamarinus* LMG 16607T, AJ877015	99	sea water
T8	DQ460768	193	γ-proteobacteria	*Enterobacter* sp. HPC64, AY996978	99	Soil contaminated with petroleum products
T9	DQ460769	195	γ-proteobacteria	*Vibrio ordalii* NCMB2168, AY628646	98	salmo salar reared at pen-site on the marine coasts of southern chile
T10	DQ460770	194	γ-proteobacteria	*Serratia* sp. D3, AY745744	99	the marine ecosystem subantartic
T11	DQ460771	194	Low G + C Gram Positive Bacteria	*Jeotgalibacillus halotolerans*, AY028925	99	the traditional Korean fermented seafood jeotgal
T12	DQ460772	194	γ-proteobacteria	*Listonella anguillarum* CW6, AY662308	100	pathogenic Vibrio anguillarum strains isolated in China
T13	DQ460773	192	γ-proteobacteria	*Vanguillarum* sp. NCMB 2130, X71818	98	fish-pathogenic vibrios

·续表·

Band	accession numbers	Length /bp	Phylogenetic affiliation	closely related species and their accession number of 16S rDNA	Similarity (%)	Source
T14	DQ460774	195	Low G + C Gram Positive Bacteria	*Enterococcus aquamarinus* LMG 16607T, AJ877015	99	sea water
T15	DQ460775	194	γ-proteobacteria	*Vibrio* sp. M12 - 2C, AY730244	99	Mono Lake, California
T16	DQ460776	194	γ-proteobacteria	Alteromonadaceae bacterium GWS-BW-H18M, AY515436	100	a tidal flat ecosystem
T17	DQ460777	193	γ-proteobacteria	*Shewanella* sp. GWS-BW-H27M, AY515438	96	oxic habitats of a tidal flat ecosystem
T18	DQ460778	192	γ-proteobacteria	*Aeromonas* sp. M10, DQ200865	98	Aeromonas sp. M10
T19	DQ460779	194	γ-proteobacteria	*Aeromonas*, DQ200865	100	Aeromonas sp. M10
T20	DQ460780	195	Low G + C Gram Positive Bacteria	*Bacillus aquimaris* MSU1110, AY647307	99	saline soil in Mahasarakham Province, Thailand
T21	DQ460781	194	γ-proteobacteria	*Aeromonas salmonicida* 3-St 2 - 6, DQ133187	100	a Trout Farm and Environments
T22	DQ460782	194	γ-proteobacteria	*Aeromonas* sp. D6, DQ103510	100	the marine ecosystem subantartic
T23	DQ460783	194	γ-proteobacteria	*Aeromonas veronii* HQ010516C - 1, DQ029351	99	Eriocheir sinensis
T24	DQ460784	193	γ-proteobacteria	*Aeromonas* sp. K649, AY362008	99	deep-water marine invertebrates
T25	DQ460786	194	γ-proteobacteria	*Vibrio ordalii* NCMB2168, AY628646	100	almo salar reared at pen-site on the marine coasts of southern chile
T26	DQ460787	194	γ-proteobacteria	*Vibrio parahaemolyticus* 4a, AY245192	100	southern rock lobsters (Jasus edwardsii)

2.3 Composition of bacterial communities in various tissues

Based on the phylogenetic analysis (shown in Fig. 2), a total of 25 species of bacteria were from the intestines, of which most species belonged to γ-Proteobacteria including *Pseudomonas* (P3, T3), *Moraxella* (P5), *Vibrio* (P4, P6, T5, T12, P10, P17, P19), *Shewanella* (P9, T4, P1, P11, P13, P2, T17), *Serratia* (T10), *Enterobacter* (T8), *Aeromonas* (T22, T26, T24, T1), and *Pseudoaltermonas* (P8). Only one genera *Arthrobacter* (P18) was from high G + C Gram positive bacteria. *Pseudomonas* (P3, T3) only presented in the intestine of puffer fishes fed with artificial diet, while *Serratia* (T10) and *Enterobacter* (T8) were specific to the group of puffer fish, which fed with natural fish and shrimp.

The ovary and liver are the two main tetrodotoxin-accumulating organs in puffer fish. A total of 30 species of bacteria from the ovary and liver were clusted into two groups, γ-Proteobacteria and low G + C Gram positive bacteria (shown in Fig. 3). γ-Proteobacteria included *Aeromonas* (T1, T21), *Shewanella* (P1, P11, P13, T16, P7, P2, P9, P14, T17), *Pseudoalteromonas* (P8), *Pseudomonas* (P3, T3), *Vibrio* (P6, P12, T6, T9, T13, T12, P10, T15), and *Moraxella* (P5). Other species were affiliated to genera *Bacillus* (P15, P16, T20), *Staphylococcus* (T2), *Jeotgalcoccus* (T11), and *Enterococcus* (T7, T14) of lower G + C Gram positive bacteria. The bacteria communities in tetrodotoxin-accumulating organs were affected greatly by the diet, especially in liver of puffer fish fed with natural diet, no bacteria could be cultured on the TCBS medium. Several genera such as *Aeromonas*, *Pseudoalteromonas*, *Bacillus*, *Enterococcus*, existed specificly in the TTX-accumulating organs of fish fed with natural diet, however, only two genera *Moraxella* and *Staphylococcus* in that fed with artificial diet.

Same situation found in skin, γ-Proteobacteria was still a dominant group, only *Jeotgalcoccus* (T11) was belonged to the group of lower G + C Gram positive bacteria. (Fig. 4). There were seven genera identified from the γ-Proteobacteria, including two kinds of *Shewanella* (P1, P11), one kind of *Pseudoalteromonas* (P8), four kinds of *Vibrio* (T12, T25, P6, P10), two kinds of *Pseudomonas* (P3, T3), one kind of *Psychrobacter* (P5), one kind of *Alteromonadaceae* (P2) and three kinds of *Aeromonas* (T18, T19, T23). The lower G + C Gram positive bacteria (T11) only existed in the skin of puffer fish fed with natural diet, while four specific genera *Pseudomonas*, *Psychrobacter*, *Alteromonadaceae*, and *Aeromonas* were found in the skin of

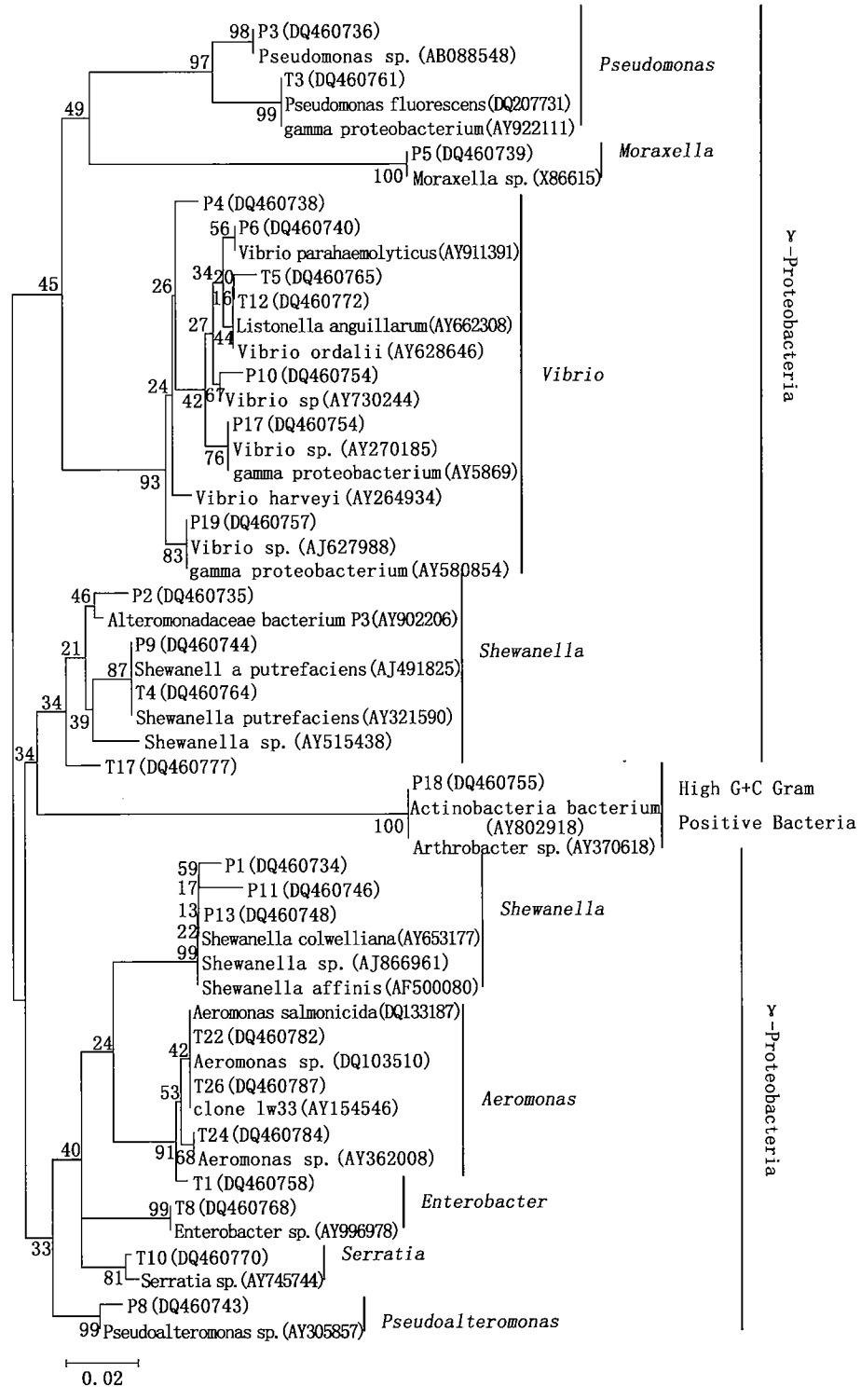

Fig. 2　Phylogenetic tree constructed based on bacterial 16S rDNA V3 region fragments from fish intestines

The trees were drawn from Clustal W generated multiple sequence alignment of nucleotide sequences using the neighbor-joining method within the MEGA (3.0) package. The topological stability of the neighbor-joining tree was evaluated by 1 000 bootstrapping replications, and the bootstrapping values are indicated by numbers at the nodes. The accession number of refer sequences of bacterial 16S rDNA V3 region fragments shown in parenthesis. The bar indicates 2% sequence variation.

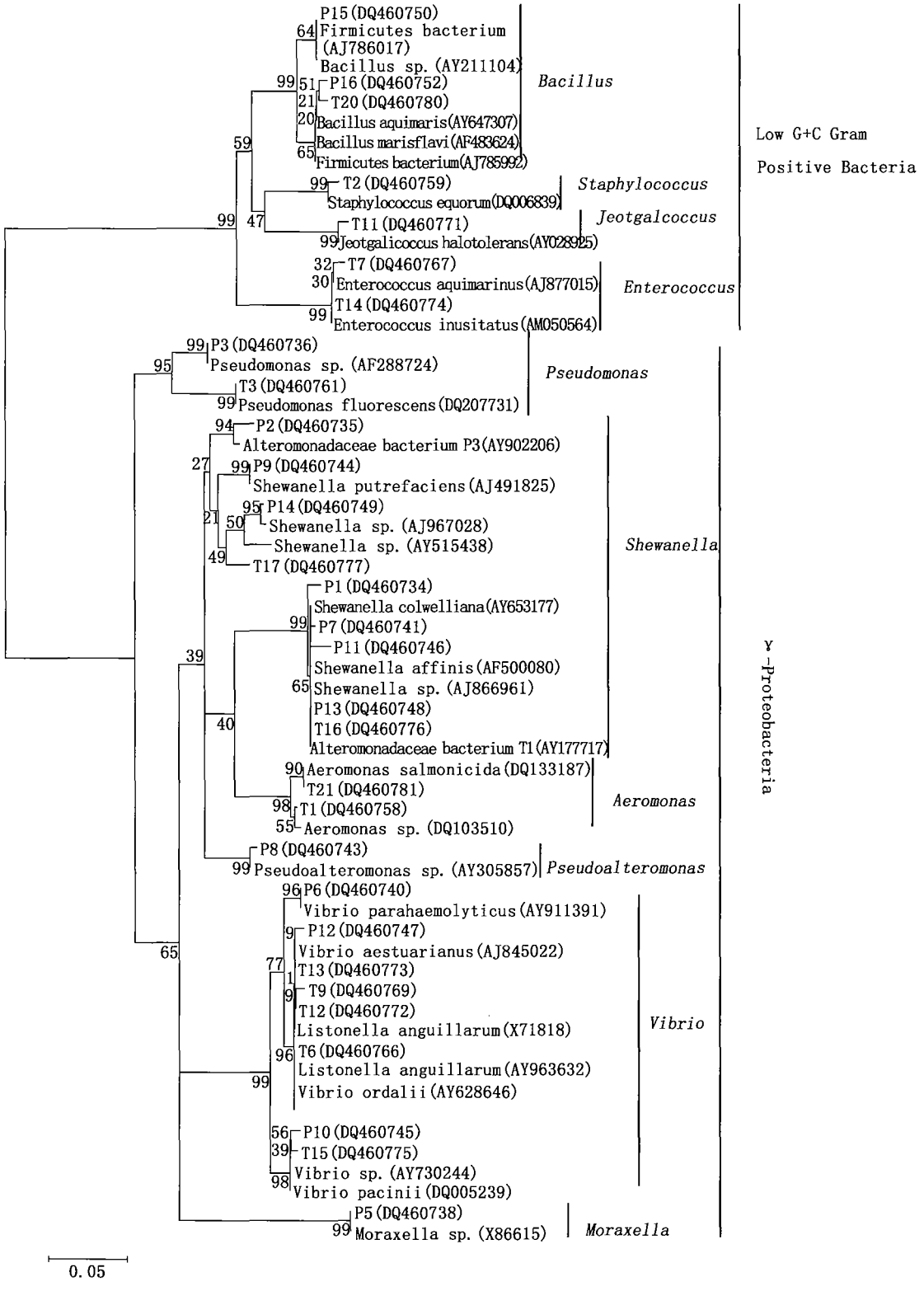

Fig. 3 Phylogenetic tree constructed based on bacterial 16S rDNA V3 region fragments from the TTX-accumulation organs ovary and liver

The trees were drawn from Clustal W generated multiple sequence alignment of nucleotide sequences using the neighbor-joining method within the MEGA (3.0) package. The topological stability of the neighbor-joining tree was evaluated by 1000 bootstrapping replications, and the bootstrapping values are indicated by numbers at the nodes. The accession number of refer sequences of bacterial 16S rDNA V3 region fragments shown in parenthesis. The bar indicates 5% sequence variation.

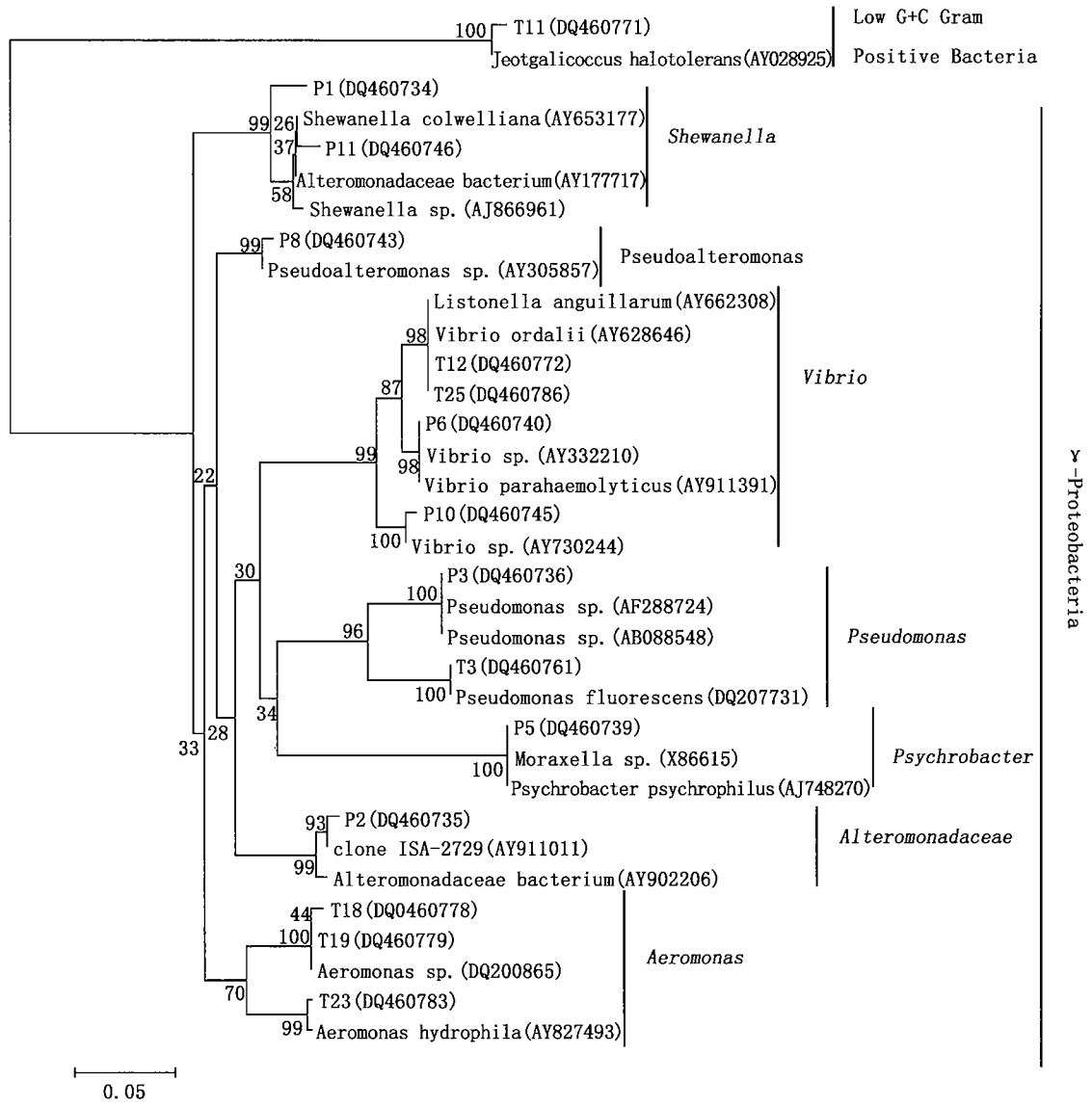

Fig. 4　Phylogenetic tree constructed based on bacterial 16S rDNA V3 region fragments from the fish skin

The trees were drawn from Clustal W generated multiple sequence alignment of nucleotide sequences using the neighbor-joining method within the MEGA (3.0) package. The topological stability of the neighbor-joining tree was evaluated by 1 000 bootstrapping replications, and the bootstrapping values are indicated by numbers at the nodes. The accession number of refer sequences of bacterial 16S rDNA V3 region fragments shown in parenthesis. The bar indicates 5% sequence variation.

fish fed with artificial diet.

3　Discussion

Most researches on the microbial community in fish have been focused on the intestines due to its importance in digestion and disease control[6-7]. The analysis presented here aimed to survey the bacterial community by sampling not only the intestines but also the ovary, liver and skin of the pufferfish *Takifugu obscurus* in relation to natural and artificial diets. On the whole, members of the gamma subclass of

Proteobacteria dominated the composition of the microbial community in puffer fish *T. obscurus*, while others were affiliated to the group of low DNA G + C content, and the group of high DNA G + C content. The bacterial composition of *T. obscurus* showed that most kinds of bacteria detected matched those that were prevalent in the marine environment[14].

Each of tissues of *T. obscurus* owned their specific bacterial community. Three genera, *Serratia*, *Enterobacter*, and *Arthrobacter*, were specific for intestine, three genera from the group of lower G + C Gram positive bacteria, *Bacillus*, *Staphylococcus*, and *Enterococcus* were specific for TTX-accumulating organ liver and ovary, while *Psychrobacter* and *Alteromonadaceae* only were found in skin. Genera like *Shewanella*, *Pseudoalteromonas*, *Vibrio*, *Pseudomonas*, and *Aeromonas*, were found in all checked tissues in this research. Obvious factor contributing to the bacterial assemblage was diet as Tanaka *et al.* reported that the bacterial diversity in the intestines of abalone fed with artificial food was higher than in individuals fed with sea algae[15]. In *T. obscurus*, we also found that the bacterial communities differed markedly between puffer fish fed natural diet and those fed with artificial diet. Only four species of total 25 species shared same in intestine, seven species of total 30 species same in TTX-producing organ, and four species of total 15 species same in skin.

The genera of most TTX-producing bacteria, which isolated from various puffer fishes, also were found in *T. obscurus*. To date, seven species were determined to be capability of TTX producing, including *Pseudomonas* spp., *Vibrio alginolyticus*, *Shewanella putrefaciens*, *Vibrio* species, *Serratia* spp., *Microbacterium arabinogalactanolyticum*, *Bacillus* spp.[16]. The genera such as *Pseudomonas*, *Vibrio* and *Shewanella*, were found in the intestine, TTX-producing organs and skin in this research. In whatever kind of tissues, the genera of TTX-producing bacterial, *Vibrio*, *Shewanella*, *Serratia*, *Bacillus*, and *Pseudomonas*, were found in fishes which fed with natural diet, while *Vibrio*, *Shewanella*, and *Pseudomonas* found in fishes fed with artificial diet. That meant whatever kinds of diet fed, the puffer fish *T. obscurus* owned the capability of TTX producing. Those results indicate that food chain accumulation of TTX contributes little to the toxicity of these fish, and remind that people still should be careful when they intend to eat the cultured puffer fish.

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暗纹东方鲀几种组织中可培养细菌的组成分析

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摘　要：采用 16S rDNA 特征序列 PCR-DGGE 法，分析了不同饵料饲养的暗纹东方鲀的表皮、肠道和河鲀毒素累积组织（肝和卵巢）中可培养细菌的群落组成。根据分子系统发育分析，共鉴定出 45 种可培养细菌，在这些细菌中，以变形细菌的 gamma 亚群占多数，其它分别隶属于低 GC 含量革兰氏阳性菌和高 GC 含量革兰氏阳性菌。摄食不同饵料的暗纹东方鲀，其肠道、表皮或河鲀毒素累积组织中的细菌菌落组成是不同的，但不管是摄食人工配合饲料或天然饵料，在暗纹东方鲀的肠道、表皮或河鲀毒素累积组织中，均发现已有报道的可产河鲀毒素的细菌类群，表明饵料来源对暗纹东方鲀的河鲀毒素产生不是必需的。

关键词：暗纹东方鲀；16S rDNA；变性梯度凝胶电泳（DGGE）；细菌组成；产 TTX 细菌

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