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Phylogenetic analysis of the pathogenic genus *Aeromonas* spp. isolated from diseased eels in China

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ABSTRACT

Analyses of 16S rRNA and housekeeping genes (HKGs) were valuated as identification markers for pathogenic *Aeromonas* isolated from diseased eels. The interrelationships of 32 *Aeromonas* strains which had been verified as pathogens to eels were studied using phylogenetic analysis with 16S rRNA and HKG sequences (*cpn60*, *gyrB*, *rpoB* and *dnaJ*) and identified by Biolog automatic microbiology analysis system (gene III). From the analysis of 5 genes, the mean gene divergences of 16S rRNA, *cpn60*, *gyrB*, *rpoB* and *dnaJ* in 32 isolates were 1.4 ± 0.2%, 7.1 ± 0.7%, 5.2 ± 0.5%, 2.2 ± 0.4% and 6.8 ± 0.5%, respectively. The results of comparative phylogeny between nucleotide based analyses (excluding the third codon position) of four HKGs with the sequences from 55 strains of *Aeromonas* (including 23 referenced strains of *Aeromonas*) showed *cpn60* and *dnaJ* have higher discriminate power than *gyrB* and *rpoB* comparing with the taxonomical identification by Biolog system. In addition, amino acid sequences of concatenated cpn60-rpoB-gyrB is a good method for *Aeromonas* pathogens identification. This study showed analysis of HKG sequences can be used as an alternative method for sound identification of bacterial pathogens isolated from diseased eels in China.

1. Introduction

The genus *Aeromonas* is a collection of Gram-negative bacteria in the natural habitats, especially freshwater, sludge and sewage, and usually does not cause clinical disease [8,32,58,70], but it may be conducive to the rapid proliferation of the bacteria under stress factors (handling, trapping, and warm water) and then facilitating disease [15,30,31,34,53,56]. Particularly, *A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. caviae*, *A. sobria* and *A. jandaei* are considered to be the most important conditional pathogens, infection with epizootic ulcerative syndrome (EUS) and a high rate of mortality in many marine and freshwater fish of the world [1,4,7,13,26,36,48,50,55,57,60]. According to the 10th edition of *Bergey's Manual of Systematic Bacteriology*, this genus comprises 14 species [37,40,44].

Analysis based on the 16S rRNA and housekeeping gene (HKG) are considered as efficient way for the identification of bacterial species [2,5,32], but it showed highly conserved sequences among different species, and some of them were discriminated only by one to several nucleotides [14,32,41,46]. DNA-DNA hybridization has been subjected to analysis the interrelationships between some present species [21,49], but discrepancies were obtained between different DNA-DNA hybridization studies and the DNA-DNA similarity between two species of *A. encheleia* (CECT4342^T) and *Aeromonas* sp. strains (HG11) was 12% and 84% by changed into in two studies respectively [11,22]. Moreover, discordances between DNA-DNA hybridization and 16S rRNA gene analysis had added more controversies regarding the species within the genus *Aeromonas* [12,38,59].

In recent years, phylogenetic analysis of sequences of HKGs had been recommended as an effective way to increase the discriminatory power over 16S rRNA sequences analysis, and phylogenetic studies based on *cpn60*, *dnaJ*, *gyrB* and *rpoB* gene sequences have shown to be useful to identify species within a genus [3,6,14,44,54]. A highly conserved protein found in bacteria is Type I chaperonin Cpn60 and gene sequences of it provided much better discriminate ability than 16S rRNA gene at the species level in genus *Aeromonas* [18,44]. The *dnaJ* gene, encoding heat shock protein 40, had been demonstrated to be a good candidate gene for identification of the species of the genera *Legionella* [35], *Streptococcus* [24] and *Aeromonas* [49]. Phylogenetic analysis of *gyrB* gene (encoded the β-subunit of DNA gyrase) discovered related species and it was also

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used to characterize novel species within a genus [32,39,52]. Yanez et al. [67] reported an excellent molecular chronometer (gyrB gene) sequence in discriminating the phylogenetic inference of 17 strains of *Aeromonas hydrophila* [62]. The rpoB gene (B subunit of DNA-dependent RNA polymerase) had also been used to identify *Aeromonas* strains [2,3,28,29,33,45].

Eel (Japanese, European and American eel) is one of the most important economic fish cultured in China, but diseases infected by pathogenic bacteria limited the productivity and further development of this industry [16,17,63,64], and some species of the genus *Aeromonas* had been implicated as pathogens in eels [10–12,25,68,69]. Traditional taxonomical identification of pathogenic bacteria in diseased eels was often influenced by changeable phenotypes [68]. Therefore, it is desirable to establish an alternative method to identify bacterial pathogens. In the present study, we used phylogenetic analysis based on *cpn60 gyrB, rpoB* and *dnaJ* amino acid sequences to identify 32 pathogenic *Aeromonas* strains isolated from diseased eels in recent 10 years. We referenced corresponding sequences of type and reference strains of *Aeromonas* described thus far. Compared to other identification method based on Biolog system and 16S rRNA, phylogenetic method is more reliable method. We investigated four independent phylogenetic trees derived from the alignments of *cpn60*, *rpoB*, *gyrB* and *dnaJ* amino acid instead of DNA sequences. The major aim of this study is to facilitate a reliable molecular method for identification of pathogenic *Aeromonas* from farmed eels in China.

2. Materials and methods

2.1. Bacterial strains

Thirty-two *Aeromonas* strains used in this study were isolated from diseased eels in China and are listed in Table 1. The strains were grown overnight at 28 °Con Tryptone soya agar base (TSA) and stored at −80 °C in saline with 20% glycerol added. Thirty-two strains were ascertained as pathogenic bacteria by challenge test as following [15,63,69]. Briefly, bacterial suspension of 32 strains was grown overnight in Tryptone soya broth (TSB) at 28 °C for 18 h, and the cells were harvested by centrifuging (3000 × g, 10 min) and washed three times with PBS. Bacteria were suspended in 10 mM PBS (pH 7.4) and adjusted to the concentration of 1.0×10^8 cfu ml^{-1}. Eels (7, 10 or 15 eels) were inoculated (*i.p* or *i.m*) with 0.1 ml bacterial suspension respectively, and then reared in separate aquaria. Mortality was recorded daily up to 14 days.

Table 2
Primers used for PCR amplification and sequencing of 16S rRNA, *cpn60*, *rpoB*, *gyrB* and *dnaJ* genes. Primer positions are given according to *E. coli* numbering. N, Any nucleotide; R, A or G; S, C or G; Y, C or T; M, A or C.

Primer	Sequence (5′ → 3′)	Reference
16S rRNA		
27f	AGAGTTTGATCCTGGCTCAG	Moreno et al. [47]
1492r	ACGGCTACCTTGTTACGACTT	Moreno et al. [47]
cpn60		
C157	GAAATYGAACTGGAAGACAA	Miñana-Galbis et al. [44]
C938	GTYGCTTTTTCCAGCTCC	Miñana-Galbis et al. [44]
rpoB		
Pasrpob-L	GCAGTGAAAGARTTCTTTGGTTC	Küpfer et al. [32]
Rpob-R	GTTGCATGTTNGNACCCAT	Küpfer et al. [32]
gyrB		
gyrB-3F	TCCGGCGGTCTGCACGGCGT	Soler et al. [62]
gyrB-14R	TTGTCCGGGTTGTACTCGTC	Soler et al. [62]
dnaJ		
Aero-dnaJF	CGAGATCAAGAAGGCGTACAAG	Nhung et al. [49]
Aero-dnaJR3	CACCACCTTGCACATCAGATC	Nhung et al. [49]

Table 1
Sources of 32 strains isolated from diseased eels used in the study.

Time	Locations	Species	Major symptoms	Organs	Strains no.	Challenge mortality[a]
2002.8	Fuqing	European eel	Intestinal edema, skin bleeding	Liver	B09	1/10
					B10	8/10
2002.9	Fuqing	European eel	Severe gill rot, sepsis	Gill	B11	15/15
				Liver	B14	8/15
2004.7	Fuqing	European eel	Liver khaki-colored, sepsis	Liver	B15	7/15
					B20	15/15
					B21	13/15
2004.8	Fuqing	European eel	Skin bleeding, sepsis	Liver	B26	9/15
					B27	11/15
					B28	13/15
2004.9	Fuqing	European eel	Skin bleeding, sepsis	Liver	B29	15/15
					B30	15/15
					B31	15/15
					B32	15/15
2008.8	Shantou	European eel	Gill rot, red-mouth	Liver	B41	1/7
2008.8	Fuzhou	Japanese eel	Skin ulceration, sepsis	Kidney	B44	7/7
2009.5	Xiamen	American eel	Gill rot, intestinal edema	Ascites	B49	7/7
				Gill	B50	7/7
2009.6	Xiamen	American eel	Gill rot, sepsis	Gill	B51	7/7
				Kidney	B52	3/7
				Heart	B53	6/7
				Gill	B55	7/7
				Liver	B56	7/7
				Liver	B57	7/7
2009.7	Xiamen	American eel	Skin ulcers, liver congestion	Liver	B59	7/7
2009.8	Xiamen	American eel	Gill rot, skin bleeding	Gill	B60	7/7
2010.7	Xiamen	American eel	Tail ulceration, liver anemia	Tail	B65	7/7
				Gill	B66	7/7
2010.8	Xiamen	American eel	Fin bleeding, gill rot, liver anemia	Gill	B67	1/7
2010.8	Xiamen	Japanese eel	Fin congestion, gill rot, liver and kidney swollen	Kidney	B69	7/7
2010.9	Xiamen	American eel	Tail ulceration, liver anemia	Liver	B70	7/7
2010.9	Shantou	European eel	Tail ulceration and bleeding	Heart	B73	2/7

[a] Numerator means dead eels, and denominator means challenged eels [10 g/eel, challenged (*i.p* or *i.m*) with 1×10^7 cfu bacteria/eel].