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New hosts and genetic diversity of *Flavobacterium columnare* isolated from Brazilian native species and Nile tilapia

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ABSTRACT: *Flavobacterium columnare* is responsible for disease outbreaks in freshwater fish farms. Several Brazilian native fish have been commercially exploited or studied for aquaculture purposes, including Amazon catfish *Leiarius marmoratus* × *Pseudoplatystoma fasciatum* and pacamã *Lophiosilurus alexandri*. This study aimed to identify the aetiology of disease outbreaks in Amazon catfish and pacamã hatcheries and to address the genetic diversity of *F. columnare* isolates obtained from diseased fish. Two outbreaks in Amazon catfish and pacamã hatcheries took place in 2010 and 2011. Four *F. columnare* strains were isolated from these fish and identified by PCR. The disease was successfully reproduced under experimental conditions for both fish species, fulfilling Koch's postulates. The genomovar of these 4 isolates and of an additional 11 isolates from Nile tilapia *Oreochromis niloticus* was determined by 16S rRNA restriction fragment length polymorphism PCR. The genetic diversity was evaluated by phylogenetic analysis of the 16S rRNA gene and repetitive extragenic palindromic PCR (REP-PCR). Most isolates (n = 13) belonged to genomovar II; the remaining 2 isolates (both from Nile tilapia) were assigned to genomovar I. Phylogenetic analysis and REP-PCR were able to demonstrate intragenomovar diversity. This is the first report of columnaris in Brazilian native Amazon catfish and pacamã. The Brazilian *F. columnare* isolates showed moderate diversity, and REP-PCR was demonstrated to be a feasible method to evaluate genetic variability in this bacterium.

KEY WORDS: Columnaris · Amazon catfish · *Leiarius marmoratus* · *Pseudoplatystoma corruscans* · Pacamã · *Lophiosilurus alexandri* · Genomovar· REP-PCR · 16S rRNA

INTRODUCTION

The fish pathogen *Flavobacterium columnare* is one of the most important aetiological agents of disease in cultured and wild freshwater fish worldwide (Declercq et al. 2013). Columnaris outbreaks in fish farms are associated with poor water conditions, high stock densities, high temperatures, and co-infection with ectoparasites (Decostere et al. 1999, Declercq et al. 2013).

Brazilian aquaculture has been growing quickly in recent years. Several native fish species have been studied and raised for commercial purposes, among them the Amazon catfish *Leiarius marmoratus* × *Pseudoplatystoma fasciatum* and pacamã *Lophiosilurus alexandri*. These species originated from the Amazon Basin and San Francisco River, respectively (Shibata 2003). Currently, the main pathogens of these fish are poorly characterized. Although columnaris is a common disease and can infect almost all

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freshwater fish, the disease is frequently misdiagnosed. The lack of growth on common agar (i.e. tryptic soy agar, sheep blood agar, and nutrient agar) used in the diagnosis of fish diseases, the necessity of a nutrient-poor medium for proper isolation (i.e. Cytophaga, Tyes, Shieh, Hsu-Shotts, or other recommended agar) (Declercq et al. 2013), and the rapid evolution of illness during outbreaks can make the diagnosis difficult (Leal et al. 2010). Therefore, a larger number of hosts may be susceptible than that currently described.

The bacterial diversity, host-associated strains, and virulence related to certain genotypes have been previously described in *F. columnare* (Olivares-Fuster et al. 2007, Shoemaker et al. 2008, LaFrentz et al. 2012, 2014). Triyanto & Wakabayashi (1999) first verified the genetic variability of the 16S rRNA of *F. columnare* strains and developed a restriction fragment length polymorphism (RFLP) method to classify the isolates in genomovars I, II, and III. Recently, LaFrentz et al. (2014) described 2 new subgroups on the basis of intragenomic sequence diversity and proposed 2 new genotypes, II-B and I/II. RFLP has been widely applied to characterize *F. columnare* strains (Michel et al. 2002, Arias et al. 2004, Darwish & Ismaiel 2005, Suomalainen et al. 2006, Avendano-Herrera et al. 2011, LaFrentz et al. 2012). In Brazil, *F. columnare* has been previously associated with disease outbreaks in farmed Nile tilapia *Oreochromis niloticus* (Figueiredo et al. 2005), the most commonly aquacultured species in the country, as well as in other native fish (Sebastião et al. 2010). However, there are no data on *F. columnare* genetic diversity and host-associated strains in Brazilian Nile tilapia and in native fish species.

We aimed with this study to characterize the aetiological agent of outbreaks of disease in Amazon catfish and pacamã hatcheries and, due to the lack of information on and the economic importance of columnaris disease for the Brazilian aquaculture, to assess the genetic diversity of *F. columnare* strains isolated from diseased cultivated fish by different typing methods.

MATERIALS AND METHODS

Outbreaks and bacteriology

In 2010 and 2011, 2 outbreaks of columnaris were investigated in a hatchery of Amazon catfish and pacamã in Brazil. The different fish species were raised in distinct indoor recirculating aquaculture systems at the same site, with aeration supplied to keep the dissolved oxygen higher than 5 mg l^{-1}. According to the farmer, the water temperature ranged from 20 to 22°C during the outbreaks. Both outbreaks occurred in the winter season. The Amazon catfish and pacamã were raised at densities of 100 and 1000 fish m^{-3}, respectively. There are no other aquaculture farms near the hatchery investigated in this study. Five diseased fish from each species were sampled and transported to the laboratory. Parasitological and bacteriological analyses were performed immediately upon arrival.

For bacteriology, swabs from the brain, kidney, liver, and subcutaneous tissues near areas of skin necrosis of diseased fish were collected aseptically and streaked onto Hsu-Shotts (MHS) agar (Bullock et al. 1986) for *Flavobacterium columnare* isolation and on 5% sheep blood agar for diagnostic evaluation of other bacterial pathogens. The plates were incubated at 25°C for 48 h. The colonies with suggestive phenotypic characteristics of *F. columnare* (yellow rhizoid colonies in Hsu-Shotts with negative growth in 5% sheep blood agar) were submitted to Gram staining and catalase and oxidase tests. Afterwards, isolates with suggestive phenotypical patterns of *F. columnare* were submitted to PCR identification following Darwish et al. (2004). After identification, the strains were maintained at –80°C in MHS broth with 15% glycerol until use.

In addition, 11 *F. columnare* isolates from Nile tilapia were included for genetic characterization (Table 1). The isolates were previously isolated from diseased fingerlings (live weight varying from 3 to 20 g) during outbreaks in 4 different tilapia farms that occurred between 2007 and 2012. The isolates that presented yellow rhizoid colonies in Hsu-Shotts were maintained at –80°C in MHS broth with 15% glycerol until use.

DNA extraction and PCR identification

The 15 isolates obtained (Table 1) were streaked onto MHS agar supplemented with tryptone 2 g l^{-1} (Leal et al. 2010) and incubated at 25°C for 48 h. Total bacterial DNA from the isolates was extracted using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. The extracted DNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific).

The identity of all of the isolates was confirmed by *F. columnare* species-specific PCR targeting the 16S rRNA gene following Darwish et al. (2004). The

Table 1. Isolate codes, host, origin, year of isolation, season of outbreaks, mortality rates, and organ of isolation of the 15 *Flavobacterium columnare* strains evaluated. Strain refers to culture collection codes from AQUAVET; all cities are in Minas Gerais State, Brazil; subcutaneous tissue was taken near areas of skin necrosis. RAS: recirculating aquaculture system; NI: not informed

Strain	Genomovar	Host	Origin				Year	Season	Mortality rate (%)	Organ of isolation
			Farm	City	Geographic coordinates	Culture system				
FC01	II	Nile tilapia	A	Esmeraldas	19°48′22″S, 44°10′38″W	RAS	2004	Winter	NI	Kidney
FC02	II	Nile tilapia	B	Lavras	21°14′40″S, 45°00′06″W	Pond	2007	Winter	12	Subcutaneous tissue
FC03	II	Amazon catfish	C	Belo Horizonte	19°52′22″S, 43°58′31″W	RAS	2010	Winter	10	Liver
FC04	II	Amazon catfish	C				2010			Subcutaneous tissue
FC05	II	Pacamã	C				2011		30	Liver
FC06	II	Pacamã	C				2011			Kidney
FC07	II	Nile tilapia	D	Morada Nova de Minas	18°35′18″S, 45°21′22″W	Cage	2012	Winter	NI	Liver
FC08	II	Nile tilapia	D							Liver
FC09	II	Nile tilapia	D							Liver
FC10	II	Nile tilapia	D							Subcutaneous tissue
FC11	II	Nile tilapia	D							Kidney
FC12	II	Nile tilapia	E	Alfenas	21°25′28″S, 45°57′21″W	Cage	2012	Winter	5	Liver
FC13	II	Nile tilapia	E							Kidney
FC14	I	Nile tilapia	E							Subcutaneous tissue
FC15	I	Nile tilapia	E							Subcutaneous tissue

primers were purchased from Integrated DNA Technologies. The PCR assays were performed in a Veriti 96 well thermal cycler (Life Technologies) using a HotStartTaq DNA polymerase kit (Qiagen) and 50 ng bacterial DNA per reaction. The PCR products were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide (0.5 µg ml^{-1} for 30 min), and visualized by UV transillumination, and images were captured using an L-Pix EX (Loccus Biotecnologia).

RFLP analysis

The *F. columnare* isolates were subjected to RFLP analysis of 16S rRNA with the *Hae*III restriction enzyme (New England Biolabs). The 16S rRNA PCR and restriction analysis were performed according to the standard protocol described by LaFrentz et al. (2014). Digested fragments were visualized by capillary electrophoresis using the QIAxcel Advanced System with QIAxcel DNA screening kit (both from Qiagen). The genomovars were determined according to the patterns described by LaFrentz et al. (2014)

Additionally, to determine the occurrence of genomovars II-B and I/II, the 16S rRNA gene of the Brazilian isolates was sequenced. The gene was amplified using primers 20F and 1500R (LaFrentz et al. 2014), and PCR products were purified with a Wizard PCR preps kit (Promega). Sequencing reactions were performed using a BigDye™ terminator cycle sequencing kit and run on an ABI 3500 genetic analyzer (both from Applied Biosystems). The contigs were assembled with the software BioEdit and submitted to *in silico* RFLP with *Hae*III using NEBcutter v2.0 (Vincze et al. 2003).

Phylogenetic analysis of the 16S rRNA gene

The 16S rRNA gene was amplified by PCR with the universal primers C70 (5′-AGA GTT TGA TYM TGG C-3′) and B37 (5′-TAC GGY TAC CTT GTT ACG A-3′) according to the method described by Fox et

al. (1995). The PCR products were purified and sequenced as previously described herein. The sequences of the Brazilian isolates were aligned in BioEdit using CLUSTALW (Thompson et al. 1994), with sequences of the following bacterial strains and species: *F. columnare* ATCC 49512 (GenBank accession number AY635167), *F. columnare* ATCC 49513 (AB023660), *F. columnare* ATCC 23463 (KC912651), *F. columnare* strain FK 401 (AB010952), *F. columnare* strain F10-HK-A (KC912679), *F. columnare* strain ALG-00-530 (KC912656), *F. columnare* strain EK-28 (AB016515), *F. columnare* LP8 (AB015480), *F. columnare* strain LV339-01 (AY842900), *F. columnare* strain PT-14-00-151 (KC912659), *F. columnare* strain GA-02-14 (KC912664), *F. columnare* strain ARS-1 (KC-912668), *F. columnare* strain PH-97028 (AB015481), *F. columnare* strain AU-98-24 (AY842899), *F. johnsoniae* ATCC 23107 (NR044738), *F. psychrophilum* ATCC 49418 (AY662493), and *F. branchiophilum* NBRC 15030 (AB680752). To eliminate poorly aligned sequences, conserved blocks were selected using the software Gblocks (Castresana 2000) and used for phylogenetic analysis. The genetic distance matrix was obtained using Kimura's 2-parameter model (Kimura 1980), and an evolutionary tree was created using the neighbour-joining method (Saitou & Nei 1987) with Mega6 (Tamura et al. 2013). Bootstrap values from 1000 replicates were displayed as percentages. In addition, the evolutionary model of all isolates of *F. columnare*, Brazilian strains, and genomovar II strains described in this work were addressed by Tajima's neutrality test using the software DnaSP v5 (Librado & Rozas 2009).

Repetitive extragenic palindromic PCR

Repetitive extragenic palindromic PCR (REP-PCR) was performed as described previously by Costa et al. (2014). The reactions were performed with a HotStartTaq polymerase kit; the mix was composed of 1× PCR buffer, 0.5 µmol l^{-1} $(GTG)_5$ primer (5'-GTG GTG GTG GTG GTG-3'), 0.2 µmol l^{-1} dNTPs, 1.5 mmol l^{-1} $MgCl_2$, 2 U of *Taq* DNA polymerase, and 35 ng of template DNA. The PCR conditions consisted of an initial step of 95°C for 15 min, followed by 30 cycles of 95°C for 30 s, 45°C for 1 min, and 72°C for 4 min; with a final elongation step of 72°C for 16 min. The PCR was performed in a Veriti 96 well thermal cycler. The PCR products were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide (0.5 µg ml^{-1} for 30 min), and visualized by UV transillumination, and images were captured using an L-Pix EX. Ladder 1 kb (Promega) was used as the molecular size standard. Each isolate was evaluated in triplicate, where each isolate was amplified and analysed on 3 independent occasions. To ensure repeatability and reproducibility as well as the accuracy of results obtained, we repeated the REP-PCR with a new DNA extraction after the first round of analysis.

The REP-PCR gels were analysed using BioNumerics v6.6 (Applied Maths). The Dice coefficient was used to analyse the similarities of the banding patterns (Dice 1945). Dendrograms were created using the unweighted pair group method with average approach. Isolates that showed similarity ≥90% were considered clonally related (Singh et al. 2006).

Fish

Two fish species were used in the *in vivo* challenge assay: Amazon catfish and pacamã. They were provided by the Aquaculture Laboratory of the Veterinary School of the Federal University of Minas Gerais (LAQUA, Brazil). A total of 27 Amazon catfish (mean ± SD weight = 5.4 ± 0.69 g) and 27 pacamãs (mean ± SD weight = 7.8 ± 1.51 g) were used in the trials. Each experimental group, comprising 6 fish, was kept in an aquarium with 57 l of water supplied with flow-through dechlorinated tap water (0.5 l h^{-1}), maintained on a 12 h light:12 h dark period at a water temperature of 25°C, and fed to apparent satiation with Nutripeixe TC40 (Purina) 2 times a day. The fish were acclimated for 21 d.

Prior to the challenge, 3 fish of each species were randomly collected and subjected to bacteriological examination. Samples of brain and kidney were aseptically collected and streaked onto 5% sheep blood agar, and the plates were incubated at 28°C for 72 h. Samples of kidney and liver were aseptically collected and streaked onto MHS, and the plates were incubated at 25°C for 72 h. In addition, samples of kidney and liver were taken and the DNA extracted as previously described, and they were tested by *F. columnare*-specific PCR (Darwish et al. 2004) to demonstrate freedom from columnaris.

Experimental infection

To fulfil Koch's postulates, Amazon catfish and pacamã fingerlings were experimentally infected with an *F. columnare* strain isolated from diseased fish of each species. The strain FC03 was selected for

the challenge assay in Amazon catfish and FC05 for the challenge assay in pacamã.

The isolates were thawed, streaked onto MHS agar supplemented with tryptone 2 g l^{-1} (Leal et al. 2010), and incubated at 25°C for 48 h. One colony of each strain was picked up, inoculated in MHS broth, and incubated at 25°C for 24 h under low agitation (140 rpm) to reach the exponential phase of the growth curve. The bacterial suspension was adjusted to an optical density of 0.240, corresponding to 10^7 CFU ml^{-1}.

Three infection routes were tested for both fish species: intraperitoneal (i.p.), intramuscular (i.m.), and immersion. Two replicate fish groups (n = 6 per group) were challenged by each infection route. The feedings were stopped 12 h previous to the challenge. Before the challenge, fish were anesthetized by immersion in a bath containing 10 mg l^{-1} benzocaine. For the i.p. and i.m. routes, fish were inoculated with 0.1 ml of a bacterial inoculum corresponding to 10^6 CFU $fish^{-1}$. For the immersion route, 100 ml of bacterial inoculum was diluted in a 10 l bucket containing 9.9 l of sterile water to reach a final concentration of 10^5 CFU ml^{-1}. The fish were maintained in the bucket for 15 min and then returned to their respective tanks and holding conditions as described previously. Fish from control groups for the i.p. and i.m. infection routes were inoculated with 0.1 ml of sterile MHS broth. The control fish for infection via immersion were immersed in a 10 l bucket containing 100 ml of sterile MHS broth and 9.9 l of sterile water; the fish were maintained in the bucket for 15 min and then returned to their tank.

The fish were monitored 4 times a day for 21 d to evaluate clinical signs of disease and mortality. All dead fish were subjected to bacteriological analysis. At the end of the experiment, all surviving fish were euthanized by benzocaine overdose and submitted to bacteriological analysis. Samples of kidney, liver, spleen, and skin lesions were collected from all dead and surviving fish for reisolation of bacteria. The isolates that presented yellow rhizoid/yellow colonies in Hsu-Shotts agar, negative growth in 5% sheep blood agar, Gram-negative long rods, and catalase- and oxidase-positive results were identified by species-specific PCR as described by Darwish et al. (2004).

Statistical analysis

The discriminatory power of each typing method (RFLP analysis, phylogenetic analysis of the 16S rRNA gene, and REP-PCR) was calculated using Simpson's index of diversity (Hunter & Gaston 1988). The adjusted Rand index was calculated to quantify the congruence among the different typing methods used (Kidd et al. 2011). These analyses were performed using the statistical software R (Chang et al. 2010).

RESULTS

Outbreak characterization and bacteriology

According to the hatchery records, there was no recent history of animal acquisition or animal transport to or from other farms. The field presentation and pathogenesis of the disease were similar in both fish species. The outbreaks were preceded by a period of anorexia followed by the observation of lethargic and sick fish. The major clinical signs observed were skin necrosis, fin erosion, gill pallor, and saddleback lesions. At necropsy, the main pathological finding in both fish species was hepatomegaly. Only fingerlings with body weight ranging from 2.5 to 15 g were affected. Mortality rates of approximately 10 and 30% were verified in the outbreaks in Amazon catfish and pacamã, respectively.

Of the 5 diseased fish tested from each species, 3 gave negative results in bacteriological testing. Four bacterial isolates were obtained from the remaining fish: 2 from Amazon catfish (1 isolate from each fish; isolated from liver and subcutaneous tissues near to areas of skin necrosis), and 2 from pacamã (1 isolate from each fish; isolated from liver and kidney). All strains were found to be Gram-negative, rod-shaped bacteria forming yellow rhizoid colonies, and were catalase positive and oxidase negative. The identity of all *Flavobacterium columnare* isolates was confirmed by species-specific PCR.

Genomovar profiles

The genomovar of the 15 Brazilian isolates of *F. columnare* was determined by RFLP with *Hae*III digestion and revealed 2 distinct restriction patterns (Fig. 1). According to the RFLP types described by LaFrentz et al. (2014), isolates FC01 to FC13 belonged to genomovar II (86.6%), and isolates FC14 and FC15 belonged to genomovar I (13.3%). Nile tilapia isolates showed 2 genomovar types, whereas isolates from native fish belonged to a single type. On

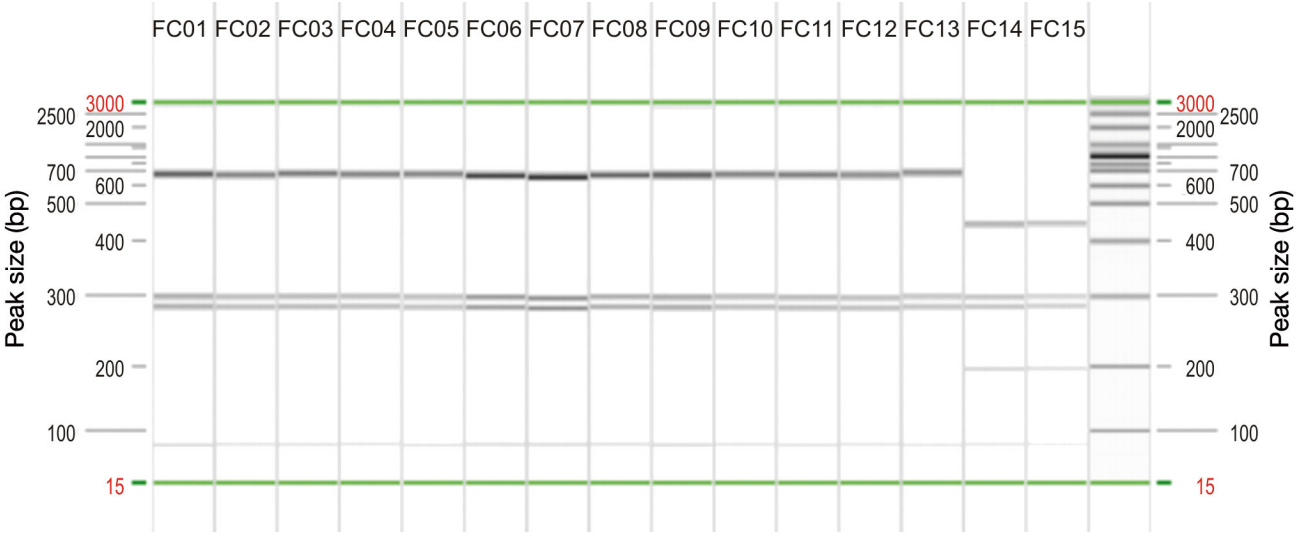

Fig. 1. Restriction fragment length polymorphism profiles after *Hae*III digestion of PCR products from 15 isolates of *Flavobacterium columnare*, visualized using a QIAxcel DNA screening kit

farm E (see Table 1), 2 different genomovar types were recovered from the same disease outbreak. RFLP presented a discriminatory power of 0.247. Conventional RFLP and its *in silico* version using 16 rRNA sequences of 15 isolates showed exactly the same results.

Phylogenetic analysis of the 16S rRNA gene

Amplification of the 16S rRNA fragments yielded a product of approximately 1500 bp. After block selection, conserved regions were concatenated, giving a total length of 779 bp, and used in the analysis. Phylogenetic analysis of the 16S rRNA gene sequences of the *F. columnare* strains resulted in the neighbour-joining tree shown in Fig. 2. The 15 Brazilian strains of *F. columnare* occurred in 2 major branches, one composed of genomovar II strains (clusters 1, 2, and 3) and the other composed of genomovar I isolates. Despite belonging to genomovar I based on RFLP analysis, isolates FC14 and FC15 clustered with strains of genomovar III.

The Brazilian strains were distributed in 4 distinct clusters. Cluster 3 contained isolates from the 3 fish species (Amazon catfish, pacamã, and Nile tilapia), whereas clusters 1, 2, and 4 were composed exclusively of Nile tilapia isolates. The strains from genomovar I occurred in a unique cluster. There was no clear relationship between genotype and host fish species. Multiple isolates from the same outbreak in tilapia on farm D grouped in a specific cluster with a strain from farm B (Table 1). In contrast, isolates from farm E presented different genomovars and belonged in different clusters. Phylogenetic analysis of the 16S rRNA gene showed a discriminatory power of 0.609.

Tajima's neutrality test showed values of –1.07844, –0.52063, and –0.002402 for all isolates of *F. columnare*, Brazilian strains, and genomovar II strains, respectively. These results were not significant and suggest a purifying/neutral model of 16S rRNA evolution in *F. columnare*.

REP-PCR profile

The REP-PCR of the 15 *F. columnare* isolates resulted in the amplification of 15 bands, ranging in size from 400 to 4500 bp. Four different REP patterns were detected, and based on the similarity threshold of ≤90%, they belonged to REP types I, II, III, and IV (Fig. 3). Repeating the REP-PCR analysis with a second DNA extraction from the same colony preparation resulted in the same allocation of isolates to REP types. REP types I and III contained the majority of isolates (6 strains in each type). The earliest isolated Brazilian strain, FC01, was the only isolate of REP type IV. The isolates from genomovar I (FC14 and FC15) presented a REP type different from the other strains. As with the phylogenetic analysis, there was no clear relationship between REP type and host origin. The REP-PCR had a discriminatory power of 0.609.

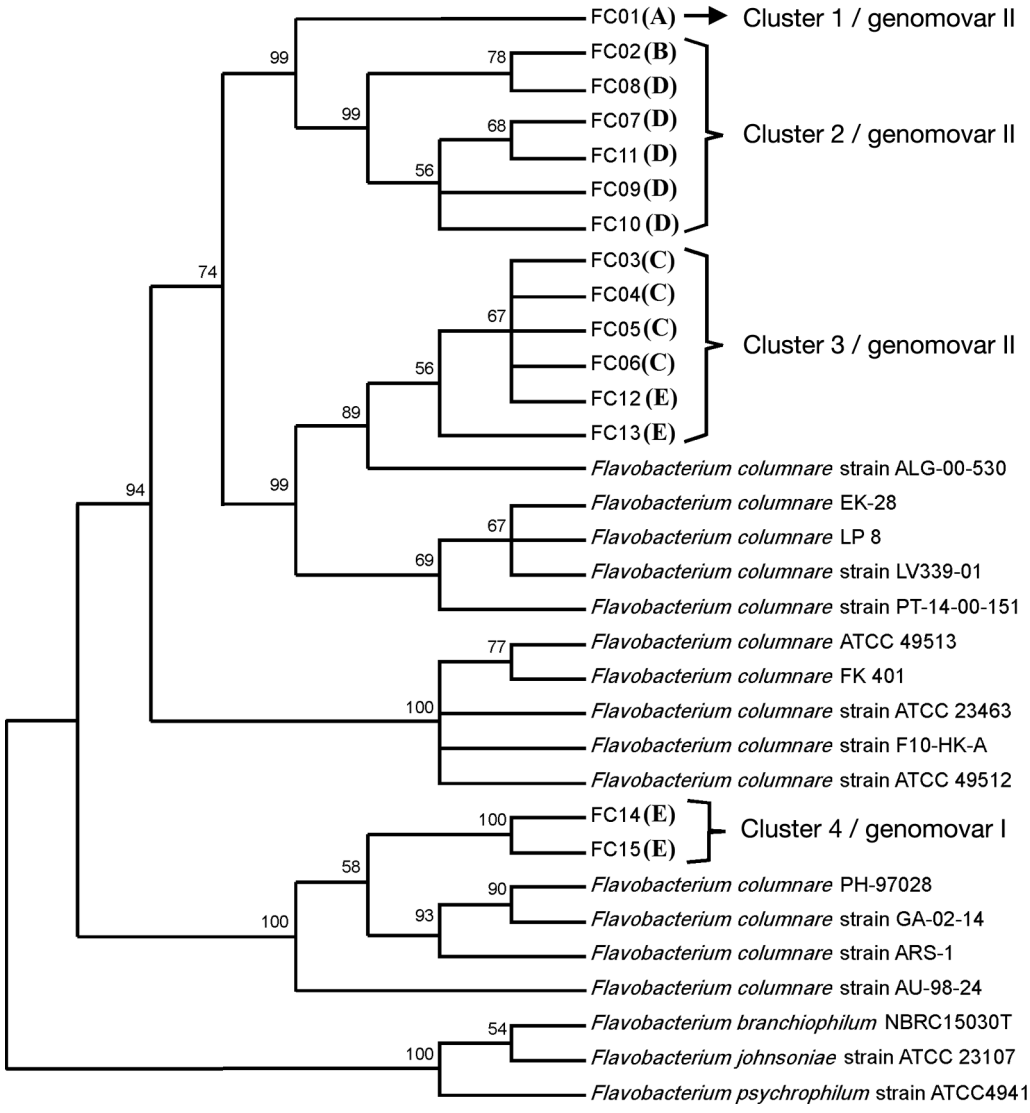

Fig. 2. Phylogenetic neighbour-joining tree based on the 16S rRNA gene sequences of 15 *Flavobacterium columnare* isolates from diseased fish from Brazilian farms (FC01 to FC15) and reference samples of *F. columnare*, *F. branchiophilum*, and *F. johnsoniae*. A bootstrap value of 1000 was used. The letters in parentheses after isolate codes represent the farms of origin

Comparison of genotyping methods

Three typing methods were evaluated in this study. All methods were able to type the strains but with varying discriminatory power. Based on Simpson's index of diversity, higher discriminatory power was obtained using REP-PCR and phylogenetic analysis of the 16S rRNA gene. The congruence between the results of the typing methods was evaluated using the adjusted Rand index (ARI). Complete concordance (ARI = 1.0) was shown between phylogenetic analysis and REP-PCR, with a direct correspondence between the isolates belonging to clusters 1, 2, 3, and 4 and REP types IV, III, I, and II, respectively. Similar results were observed between conventional RFLP and *in silico* RFLP. However, the RFLP showed a low discriminatory power and concordance with phylogenetic analysis and REP-PCR, with an ARI value of 0.242. Brazilian *F. columnare* isolates showed a moderate genetic diversity, as defined by the Simpson's index of diversity (Hunter & Gaston 1988), on the basis of the phylogenetic and REP-PCR analysis, with clear intragenomovar variability not traceable by RFLP.

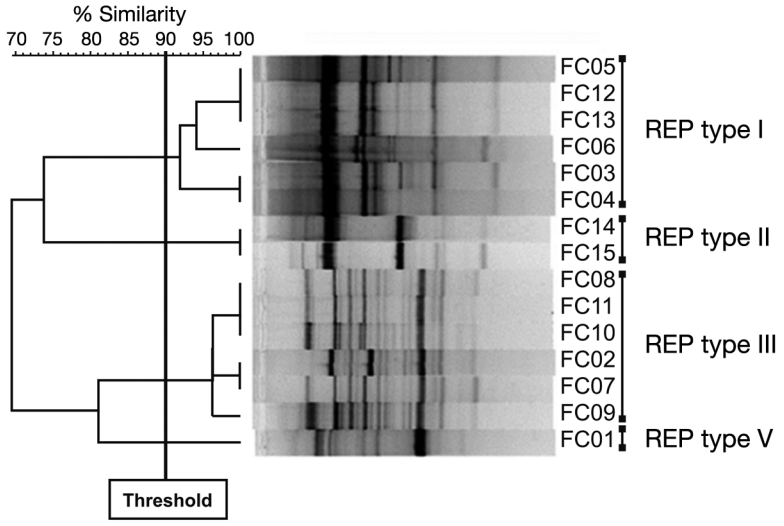

Fig. 3. Repetitive extragenic palindromic (REP) PCR results for 15 *Flavobacterium columnare* samples isolated from different fish species

Challenge assay

The disease was successfully reproduced under all experimental conditions in both Brazilian native fish species. No clinical signs or mortality were observed in the control groups. The main clinical signs initially observed in Amazon catfish were anorexia and lethargy, and these evolved to skin necrosis (saddleback lesions) (Fig. 4), fin erosion (Fig. 4), ascites, and death of the fish. At necropsy, haemorrhage in the liver and hepatomegaly were observed. A mortality rate of 100% was observed in all groups challenged by different infection routes after 112 h post infection (hpi). For the i.p., i.m., and immersion challenges, the first mortalities were recorded at 24, 44, and 48 hpi, respectively. Similar clinical signs were verified in fish challenged by different infection routes.

The main clinical signs initially observed in pacamã were anorexia and lethargy, and these evolved to skin depigmentation, skin necrosis (saddleback lesions), fin erosion, and death of the fish. At necropsy, visceral bleeding, hepatomegaly, pale liver, and splenomegaly were observed. The first mortality was recorded at 72 hpi for the i.p. and i.m. challenges. A mortality rate of 100% was observed with the i.p. challenge; the last dead fish was observed at 99 hpi. The i.m. and immersion infection routes produced 66.7% mortality, beginning at 48 hpi. *F. columnare* was reisolated from the kidney, liver, spleen, and skin lesions from all dead fish. All isolates presented positive results in *F. columnare*-specific PCR. The fish challenged by the i.m. and immersion routes that remained alive until the end of the experimental period (21 d post infection) presented negative bacteriology results.

DISCUSSION

Columnaris disease affects almost all freshwater fish species in culture conditions around the world and is responsible for high economic losses to fish farmers (Olivares-Fuster et al. 2007, Declercq et al. 2013). In Brazil, the disease has already been reported in Nile tilapia, piracanjuba *Brycon orbignyanus*, pacu *Piaractus mesopotamicus*, tambaqui *Colossoma macro-*

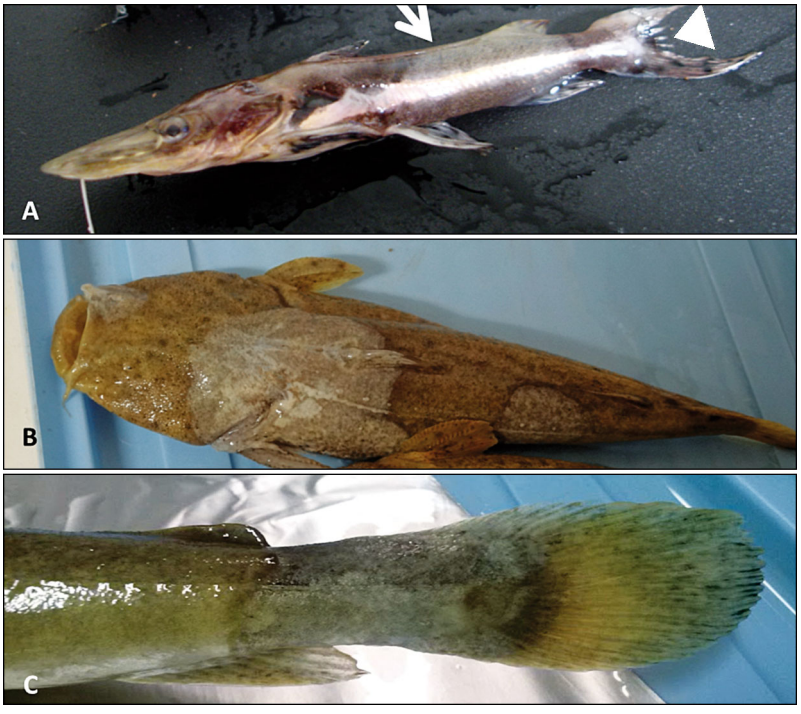

Fig. 4. Diseased fish experimentally challenged with *Flavobacterium columnare* isolates FC03 and FC05. (A) Amazon catfish with skin necrosis (arrow) and fin erosion (arrowhead); (B) pacamã with skin necrosis in dorsal region and head; (C) pacamã with skin necrosis in caudal region

pomum, cascudo *Hypostomus plecostomus*, and matrinxã *B. amazonicus* farms (Figueiredo et al. 2005, Sebastião et al. 2010). In 2010 and 2011, 2 outbreaks associated with high mortality were observed in a hatchery of Amazon catfish and pacamã. From these cases, we have described for the first time *Flavobacterium columnare* as the aetiological agent of disease in these native Brazilian fish species from the Amazon and San Francisco River basins.

The main clinical signs noted in diseased Amazon catfish and pacamã during the outbreaks were skin necrosis, fin erosion, gill pallor, and saddleback lesions. These classic signs of columnaris are similar to those previously reported in other cases of the disease in Brazilian native fish (Pilarski et al. 2008), Nile tilapia (Figueiredo et al. 2005), salmonids (Suomalainen et al. 2006, Avendano-Herrera et al. 2011), and channel catfish *Ictalurus punctatus* (Bader et al. 2006). The disease was successfully reproduced under laboratory conditions, thus fulfilling Koch's postulates. Amazon catfish and pacamã fingerlings showed the same lesions when challenged by i.p. injection, i.m. injection, and immersion with 2 Brazilian *F. columnare* isolates (FC03 and FC05) isolated from naturally infected individuals of the same fish species. Those strains seem to be highly virulent to their hosts of origin, regardless of the route of infection. We used median lethal dose (LD_{50}) data of *F. columnare* for zebrafish as a reference dosage to perform the challenge assays. Despite being the same bacterial species, differences in host susceptibility and pathogenicity of the isolates could affect virulence in experimental infection trials. Therefore, to determine the real virulence of FC03 and FC05 to Amazon catfish and pacamã, respectively, LD_{50} studies should be carried out.

In spite of their common occurrence in Brazil, there are no data on the genetic diversity of *F. columnare* strains isolated from diseased fish of different species and geographical origins. Based on RFLP analysis, the majority of Brazilian isolates belonged to genomovar II, and just 2 strains belonged to genomovar I. Genomovar II was associated with infections in the 3 fish species evaluated in the present work. This is the principal genomovar that has been associated with outbreaks in warm-water fish farms (Arias et al. 2004, Olivares-Fuster et al. 2007, LaFrentz et al. 2012). Similar to our results, Dong et al. (2015) found genomovar II to be mainly responsible for cases of infection in red tilapia *Oreochromis* sp., koi carp *Cyprinus carpio*, striped catfish *Pangasianodon hypophthalmus*, and Nile tilapia in Thailand and Vietnam. Currently, the genetic and phenotypic determinants that confer the ability of strains of that genomovar to cause disease preferentially in warm-water fish are unclear. This should be addressed in future studies.

Genomovar I has a large geographical distribution, and it has been previously described in Europe, America, and Asia (LaFrentz et al. 2012). Here, we present the first description of *F. columnare* genomovar I isolates in South America in addition to those associated with outbreaks in the tropical fish Nile tilapia. Dong et al. (2015) also recently described the occurrence of genomovar I infection in red tilapia in Thailand. This genomovar has been widely isolated from cold-water fish such as Coho salmon *Oncorhynchus kisutch*, Chinook salmon *O. tshawytscha*, sockeye *O. nerka*, Atlantic salmon *Salmo salar*, brown trout *S. trutta*, brook trout *S. fontinalis*, and Arctic charr *Salvelinus alpinus* (LaFrentz et al. 2012). In addition, the genomovar was previously isolated from the tropical species common carp and channel catfish but with significantly lower frequency and lower virulence. Triyanto & Wakabayashi (1999) reported that genomovar I is able to grow in temperature ranges from 15 to 37°C; in contrast, genomovar II is unable to grow at 15°C. The absence of outbreaks caused by genomovar II on cold-water fish farms can be explained by its inability to grow at low temperatures. However, the lower virulence of genomovar I strains in tropical fish is poorly understood.

Olivares-Fuster et al. (2007) described the occurrence of genomovars I and II in the same geographical region. However, the occurrence of 2 genomovars in the same clinical case was not reported before 2014. Here, different genomovars were involved simultaneously in an outbreak affecting a Nile tilapia farm (farm E) in Brazil. Similarly, Dong et al. (2015) described the co-occurrence of genomovars I and II on a red tilapia farm. Both studies contemporaneously characterized this trait. The main factors predisposing to or affecting the occurrence of multiple genomovars on tilapia farms around the world are unclear.

The initially proposed hypothesis of host or geographically associated genomovars has been refuted by many authors. Arias et al. (2004) isolated different genomovars from channel catfish and concluded that they are widely distributed in fish farming regions of the USA. Darwish & Ismaiel (2005) isolated 3 genomovars from wild fish and reported 2 genomovars (II and III) at the same point of the Mississippi River; however, those authors were unable to correlate the host species or geographical origin with the genomovars found. Olivares-Fuster et al. (2007) found no

correlation between 90 *F. columnare* isolates of genomovars I and II with 4 hosts: channel catfish, threadfin shad *Dorosoma petenense*, blue catfish *I. furcatus*, and freshwater drum *Aplodinotus grunniens*. Similarly, there was no clear relationship between genomovar, geographical origin, and fish species in Brazilian cases of columnaris studied herein.

Sequencing of the 16S rRNA gene is one of the most feasible and extensively used molecular tools in bacterial phylogeny (Almeida & Araujo 2013). Here, this methodology was applied to address the genetic relationships and diversity of reference isolates (from different regions, species, and genomovars) and Brazilian *F. columnare* strains. Our isolates belonged to 4 cluster lineages, with genomovar II strains grouping together with reference strains of the same genomovar. Clear variability was observed within genomovar II for Brazilian isolates of *F. columnare*. These data suggest that these bacterial populations are facing evolutionary and genetic events in the 16S rRNA gene that are not reflected in their RFLP patterns.

Surprisingly, the Brazilian genomovar I isolates clustered with genomovar III isolates based on rRNA sequence data. Similar results were obtained by Dong et al. (2015), who evaluated 16S to 23S rRNA interspacer region variability in *F. columnare* isolates from different species. These authors argue that in spite of differences in restriction sites of *Hae*III, genomovars I and III may present high similarity in their RNA operons, which promotes phylogenetic similarity. Another explanation for this phenomenon is the intragenomic heterogeneity described in *F. columnare* strains (LaFrentz et al. 2014), which may have caused some potential bias in this analysis. This could be especially interesting for strain ARS-1, evaluated herein and previously. It was first classified as genomovar I (Arias et al. 2004) and afterwards reclassified as genomovar III (LaFrentz et al. 2014). LaFrentz et al. (2014) showed that for this strain, different cloned plasmids generated for 16S rRNA sequencing can harbour sequences from distinct 16S rRNA alleles presenting RFLP patterns I, III, and IV. Thus, according to the sequence used, different phylogenetic results could be obtained for the same strain. This could be a common behaviour for genomovar III sequences available in GenBank, evaluated here and in other work (Dong et al. 2015).

The REP-PCR method has been used successfully to determine genetic variability in different fish pathogens such as *Streptococcus dysgalactiae* (Costa et al. 2014) and *Francisella noatunensis* subsp. *orientalis* (Leal et al. 2014). This genotyping method has the merit of being less labour intensive and faster to complete (Wong & Lin 2001). REP-PCR was used in the present study to genotype *F. columnare* strains isolated from diseased fish in Brazil. Four different genetic patterns were obtained. This technique presented moderate discriminatory power, but it was able to show variability within genomovar II. Its results presented 100% congruence with phylogenetic analysis of the 16S rRNA gene, a more accurate method. Thus, REP-PCR may be a feasible alternative to track and evaluate the genetic diversity of *F. columnare* strains. It could be combined with RFLP typing to determine genetic variability, and the main clones responsible for outbreaks within or between farms, and could provide essential information for monitoring and control programmes for this pathogen at the farm level.

In conclusion, this is the first report of outbreaks caused by *F. columnare* in Amazon catfish and pacamã in Brazil, as well as the occurrence of infection caused by genomovar I in Nile tilapia and concomitant genomovars in the same columnaris case. In addition, REP-PCR was shown to be a feasible method to evaluate intragenomovar variability in *F. columnare* from warm-water fish.

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