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Full Length Research Paper

Isolation and molecular characterization of *Flavobacterium columnare* strains from fish in Brazil

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***Flavobacterium columnare*, the etiologic agent of columnaris disease, has a broad geographical distribution and accounts for a large number of mortalities in fish species. This study aimed to generate a faster method for diagnosis of columnaris through isolation and characterization of the *F. columnare* 16S rDNA gene from bacteria isolated from Nile tilapia *(Oreochromis niloticus),* tambaqui *(Colossoma macropomum)* and matrinxã (*Brycon amazonicus*). The bacteria were characterized biochemically and by PCR-RFLP. For isolation, rasping with "swab" was performed directly on the characteristic lesions and the cephalic kidney of the fish then transferred to culture medium suitable for *Flavobacterium.* DNA was extracted for PCR and digestion with restriction enzymes. Altogether, 37 isolates were obtained. Biochemical assays included testing of absorption of Congo red, production of flexirrubin, production of H_2S, nitrate reduction and motility. The results indicated that the isolates can be classified as *F. columnare.* The phylogram generated by the PCR-RFLP technique showed three main branches among of the *F. columnare* isolates. Therefore, the use of PCR-RFLP for identification of the bacteria was shown to be a more efficient and rapid tool than current biochemical techniques, which are time consuming and often inconclusive.**

Key words: Fish, *Flavobacterium columnare,* PCR-RFLP, 16S rDNA.

INTRODUCTION

Flavobacterium columnare, which is responsible for columnaris disease in fish, is an opportunistic bacterium with a broad geographical distribution. It makes up part of the normal microbiotic of the water, skin and gills of a fish. It is commonly observed in fresh-water fish culture and is described as a causal agent of large numbers of mortalities in trout (*Oncorhynchus mykiss*), salmon (*Salmo salar*), carp (*Cyprinus carpio*), tilapia (Oreochromis niloticus), perch (*Perca fluviatilis*) and catfish (*Ictalurus punctatus*) (Bernardet and Grimont, 1989).

Columnaris disease is characterized by grayish dots or yellowish areas of erosion that are usually surrounded by a hyperemic reddish zone on the head, body surface and gills. These sites display progressive necrosis involving the epidermis, dermis and muscles, and they often lead to systemic infection (Decostere et al., 1999).

In Brazil, many columnaris outbreaks have been obse-rved, especially during the changing of seasons when daily water temperature variation is greater than 5°C and water quality is inadequate. During such times there is

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often a reduction of the dissolved oxygen concentration, an excess of organic matter and inadequate management. Besides being a serious health problem, this disease reduces production due to high and rapid mortality of fingerlings, decimation of shoals and non-utilization of the product due to the lesions that it causes. Despite this fact, there are few studies in the Brazilian literature addressing columnaris disease (Pilarski et al., 2008).

Species that exhibit rapid growth, good feed conversion, easily induced reproduction and features appropriate for sport fishing have stimulated interest in fish culture. For this study the following species were selected: matrinxã *(Brycon amazonicus)*, a species of the Bryconinae subfamily from the Amazon Basin (Tavares-Dias et al., 1999); tambaqui *(Colossoma macropomum),* a freshwater teleost fish belonging to the order Characiformes, family Serrasalmidae (Géry, 1977), which is native to the Amazon and Orinoco basins and their tributaries (Chagas and Val, 2003); and the Nile tilapia *(O. niloticus),* which has become the most cultivated species in Brazil over the last decade, accounting for approximately 40% of the national aquaculture (Zimmermann and Hasper, 2003; Marengoni, 2006).

Molecular characterization has been used in epidemiological studies to identify different pathogen genotypes in a safe and highly efficient manner. Furthermore, these techniques can be performed faster than other methods. Recognition of the prevalence of a particular genotype and its patterns in an infection can lead to the development of more effective methods for ichtiopathology control (Darwish and Ismaiel, 2005; Olivares-Fuster et al., 2007b; Figueiredo and Leal, 2008).

Several studies describe the genetic variation observed by PCR and RFLP analysis of *F. columnare* strains found throughout the world (Song et al., 1998; Bernardet and Grimont 1989; Toyama et al., 1996).

Therefore, the objective of this study was to isolate and characterize strains of *F. columnare* using molecular and biochemical methods. The intraspecific variation of the 16S rDNA gene using the PCR-RFLP technique was used in cultured specimens of tilapia *(O. niloticus),* matrinxã *(Brycon amazonicus)* and tambaqui *(C. macropomum)* collected from various regions in the Southeastern Brazil, with characteristic clinical signs of columnaris disease.

MATERIALS AND METHODS

The study was conducted at the Laboratory of Bacteria Genetics and Applied Biotechnology, belonging to the Department of Applied Biology, Faculty of Agriculture and Veterinary Sciences - UNESP, Jaboticabal Campus. Isolation of *F. columnare* strains occurred in the Ichtiopathology Laboratory of the Department of Veterinary Pathology, Faculty of Agriculture and Veterinary Sciences - UNESP, Jaboticabal Campus.

Bacterial isolation

Samples of *F. columnare* were taken from 18 specimens of tilapia *(O. niloticus),* matrinxã *(B. amazonicus*) and tambaqui *(C. macropomum)* from different fish farms in the region of São Paulo, Brazil. The fish used in this study were clinically diagnosed as suffering from columnaris disease by the Pathology Laboratory of Aquatic Organisms (LAPOA-CAUNESP-Jaboticabal, SP).

For isolation of bacteria, a sterile swab was rasped in the characteristic lesions and in the kidneys of the fishes. The material was then immediately transferred to a culture medium (liquid and solid) favorable for growth of *F. columnare*, modified by Pilarski et al., (2008) and incubated at 30°C for 48 h.

The bacterial isolates were grown on solid medium that was composed of 100 ml natural fish fillet, 5.0 mL yeast extract (yeast Fleishemann), 0.02 g sodium acetate and 2.5 g agar at pH 6.8. Media were autoclaved at 121°C for 15 min in 100 ml, distributed in Petri dishes for plating and incubated for 48 h in a bacteriological incubator at 30°C.

The characteristic colonies were inoculated in flasks containing 6. 0 ml of liquid medium which consisted of 100 ml natural fish fillet, 5 ml yeast extract and 0.02 g sodium acetate at pH 6.8. This medium was autoclaved at 121°C for 15 min and incubated at 30°C for 48 h.

Biochemical characterization

The biochemical tests for identification of *F. columnare* isolates were performed using kits for bacterial biochemistry (LaborClin®), in which included assays for desamination of L-tryptophane, utilization of glucose as a sole carbon and energy source, gas and H_2S production, decarboxylation of ornithine, indole, rhamnose, citrate and lysine, motility, production of flexirrubin type of pigments, Congo Red adsorption, nitrate reduction, TSI (Triple Sugar Iron Agar), esculin, mannitol, inositol, arginine, sucrose, lysine, starch hydrolysis and catalase.

DNA extraction and PCR-RFLP

DNA was extracted according to the method described by Wilson (1987) with modifications including the addition of lysozyme for cell wall degradation, proteinase K to degrade proteins, a solution of CTAB/NaCl and treatment with RNase A at the end of the process for DNA purification.

DNA from the new isolates of *F. columnare* was analyzed by amplification of the conserved region of 16S rRNA using the PCR method. Two strains of *F. columnare* (referred to as 38 and 39), one strain of *Aeromonas hydrophila* and one strain of *Streptococcus agalactiae* belonging to the collection of the Pathology Laboratory of Aquatic Organisms (LAPOA-CAUNESP - Jaboticabal / SP) were used as controls.

The sequences of oligonucleotide primers used for amplification of the ribosomal subunit 16S rDNA were made according to Kuske et al. (1997) as follows: PAF 5'AGAGTTTAGTCCTGGCTCAG 3' (located in *E. coli* bases 8 - 27) and PC5B 5'TACCTTGTTACGACTT 3' (located in *E. coli* bases 1507 - 1492), with approximately 1500 bp.

The amplification reactions were conducted in a reaction volume of 20 ∝l containing 110 ng DNA to be amplified, 0.5 ∝l solution of

dNTPs (10 mM), 0.6 ∝l $MgCl_2$ (1.25 mM), 2.0 ∝l buffer solution (10X) for the PCR reaction, 1.0 ∝l of each primer (10 pmol/∝l), 5.0 U *Taq* polymerase and Milli Q distilled water (previously sterilized, q.s.p.). All amplification reactions were performed in sterile tubes containing no genetic material.

The cycle of amplification was performed in a MJ Research, Inc., PTC-100 TM model thermal cycler equipped with a "Hot Bonnet" circuit. The program initially consisted of the following steps: 94°C for 2 min for denaturation, then 35 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 1 min, followed by an extension step at 72°C for 5 min. Samples were then maintained at 10°C until removal of the DIFFERENT LETTER.

PCR products were submitted to electrophoresis in 1.5% agarose gels for 1 h, 30 min at 90 V and stained with 5 µg/ml ethidium bromides to visualize the band of 1500 bp. In all electrophoresis experiments, a DNA sample of known molecular weight was used (1 Kb ladder purchased from Fermentas®), which served as a reference for calculation of the molecular weights of the fragments obtained in the PCRs.

The amplified DNA fragments were separately cleaved with five restriction enzymes including *EcoRI, MboI, PstI, HhaI* and *HpaII* according to the manufacturer's instructions. To visualize the genetic profile generated by digestion with these restriction endonucleases, the reactions were subjected to electrophoresis under the exact same conditions mentioned above.

A binary matrix was constructed based on the bands representing fragment polymorphisms that were generated by digestion of PCR with restriction enzymes (RFLP-PCR).

This matrix was used to construct a table of genetic similarity and a phylogram with the software FREETREE version 0.9.1.50, using UPGMA grouping and the Jaccard coefficient. Its visualization was made possible with the aid of the software TREEVIEW, bootstrapped 1000 times.

RESULTS

Bacterial isolates

The procedure for isolation of *F. columnare* yielded 37 new isolates, listed in Table 1. Among these, 22 were derived from tambaqui, 7 were from tilapia and 8 were from matrinxã. These isolates were characterized as *F. columnare* based on the characteristics of the colonies and their culture and biochemical features.

When the bacteria were cultured in liquid media, we observed the development of bacilli that were thin, long and exhibiting flexing movements by gliding. When grown on a solid medium, the colonies produced by strains were flat, rather small, yellow-orange and had rhizoid edges.

By gram staining, all strains were gram-negative, filamentous bacteria showed morphology of thin bacilli. The bacteria were grouped in columns in smears.

Biochemical tests

After a preliminary diagnosis of the isolates conducted by observation of colony morphology and behavior in a hanging drop under a microscope, 22 biochemical tests were performed and their results are given in Table 2.

The TSI test showed four distinct behaviors among the isolates. A total of 49% of the isolates showed an alkaline response and production of H_2S; 8% presented little fermentation, alkaline character and production of H_2S; 27% performed anaerobic fermentation and had alkaline as well as acid characteristics in the test tube; 16% showed total fermentation.

The fact that the samples were positive for absorption of Congo red; produced flexirrubin, H_2S showed nitrates reduction and motility indicates that the isolates could be classified as *F. columnare* (Table 2).

Of the 22 biochemical tests, which required seven days for completion, 12 resulted in the same response for 100% of the isolates. The OTHER TESTS showed variations between strains and could therefore not be used as standards for the identification of the species.

PCR-RFLP technique

The PCR-RFLP technique was used to develop a faster and more conclusive test.

The restriction of the amplified products with *EcoRI* generated two fragments between 900 and 700 bp. These fragments were produced by *EcoRI* digestion of genetic material from all of the microorganisms analyzed, resulting in a single genetic profile. Digestion with *EcoRI* was not even able to differentiate between the controls strains of *Aeromonas hydrophila* and *Streptococcus agalactiae* (Figure 1).

Restriction of amplified products with *PstI* generated two fragments between 1000 and 700 bp that were only observed in isolates 7, 8, 9, 11, 19, 20, 21, 38 and 39 of *F. columnare.* The other isolates were not cleaved by this enzyme.

The restriction of amplified products with *HhaI* produced four fragments between 700 and 200 bp in isolates 1 - 37 of *F. columnare*, three fragments between 1000 and 300 bp in strains 38 and 39 of *F. columnare,* three fragments between 600 and 300 bp in *A. hydrophila* and four fragments between 700 and 200 bp in *S. agalactiae.* These fragments were of similar molecular size to those observed in *F. columnare.*

Restriction with *MboI* produced four fragments between 1000 and 100 bp in the 37 new isolates of *F. columnare*, no cleavage in strains 38 and 39 of *F. columnare*, no cleavage in *A. hydrophila* and four fragments in *S. agalactiae* that were of different molecular sizes relative to those produced in *F. columnare.*

Restriction of the amplified products with *HpaII* generated four fragments between 750 and 100 bp in isolates of *F. columnare,* and the genetic profile did not differ from the fragments produced by digestion of sequences amplified from other species. Thus, there is

Table 1. Number, name and origin of the new *Flavobacterium columnare* strains isolated from the tropical fishes tilapia, matrinxã and tambaqui naturally infected with columnaris disease in Brazil.

Indication	Isolate name	Origen
1	F3	KIDNEY MATRINXÃ
2	F5	SKIN TAMBAQUI
3	F7	SKIN TAMBAQUI
4	F8	SKIN TAMBAQUI
5	F9	SKIN TAMBAQUI
6	F10	SKIN TAMBAQUI
7	F11	SKIN TAMBAQUI
8	F15	KIDNEY MATRINXÃ
9	F16	KIDNEY MATRINXÃ
10	F17	KIDNEY TAMBAQUI
11	F18	SKIN TAMBAQUI
12	F19	SKIN TAMBAQUI
13	F20	SKIN TAMBAQUI
14	F21	SKIN TAMBAQUI
15	F22	SKIN TAMBAQUI
16	F23	SKIN TILAPIA
17	F24	KIDNEY TILAPIA
18	F25	SKIN MATRINXÃ
19	F26	SKIN MATRINXÃ
20	F27	SKIN MATRINXÃ
21	F28	SKIN TAMBAQUI
22	F29	SKIN TILAPIA
23	F32	SKIN TILAPIA
24	F33	SKIN TILAPIA
25	F35	SKIN TAMBAQUI
25	F36	SKIN TILAPIA
27	F37	SKIN TAMBAQUI
28	F38	SKIN TAMBAQUI
29	F39	SKIN TAMBAQUI
30	F40	SKIN TAMBAQUI
31	F41	SKIN TAMBAQUI
32	F42	SKIN TAMBAQUI
33	F43	SKIN TILAPIA
34	F45	SKIN TAMBAQUI
35	F46	SKIN MATRINXÃ
36	F47	SKIN TAMBAQUI
37	F48	SKIN MATRINXÃ

no possibility of discrimination between the species studied by means of restriction with this enzyme. Analysis of the phylogram (Figure 2) and the similarity matrix revealed three main branches between the new isolates of *F. columnare* in addition to the control group of this species. The similarity between species in group I was greater than or equal to 81%; in group II, the similarity was greater than or equal to 93%; in group III, the isolates shared 94% similarity or greater, and in the control group of *F. columnare,* the similarity was 100%.

Aeromonas hydrophila differed from group I of *F. columnare* by 65.5% on average. This species differed from group II by 58%, from group III by 55% and from the control group of *F. columnare* by 73%. *Streptococcus*

Table 2. Biochemical characteristics of *Flavobacterium columnare* strains isolated from the tropical fishes tilapia, matrinxã and tambaqui naturally infected with columnaris disease in Brazil.

Characteristics	Results (%)
Starch hydrolysis	+19 / - 81
Arginine	+ 92 / - 8
Catalase	+ 100
Citrate	+43/ - 57
L-Tryptophane desamination	+63 / - 37
Lysine decarboxylation	+ 90/ - 10
Ornithine decarboxylation	+100
Esculin	+59 / - 41
Glucose fermentation	+100
Flexirrubin-type pigment	+100
Gas from glucose	- 100
Gelatin hydrolysis	+100
Gram staining	-100
Indole production	+100
Inositol	+89/ - 11
Mannitol	+89/ - 11
Gliding movements	+100
H_2S and gas production	+66 / - 34
Nitrate reduction	+100
Rhamnose	+10 / - 90
Sucrose	+100
Congo red test	+100

(+) positive response, (-) negative response, (+/-)part of isolates a positive response and the other part, negative.

agalactiae differed by only 27% on average from group I of *F. columnare*, by 21% compared from group II, by 23% from group III and by 56% from the control group of *F. columnare.* The genetic difference between strains of *A. hydrophila* and *S. agalactiae* was 53%.

Regarding the preference of each *F. columnare* subgroup for the studied fish species, we found that for isolates belonging to subgroup I, 54% were from tambaqui, 31% were from tilapia and 15% were from matrinxãs. In subgroup II, 83% were from tambaqui and 17% were from tilapia. Finally, 57% of the isolates in subgroup III were derived from matrinxãs, 29% were from tambaquis and 14% were from tilapia. In the control group of *F. columnare,* a strain was isolated from Nile tilapia and the other was from tambaqui.

DISCUSSION

Information on the occurrence, pathogenicity and isolation of *F. columnare* in fish cultures in Brazil is scarce. Most of the publications on isolation and characterization

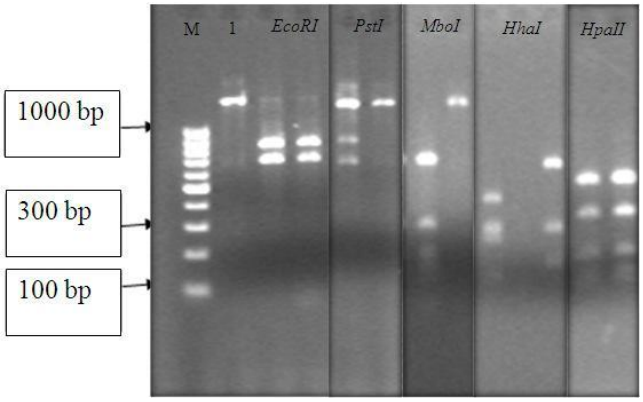

Figure 1. Gel containing the polymorphisms found among the PCR products of the 16S rDNA gene amplified from the DNA of *Flavobacterium columnare* isolates and digested with the restriction endonucleases *EcoRI, MboI, PstI, HhaI* and *HpaII*; M refers to 1 kb DNA ladder molecular marker; 1 refers to the amplified gene, without addition of restriction enzymes.

of this bacterium are carried out in fishes from temperate waters (Pilarski et al., 2008).

The lack of a specific medium for culturing *F. columnare* is another obstacle for isolation of the bacterium. Decostere et al. (1998) reported on the difficulty of obtaining pure colonies.

In this work we used the Carlson and Pacha (1968) medium modified by Pilarski et al. (2008), which enabled the development of *Flavobacterium* colonies, as well as probable strains of *Aeromonas* and *Pseudomonas,* which grew in an overlapping pattern. A large number of replications were required to purify the colonies of the desired species.

The antibiotic tobramycin was not used to eliminate contaminating species as was previously described by Decostere et al. (1998) in order not to induce the appearance of L form bacteria (Davis et al., 1973).

The pure *F. columnare* colonies were studied with respect to their morphophysiological and biochemical characteristics as described by the Decostere et al. (1998). Their molecular characteristics (PCR-RFLP of 16S rDNA) were assessed according to Arias et al. (2004) and Figueiredo et al. (2005).

In accordance with the results described by Pilarski et al. (2008), the 37 isolates found in this study showed rod-shaped, Gram-negative, long, slender and aerobic cells with optimum growth at 30°C.

In liquid medium, the isolates showed sliding motility in a hanging drop analysis. On solid medium, the isolates produced colonies of yellow-grayish color with irregular borders. The colonies had the form of roots and were not strongly adherent to the Agar. Similar results were obtained by Shamsudin and Plumb (1996), Descostere et al. (1998) and Pilarski et al. (2008).

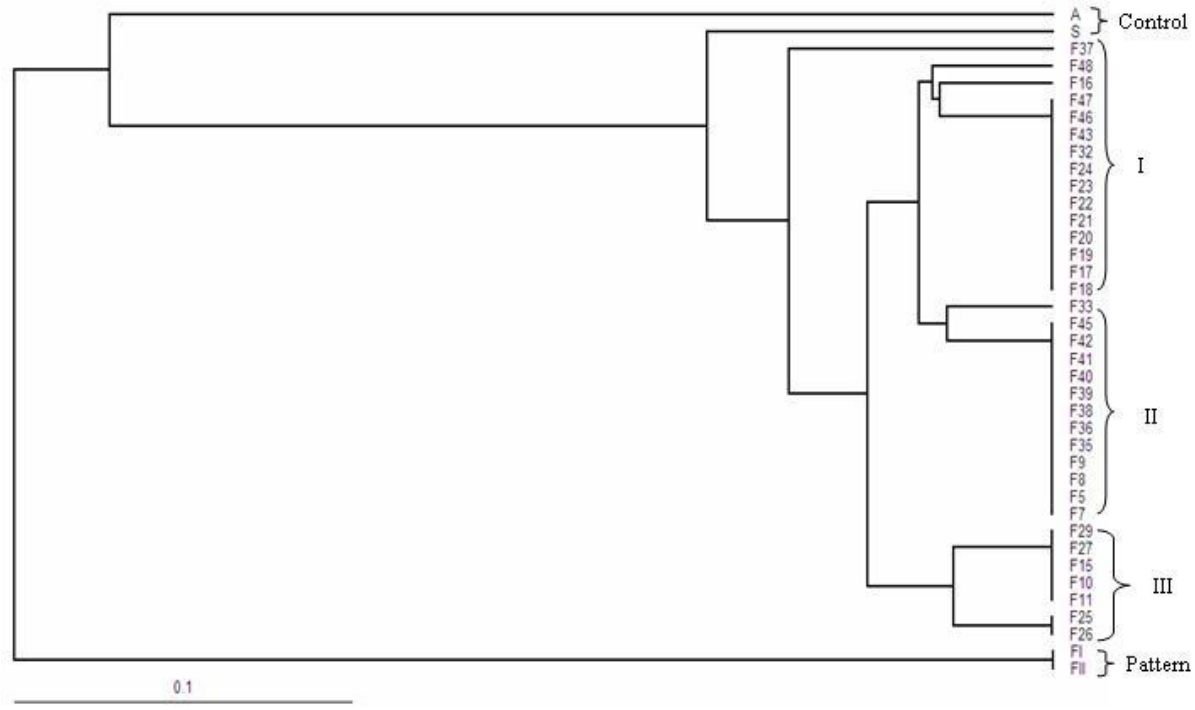

Figure 2. Phylogram obtained by analysis of PCR products amplified from DNA isolates of *Flavobacterium columnare*(F), *Aeromonas hydrophila* (A) and *Streptococcus agalactiae* (S*)* and digested with the restriction endonucleases *EcoRI, MboI, PstI, HhaI* and *HpaII*.

Bernadet and Grimont. (1989), Griffin (1992), Shamsudin and Plumb (1996), Descostere et al. (1998) and Pilarski et al. (2008) have reported that cultures grown on solid medium showed an organoleptic property similar to the smell of fruit. Among the isolates described in this work, this feature was not noticeable.

Regarding the biochemical characterization, a wide variety of behaviors could be observed among the various isolates. Among the tests for characteristics that are specific to *F. columnare* (motility, nitrate reduction, flexirrubin production, absorption of Congo Red, fermentation of glucose and sucrose, desamination of L-tryptophan, gelatin and catalase hydrolysis), all isolates tested uniformly positive regardless of the fish species from which they were collected. This result is consistent with results reported by Pilarski et al. (2008).

The absorption of Congo red, which occurred in all isolates, is indicative of galactosamine glucan production by *F. columnare*, and this fact is corroborated by Bernadet et al. (1989), Griffin (1992), Decostere et al (1999) and Pilarski et al. (2008).

All isolates in this study were positive for gelatin hydrolysis, and this is one of the main features of the bacterium *F. columnare.* Because the production of certain enzymes promotes the degradation of macromolecules in the medium such as gelatin, there is a clearing of the medium in which there is bacterial growth (Bernadet and Grimont 1989; Griffin, 1992; Pilarski et al., 2008).

Among the molecular techniques, PCR is undoubtedly the most efficient tool for the diagnosis of columnaris. Several protocols were applied, but due to the lack of data on the genome sequence of *F. columnare* from water and tropical climate hosts, the use of specific primers for conserved regions of bacterial DNA is the most accessible technique (Bader et al., 2003; Darwish et al., 2004; Figueiredo and Leal, 2008).

Thus, using PCR-RFLP of the 16S rRNA gene for identification and differentiation of *F. columnare* isolates from existing strains such as *A. hydrophila* and *S. agalactiae,* it was possible to map genetic profiles of each microorganism in relation to the applied restriction enzyme (Figure 1).

Restriction of the amplified products with *EcoRI* and *HpaII* showed only one genetic profile for the three species in question and did not allow for differentiation. While restriction with *PstI* allowed for the detection of two genetic profiles of *F. columnare* (Figure 1). However, according to a simulation performed with the software "pdraw32" on one 16S gene of *F. columnare* that has been partially sequenced (deposited in the National

Center of Genes and Genomes Information), the enzyme *PstI* has no restriction site in this gene. The fact that the enzyme recognized a site in some isolates suggests the presence of a mutation.

After restriction with *MboI*, two genetic profiles of *F. columnare* were observed and these were distinct from the profiles of *A. hydrophila* and *S. agalactiae.* Similar results were also observed after restriction of the amplified products with *HhaI,* but there was no difference in the profile of *S. agalactiae* (Figure 1).

The phylogram (Figure 2) shows three main branches between the isolates of *F. columnare* in addition to the grouping of *F. columnare* strains that was used as a control. Several authors have found intraspecific variation between the different *F. columnare* isolates that were studied. Shoemaker et al. (2007) found two subgroups among his isolates using the ISR-SSCP technique; Arias et al. (2004) and Darwish and Ismaiel (2005) described three *F. columnare* subgroups when using PCR-RFLP; and Flemming et al. (2007) identified two *Flavobacterium johnsoniae* subgroups by PCR-RFLP with the enzyme *HaeIII.*

Amplification of the 16S rDNA gene region and the respective application of RFLP with the enzymes *EcoRI, MboI, PstI, HhaI* and *HpaII* is a simple technique to be performed for the characterization of large numbers of isolates; nevertheless, the RFLP patterns produced depend on the variability of the sequences produced in determined regions of the gene.

Data from this study show the existing genetic variation within a supposedly conserved gene, 16S rDNA, and they can explain the difficulty of establishing diagnostic protocols for molecular characterization of the studied species. Analysis of the phylogram and its ramifications also suggests the existence of subspecies of *F. columnare.*

The results are in accordance with Sebastião et al., 2007 (unpublished data) who showed by means of the RAPD-PCR technique that both strains are 78% similar and sufficient for grouping.

Dependence of the molecular technique on certain regions of the gene makes comparisons between isolates from different studies a challenge unless similar methods are applied in the analysis (Darwish and Ismaiel, 2005).

Although the number of fish samples was small (18 samples), this study represents an initial attempt to correlate the genetic subgroups of *F. columnare* with their host species, that is, tambaquis are more susceptible to subgroups I and II of tropical *F. columnare* and matrinxã is more sensitive to colonization by strains belonging to the bacteria of subgroup III. No assumptions can be made regarding the control group because it only contains two strains of different origins. Similar relationships have also been observed by Olivares-Fuster et al. (2007a), but for temperate climate species.

Conclusion

Therefore, based on 37 isolates obtained from three fish species (matrinxã, tambaqui and Nile tilapia), the existence of broad genetic diversity in tropical *F. columnare* strains was verified, despite the similarity of phenotypic and biochemical characteristics. For Brazilian aquaculture, this work signifies a first step in identifying the existing *F. columnare* strains for a faster and more precise diagnosis and the correlation of three genetic subgroups with host species.

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