DNA fingerprinting of *Flavobacterium columnare* using RAPD-PCR

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Abstract In the present study, DNA fingerprinting of eight strains of *Flavobacterium columnare* was done by random amplification of polymorphic DNA (RAPD) fingerprinting method. The strains were collected from Fish Health Management Division, Central Institute of Freshwater Aquaculture, Bhubaneswar, India. A total number of 160 primers were screened for RAPD-PCR, of which 10 primers yielded amplification with all the strains. The molecular weight of amplified bands varied from 0.29–2.63 Kb. The number of bands varied from 1 to 8. Unique band was seen with primer OPY-15 with molecular weight 0.75 Kb that can be used for epidemiological study. Genetic variability was investigated using NTSYS software. Highest genetic similarity was found between MS1 and MS3 followed by MS5 and MS7. Minimum genetic similarity was found between MS2 and MS8. Phylogenetic tree was constructed using UPGMA and neighbor joining methods.

Keywords *Flavobacterium columnare* · DNA fingerprinting · RAPD-PCR · Phylogeny

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Introduction

Flavobacterium columnare is recognized as a pathogen of freshwater fish with a worldwide distribution [1–5]. It was formerly called *Flexibacter columnaris*, but in 1996 it was transferred to the genus *Flavobacterium* [6]. *F. columnare* (*Flexibacterium columnaris*) is recognized as a freshwater fish pathogen and is an important cause of gill and skin disease often resulting in high mortality [1–2]. It is the etiological agent of columnaris disease, which is manifested as chronic ulcerative condition beginning as a localized infection on the body surface and gills. As the disease progresses, the lesions spread and may cover most of the body surface. In some cases, the disease is manifested by symptoms such as extensive gill necrosis, particularly if a high virulent strain is involved in the pathogenesis process. In this case, external lesions may not be evident and the fish die acutely after showing signs of suffocation. In identifying fish diseases, molecular techniques are potentially more sensitive than conventional methods such as culture, serology and histology [7]. Randomly amplified polymorphic DNA (RAPD) fingerprinting blended with PCR is an important molecular diagnostic tool that can be adopted for quick and precise identification of *F. columnare* and to compare strains of numerous bacterial species because of the generic capabilities of PCR system [2, 8, 9]. This method is quite simple, relatively economical, reliable and is particularly suitable when the quantity of the genomic DNA is limited and where prior information related to structure of the genomic DNA is lacking. RAPD-PCR is based on the ability of a single primer of arbitrary nucleotide sequence to generate polymorphic amplified products for any genome in question. These amplified products produce specific

genomic fingerprints on gel electrophoresis that could generate a species-specific or strain-specific pattern. There are evidences that molecular techniques have been used for bacterial pathogens for identification.

Stress induced columnaris disease in rohu (*Labeo rohita*) in India was studied by Kumar et al. [10]. *Catla catla*, *Labeo rohita*, *Cirrhinus mrigala*, *Anabas testudineus* and *Clarias batrachus* are commercially important freshwater food fishes and cultured extensively in our country. These fishes are susceptible to columnaris disease. During 2003–04, there was an outbreak of columnaris disease in Kolleru lake area of Andhra Pradesh that caused massive mortality of Indian major carp. Faster diagnosis of the disease becomes a concern in this type of cases. The conventional methods of typing and identifying several pathogenic strains of *F. columnare* through phenotypic characteristics, biochemical tests and antimicrobial susceptibility assays are time-consuming, difficult to perform, poorly sensitive and not completely reliable. The recent introduction of genotypic tests such as restriction endonuclease analysis (REA), restriction fragment length polymorphism (RFLP), pulse field gel electrophoresis (PFGE) and RAPD fingerprinting have overcome these limitations of conventional tests. In comparison with other molecular typing methods, RAPD is faster and less labour-intensive, only small amount of template DNA is required for the amplification reactions and in some cases, the method give better discrimination among bacterial strains [11]. Moreover, this technique of genetic characterization is most applicable where the genome of organisms is unknown. The PCR-based RAPD fingerprinting generates distinct DNA profile with the help of short oligonucleotide primers (arbitrary) and these resultant amplified DNA profiles are referred as RAPD markers, which are inherited in a Mendelian fashion [12]. The degree of similarity of RAPD banding pattern could be used to estimate genetic variation and nucleotide diversity. The use of RAPD assay analysis in genetic variation was successfully applied in several bacterial species/isolates/strains such as *Streptococcus* spp. [13], *Staphylococcus* spp. [14] and *Pseudomonas aeruginosa* [15]. Therefore, the RAPD markers are very helpful in estimating interspecies genetic variation and phylogenetic studies in bacteria. As there was scanty work done on the molecular characterization of this species, an attempt was made to characterize *F. columnare* at the molecular level that will aid in faster diagnosis of the disease. The present investigation deals with the molecular characterization and the phylogenetic relationship among the different *F. columnare* strains collected from diseased freshwater fishes of Eastern India that was isolated and maintained at Central Institute of Freshwater Aquaculture (CIFA).

Materials and methods

Collection of strains

The *F. columnare* strains were obtained from Fish Health Management Division, CIFA. There were eight strains from 78 isolates and grouped after biochemical characterization [16]. The strains were coded as MS1, MS2, MS3, MS5, MS6, MS7, MS8 and MS9 respectively. The biochemical profiles of the strains are given in Table 1.

Genomic DNA Isolation

The protocol described by Murray and Thompson [17] for isolation of genomic DNA was followed with modifications.

PCR reaction

The RAPD technique was standardized via PCR, using various 10 decamer arbitrary random primers obtained from Operon Technology, USA. In the present study, eight sets of primers were screened. In each set 20 arbitrary primers were present. So altogether 160 arbitrary primers were screened using the strain MS9 of *F. columnare*. The various primers that were used belonged to OPA, OPB, OPC, OPE, OPF, OPJ, OPK and OPY series. All the primers were 10 nucleotides in length and had a GC content of 60%. PCR was standardized by varying the annealing temperature from 30–45°C and DNA concentration from 5–50 ng. The reaction was carried out in a reaction volume of 25 µl containing 2.5 µl 10 X buffer (MBI Fermentas, Cat No 4303), 2.5 µl of MgCl₂, 2.5 µl dNTP (mix of all 4 dNTPs, cat No. FC23 was procured from Bangalore Genei Pvt. Ltd, 5 pmol primer (160 different oligonucleotide primers obtained from Operon Technologies Inc., Alameda, USA), 1U Taq DNA polymerase (Bangalore Genei Pvt. Ltd, with cat No. MME5J) and 25 ng genomic DNA. The tubes were tapped gently and spinned for a few seconds. Then they were placed in the thermal cycler with programmes as: one cycle of initial denaturation step at 94°C for 4 minutes followed by 45 seconds of 94°C (denaturing temperature), 36°C (annealing temperature) for 45 seconds and 72°C (extension temperature) for 90 seconds, the total number of cycles being 35. The cycling was concluded by an additional final extension at 72°C for 7 min and the products were stored at 4°C until further analysis. The PCR-amplified products were analyzed through 1.8% agarose gel electrophoresis following the protocol as described by Sambrook et al. [18]. The fingerprinting patterns obtained was then photographed onto gel documentation unit and the bands were analyzed.

Analysis

The presence of a band was scored as 1 and absence was scored as 0. This 1-0 data was used in NTSYS (Numerical taxonomy system) software to get genetic similarity between the 8 strains of *F. columnare*. Phylogenetic tree was constructed using the following methods:

Unweighted pair group method with arithmetic averages (UPGMA)

This method, one of the oldest techniques to be used in phylogenetic analysis, is an offshoot of the linkage methods that were popular in numerical taxonomy studies. Its simplicity and ease of interpretation has made its survival in phylogenetic studies. It works on the assumption that the rates of evolution in all lineages are same and gives output clustering in increasing order of distance.

Neighbor-Joining (NJ) method

Estimation of minimum evolution phylogeny by heuristic method is done by this method. It works on the assumption of unequal rates of evolution.

Results and discussion

Band analysis

The sets of primers that were screened are OPA, OPB, OPC, OPE, OPF, OPJ, OPK and OPY. Out of these primers,

the primers that produced three or more than three bands were selected for future PCR analysis. Based on the ability to produce consistent and distinguishable fragment pattern, 34 primers were selected. All the selected primers were tested on the eight strains of *F. columnare*. With each primer, the number of amplified bands varied from a maximum of eight to a minimum of one band and the molecular weight of amplified fragment varied from 0.29Kb to 2.63Kb. The summary of number of bands amplified and molecular weight range of bands are presented in the Table 2. The unique bands, i.e. the bands, which is present in all the strains for the particular primer was found out. It was found out that all the strains amplified for the primer OPY15 (Fig. 1) and the bands produced had the molecular weight 0.75 Kb. In case of OPJ19 (Fig. 2) a major band of molecular weight 1.06 kb common to all the strains, was found missing in case of strain MS2. In case of OPY16 (Fig. 3), a band of molecular weight 1.08 kb common to all the strains was found missing in case of strain MS1 and MS8.

Genetic similarity

From the genetic similarity value (Table 3), it was found that there is maximum similarity between strains MS1 and MS3 and minimum similarity was found between strains MS2 and MS8.

Table 1 Biochemical properties of the 8 strains of *F. columnare*

Tests	Isolate No.							
	MS1	MS2	MS3	MS5	MS6	MS7	MS8	MS9
Catalase	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+
H ₂ S	-	-	-	-	-	-	-	-
Gas	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+
Indole	-	-	-	-	-	-	-	-
Nitrate	+	+	+	-	+	+	+	+
Citrate	-	+	-	-	+	-	-	-
Urease	-	-	-	-	-	-	-	-
MR	+	-	-	-	-	-	-	+
O/F	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
VP	-	-	-	-	-	-	-	-
Casein	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+
Starch	-	-	+	+	+	+	-	+
Lysine	+	-	-	-	-	-	-	-

Table 2 Summary of the molecular weights range of amplified products (primer-wise and strain-wise) of 8 isolates of *F. columnare* obtained from decamer primer of random sequence

Primer	Molecular weight range (Kbp) of amplified DNA fragment							
	1	2	3	4	5	6	7	8
OPE15	0.63–2	0.63–1.12	0.57–2.0	0.59–1.12	0.59–1.12	0.59–0.63	1.25–2.0	0.63–2.0
OPE19	0.82	–	0.59–0.82	0.62–1.07	0.61–0.82	0.61	0.64–2.07	0.57–0.82
OPF12	0.65	0.66–0.77	–	0.62–0.87	–	0.67–0.78	0.88–2.01	0.62–2.01
OPJ20	0.29–2.26	–	0.29–2.26	0.35–1.14	–	0.27–1.14	0.27–0.96	0.32–0.48
OPJ19	1.06	–	1.06	0.62–1.06	0.62–1.97	0.63–1.06	0.61–1.06	1.06–1.29
OPK17	1.23	–	1.24	1.25	0.61	1.23	1.21	1.20
OPY13	0.64–1.34	–	0.84–1.35	0.39–2.06	0.69–1.05	0.69–0.82	0.70–1.53	0.85
OPY15	0.44–1.30	0.75	0.75	0.75	0.68–0.75	0.68–0.75	0.44–0.75	0.68–0.75
OPY16	0.75–2.34	0.89–1.08	1.08–2.34	0.89–1.08	0.89–1.08	0.89–1.08	0.75–1.57	0.89–1.08
OPY17	0.29–0.78	–	0.29–0.78	0.34–0.96	0.34–0.96	0.34–0.96	0.40–1.12	0.35–2.63
Total no. of amplified bands	38	7	29	36	21	26	31	32

Table 3 Similarity matrix between the different strains of *F. Columnare* from RAPD PCR

	MS1	MS2	MS3	MS5	MS6	MS7	MS8	MS9
MS1	1.000000							
MS2	0.119048	1.000000						
MS3	0.581395	0.156250	1.000000					
MS5	0.250000	0.189189	0.300000	1.000000				
MS6	0.176471	0.208333	0.282051	0.390244	1.000000			
MS7	0.203704	0.214286	0.279070	0.550000	0.468750	1.000000		
MS8	0.228070	0.026316	0.176471	0.218182	0.106383	0.212766	1.000000	
MS9	0.203390	0.212121	0.297872	0.333333	0.261905	0.260870	0.188679	1.000000

Fig. 1 RAPD patterns obtained with OPY15 primer using eight strains of *F. columnare* **Fig. 2** RAPD patterns obtained with OPJ19 primer using eight strains of *F. columnare*

Phylogenetic analysis

UPGMA method

Genetic tree was made by unweighted pair group method with arithmetic average (UPGMA) method in NTSYS software (Fig. 4). It was seen that strains MS5 and MS7 are

genetically close to each other. MS6 is found to be related to MS5 and MS7. MS9 is closely related to this group (MS6, MS5 and MS7). Again this group is related to the group of MS1 and MS3, which are having maximum genetic similarity. MS8 is genetically similar to the above said strains whereas MS2 is far from all the strains.

Neighbour joining method

In the neighbor joining method (Fig. 5) it was seen that MS1, MS3 and MS8 are closely related to each other while the others form a different group. In the second group, MS5 and MS7 are very close to each other followed by MS6. MS2 and MS9 are closer to MS5 and MS6 respectively.

The RAPD-PCR results obtained in this study on eight strains of *F. columnare* showed a wide variety of profiles on gel electrophoresis, depending on the primer used and strain investigated. The results obtained in this study have clearly proved that RAPD technique is used to generate consistent, reproducible and distinct DNA profiles in eight strains of *F. columnare*. This differentiation was in accordance with the studies in *Vibrio angillarum* [19], *Escherichia coli* [20], *Salmonella* spp. [21], *Aeromonas salmonicida* [22], *Piscirickettsia salmonis* [23], *Flavobacterium psychrophilum* [24], *Vibrio vulnificus* [25], *Vibrio parahaemolyticus* [26], *Vibrio cholerae* [27], *Clostridium botulinum* [28] and *Renibacterium salmoninarum* [29].

Fig. 3 RAPD patterns obtained with OPY16 primer using 8 strains of *F. columnare*

Fig. 4 Dendrogram (UPGMA) for eight strains of *F. columnare* based on RAPD bands

Fig. 5 Dendrogram (neighbor joining tree) for eight strains of *F. columnare* based on RAPD bands

One of the primers, OPY15, produced amplified fragment common to all the strain with molecular weight 0.75 Kb referred to as the unique band. The presence of unique band will definitely aid in epidemiological studies. Moreover, these bands could be cloned and sequenced in future, for designing suitable primers to be used in PCR based diagnosis. RAPD-PCR had already been used in the epidemiological studies of microorganisms such as *Campylobacter* [30] and *Aeromonas salmonicida* subsp. *salmonicida* [31].

At this stage, the common bands generated by the primer cannot be called as species-specific marker. To validate the species-specific marker it is essential to test with other pathogenic/non-pathogenic microorganism and/or it should be cloned and sequenced. Oakey et al. [32] found single RAPD, when tested showed multiple fragments of similar size, but different in nucleotide sequence. RAPD fingerprinting under identical PCR condition using short oligonucleotide primers can generate polymorphic DNA banding profile. The highest GS value was found between strain MS1 and MS3 followed by MS5 and MS7. Minimum GS was found between strain MS2 and MS8. This study provides evidence that RAPD could be used for the assessment of genetic diversity and differentiation of eight strains of *F. columnare*. This assessment has also made utility of RAPD for phylogenetic relationship among the strains.

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