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Simultaneous detection of *Flavobacterium columnare* and *Saprolegnia ferax* during disease outbreak in cultured pacu, *Piaractus brachypomus* (Cuvier, 1818) from India

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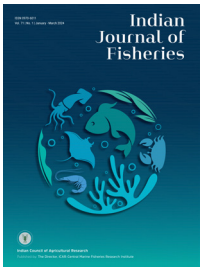

Abstract

The present study reports the simultaneous detection of *Flavobacterium columnare* and *Saprolegnia ferax* in cultured pacu (*Piaractus brachypomus*), during winter. The gross lesions in moribund pacu showed erosion of fins, cotton-wool like growth on the body surface and prominent discolouration on skin and gills. Wet mount from the gill tissue showed haystack-like appearance, presumptive of columnaris disease. *F. columnare* was detected in gill tissues of diseased pacu by PCR using specific primers. Phylogenetic analysis of 16s rDNA amplified from DNA of diseased pacu gills revealed that the sequence belonged to genetic group 2 of *F. columnare*. Histopathological findings of the diseased fish showed extensive damage in gills. In addition, wet mount of surface lesions revealed long thin aseptate hyphae presumptive of oomycete infection. Skin and fin tissue from diseased pacu inoculated on GPY agar plates showed cotton wool like growth. The isolated oomycete was purified and identified as *S. ferax* through amplification and sequencing of ITS region. These results attribute the mortality of pacu to infection with *F. columnare* and *S. ferax*. Considering the increase of pacu culture in many countries, and specially its polyculture with major carps in India, the findings of the present study necessitate early detection of these pathogens for health management of pacu.

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Introduction

Piaractus brachypomus, commonly known as pacu, is a native fish of South America. In many countries like Bolivia, Brazil, Colombia, Ecuador, Peru, and Venezuela, pacu is cultured as high-value species for human consumption (Lochmann *et al.,* 2009). Pacu was introduced to India from Bangladesh as an ornamental species in 2003-2004 (Chattarjee and Mazumdar, 2009). Subsequently, seed production and culture of pacu was initiated in many regions of India (Singh, 2014). The species has been reported to be compatible in polyculture with Indian major carps, the major cultured species of the Indian sub-continent (Kumar *et al.,* 2018). The average production of pacu in the surveyed areas from Andhra Pradesh was 10,409 to 12,838 kg ha^{-1} in polyculture, and 7125 kg ha^{-1} in monoculture (Seshagiri *et al.,* 2022). Over the years, the area under culture and production of pacu has increased in several states. A shorter culture period, faster growth, and strong market demand have made pacu culture popular among the farmers. The species has emerged as a promising candidate for diversification of aquaculture in India (Seshagiri *et al.,* 2022). In 2024, Government of India has also formulated guidelines for culture of pacu in India.

Diseases are considered as a significant constraint for the growth of aquaculture and among them, bacterial diseases are the most significant. The bacterium *F. columnare* causing columnaris disease has been recognised as an important freshwater fish pathogen worldwide. This disease has been reported from many tropical as well as temperate freshwater fish species, such as black mollies (Decostere *et al.,* 1999),

perch (Morley and Lewis, 2010), channel catfish (Hawke and Thune, 1992), tilapia (Figueiredo *et al.,* 2005), trout (Suomalainen *et al.,* 2009), freshwater striped catfish (Tien *et al.,* 2012), catla (Verma and Rathore, 2013) and goldfish (Verma *et al.,* 2015). Among oomycetous pathogens, members of *Saprolegniaceae* family, are responsible for tremendous losses in wild and cultured fish species (Bruno *et al.,* 2011; Ravindra *et al.,* 2022). Oomycetes are generally considered as agents of secondary infections arising from conditions such as bacterial infection, poor husbandry practices, and infestations by parasites and social interactions (Mastan *et al.,* 2012).

Co-infections are relatively common in fish culture systems as they are exposed to multiple pathogens. Co-infections have an impact on disease susceptibility and severity of the disease. Infections are the outcomes of the complex interactions between pathogens, driven by factors such as intensive farming and environmental conditions. There are reports of viral and bacterial co-infections in aquacultures species (Swaminathan *et al.,* 2021; Vega-Heredia *et al.,* 2024). Several co-infections involving *F. columnare* have been reported in cultured fish species. These include *F. columnare* and *E. ictaluri* in striped catfish, *Pangasianodon hypophthalmus* in Thailand (Dong *et al.,* 2015b), *F. columnare* and *Dactylogyrus intermedius* in goldfish, *Carassius auratus* (Zhang *et al.,* 2015) and *F. columnare* and *Argulus coregoni* in rainbow trout (Bandilla *et al.,* 2006). Fungi mainly *Fusarium* have also been implicated along with bacteria in co-infections (Cutuli *et al.,* 2015). In addition, *Saprolegnia* sp. have also been associated with primary bacterial infection, *Aeromonas liquefaciens* in Japanese eel, *Anguilla japonica* (Egusa and Nishikawa, 1965) and with parasite, *Gyrodactylus salaris* in Atlantic salmon (Johnsen, 1978). Co-infection of *Chryseobacterium* species and *Aeromonas hydrophila* in pacu is reported from India (Abraham *et al.,* 2017). Additionally, there has been report of susceptibility of pacu to *Aeromonas* infection and parasitic infestations (Medina-Morillo *et al.,* 2023; Oliveira *et al.,* 2025).

In the present study, a case of large-scale mortality in farmed pacu during the winter season was investigated. The disease was diagnosed to be infection with *F. columnare* and *S. ferax* by both conventional and molecular methods.

Materials and methods

Sampling and disease investigation

During the winter month (water temperature 20±1°C), persistent mortality in *P. brachypomus* (500-600 g) was noticed over a period of one week in one of the earthen ponds of ICAR- National Bureau of Fish Genetic Resources (ICAR-NBFGR), Lucknow, with a cumulative mortality of around 40% of the total stocking. The moribund pacu (n=10) showing clinical signs were collected from the affected ponds, brought to the laboratory in live condition and processed for bacteriological, mycological and virological examinations. Wet mounts of skin and gills were prepared and observed under light microscope (400X) for presence of parasites and bacterial aggregations. In addition, gills, liver and kidney were collected in 95% ethanol for DNA isolation, and in 10% (v/v) neutral buffered formalin for histopathology. All the above procedures were also followed for apparently healthy pacu fish collected from a nearby pond that did not show any mortality or morbidity. These fish are referred as control fish, hereafter. All the experimental procedures in the present study were performed according to the guidelines of Committee for Control and Supervision of Experiments on Animals, Govt. of India on care and use of animals for scientific research.

Bacterial isolation

As the clinical signs were suggestive of columnaris disease, skin, gill lesions and kidney of moribund and control pacu were streaked on tryptone-yeast extract-salts (TYES) agar (Holt 1988) for the isolation of *F. columnare*. Additionally, Tryptic soya agar (TSA) plates were also streaked for isolation of any other bacterial pathogens. Inoculated plates were incubated at 28°C for 24-72 h for the growth of the target bacteria. An earlier isolate of *F. columnare* strain RDC-1 stored at -80°C in 20% glycerol stock in Fish Health Management and Exotics Division at ICAR-NBFGR, was revived on TYES agar and used as a positive control.

Isolation of oomycete

The affected fish were also showing cotton wool-like growth on skin and caudal fins, so oomycete isolation was attempted as per Lilley *et al.* (1998). Briefly, skin and caudal fin showing cotton wool-like growth were aseptically cut and spot placed on agar plate of glucose-peptone-yeast (GPY) medium supplemented with penicillin and streptomycin and incubated at 20°C. After two days, the marginal hyphae were transferred to new sterile GPY agar plates. The culture was preserved on Peptone and Glucose-1 (PG-1) medium for further use.

Virological examination

To confirm presence of any viral etiology in moribund pacu fish, experimental infection was performed as per Sahoo *et al.* (2016). Briefly, organ pools of anterior kidney, spleen and heart tissues were homogenised in L-15 medium at a final dilution of 1/10 (w/v) using a mortar and pestle. Homogenate was centrifuged at 4000 *g* for 15 min at 4°C, and supernatant was filtered using 0.22 µ filter for removal of bacterial contamination. Filtrate was injected intraperitoneally in healthy pacu fish @100 µl per fish. Injected fish were observed for 10 days for any mortality or morbidity associated with viral etiology.

Histopathology

Formalin fixed tissue samples were processed following standard protocols (Luna, 1968) and paraffin blocks were prepared. Thereafter, sections were cut at 5 µm thickness, stained with haematoxylin and eosin (H and E) and examined under the microscope for histopathological changes.

DNA extraction

Genomic DNA from collected tissues, cultured oomycete mycelia and *F. columnare* (RDC-1 strain) were extracted using DNeasy Blood and Tissue Kit (Qiagen) as per the manufacturer's protocol.

Polymerase chain reaction (PCR)

Detection of *F. columnare*

Detection of *F. columnare* in gills, liver and kidney of moribund and control fish was performed using the primer pair, ColF (5'-CAGTGGTGAAATCTGGT-3')

and ColR (5'-GCTCCTACTTGCGTAGT-3') (Darwish *et al.,* 2004). A 25 µl of amplification reaction mix contained 100 ng of extracted genomic DNA, 0.5 µM of each primer, 100 µM of each dNTPs, 4.0 µM of $MgCl_2$ and 1.0 unit of Taq polymerase (Thermo Scientific) in 1x reaction buffer. The amplification conditions consisted of initial denaturation at 94°C for 5.0 min followed by 35 cycles of denaturation, primer annealing and DNA polymerization steps at 94°C for 30 s, 45°C for 1.0 min and 72°C for 1.0 min, respectively with final polymerisation extension at 72°C for 8.0 min in a thermal cycler (Veriti® Thermal Cycler, Invitrogen Inc.). Amplified products were subjected to electrophoresis in 1.5% agarose gel containing 0.5 µg ml^{-1} ethidium bromide and photographed under gel documentation system (Gel Doc EZ Imager, Bio-Rad). DNA from *F. columnare* (RDC-1 strain) was used as positive control during PCR reactions.

Genetic group confirmation of *F. columnare*

For genetic group confirmation of *F. columnare* detected in moribund pacu, partial 16srDNA of *F. columnare* was amplified from genomic DNA of gill tissue using primer sets; FvpF1 (5'-GCCCAGAGAAATTTGGAT-3') and FvpR1 (5'-TGCGATTACTAGCGAATCC-3') (Bader *et al.,* 2003). The thermal cycling conditions followed were initial denaturation at 94°C for 5.0 min followed by 35 cycles of denaturation, primer annealing and DNA polymerisation steps at 94°C for 30 s, 59°C for 1.0 min and 72°C for 1.5 min, respectively, followed by final extension at 72°C for 8.0 min. The resultant PCR amplicon of 1193 bp fragment was excised from gel and purified by GeneJET Gel Extraction Kit (Thermo Scientific), as per manufacturer's protocol and sequenced using Sanger sequencing in 3500 Genetic Analyser (Applied Biosystems- Hitachi) at ICAR-NBFGR, India.

Molecular identification of oomycete

Molecular identification of oomycete isolated from moribund pacu was done by amplification of internal transcribed spacer (ITS) region with specific primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as per White *et al.* (1990). PCR amplification conditions were similar as given above except primer annealing at 55°C for 2 min, and cyclic extension at 72°C for 2 min. Resulting amplicon was gel purified and sequenced using Sanger sequencing method.

Sequence and phylogenetic analysis

The reads obtained from 5' and 3' ends of 16s rDNA sequence of *F. columnare* amplified from DNA of diseased pacu gills (FCPG); and ITS region of oomycete from diseased pacu were assembled and consensus sequences were generated. The sequences were analysed and submitted to GenBank (Accession number MF990560 for *F. columnare* and MF962873 for *S. ferax*). For phylogenetic analysis of genetic group of FCPG, a total of twenty 16s rDNA sequences of different genetic groups of *F. columnare* were obtained from NCBI database and analysed following the method of LaFrentz *et al.* (2018). Similarly, 10 ITS gene sequences of *Saprolegnia* spp were used for molecular identification of the isolated oomycete using BLAST and its phylogenetic analysis. Briefly, multiple alignment of obtained sequences was done with respective reference sequences using ClustalW and phylogenetic trees were constructed using maximum composite likelihood method in MEGA 7.0 software (Kumar *et al.,* 2016). Evaluation of tree topology was done using 1000 bootstrap replicates. To root the trees, sequences from *Chryseobacterium soldanellicola* and *Phytophthora katsurae* were used as outgroups for *F. columnare* and oomycete, respectively.

Results

Disease investigation

Moribund pacu exhibited cotton wool-like growth on body surface, especially around the head, dorsal surface and caudal fins, and pale yellow discolouration of gill filaments (Fig. 1). They were lethargic with excess mucus all over the body. Wet mount of the skin lesions revealed aseptate hyphae, while the gill lesions exhibited long rods in haystack-like arrangements typical of columnaris disease. No external parasites were observed in wet mount preparations of skin and gills of moribund fish. Mortality or morbidity was not noticed in any fish injected with filtered pooled tissue homogenate thereby ruling out viral etiology of the disease outbreak in pacu.

Bacterial/oomycete isolation

TYES plates streaked from gill lesions of moribund fish showed mixed growth of greyish white transparent colonies and yellow colonies with rhizoid margins. TYES plates from skin of moribund fish; and control fish (skin and gills) showed predominant growth of greyish white transparent colonies. Based on the typical colony morphology, the yellow colonies with rhizoid margins were suspected to be colonies of *F. columnare*. Gram staining of these colonies revealed presence of Gram-negative, long and thin rods without spore formation, typically associated with *F. columnare*. However, several attempts to purify and sub-culture these colonies from mixed bacterial growth were not successful in TYES, Shieh and cytophaga agar or broth. TYES plates and TSA plates inoculated from kidney of moribund and control fish did not result in any bacterial growth. TSA plates inoculated from gills and skin of both moribund fish and control fish showed predominant growth of medium sized, greyish white and transparent colonies. All such colonies obtained

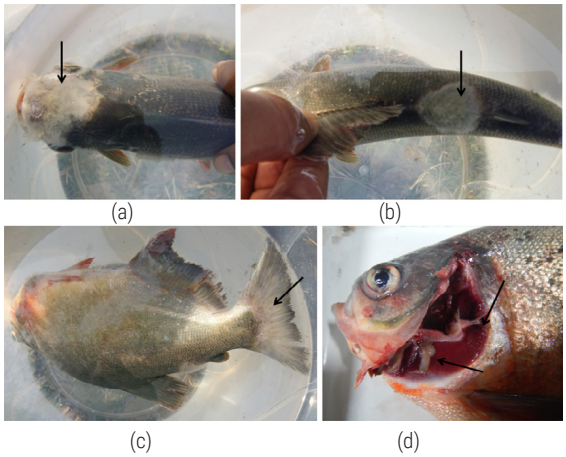


Fig. 1. Moribund pacu showing cotton wool-like growth on head, body surface and tail (a, b and c); (d) Pale-yellow discolouration (arrow) of gill filaments (operculum removed).

on TSA or TYES plates were tested to be Gram-negative short rods, oxidase positive and glucose non-fermenters. These colonies were presumed to be part of normal environmental microflora and were not processed further. The GPY agar plates inoculated with skin and caudal fin showed whitish cotton like mycelium growth after two days. The mycelium presented long thin non-septate hyphae under light microscope, and were indicative of oomycetes.

Histopathology

Histopathology of the gills of moribund pacu revealed desquamation of epithelial cells in the lamellar tissue, increase in number of mucous cells in the epithelium covering the tip of primary gill filaments, extensive haemorrhages, loss of cartilaginous tissue and inflammatory exudate between secondary lamellae (Fig. 2). No significant changes were seen in kidney and liver tissues of the diseased pacu.

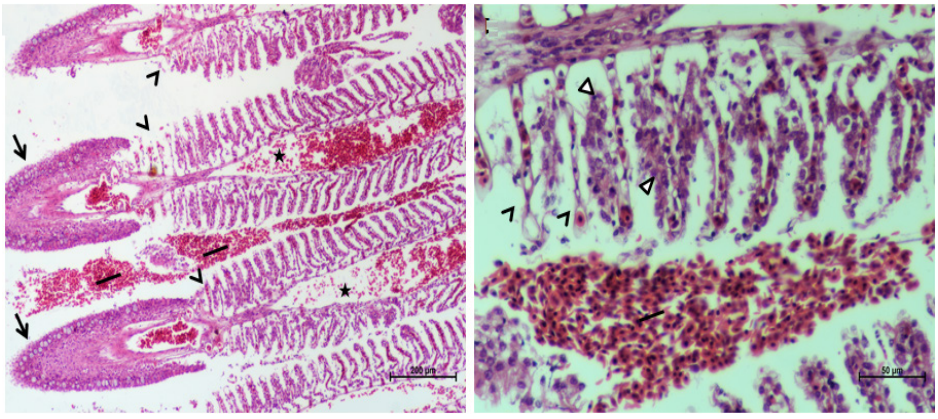
PCR amplification

Detection of *F. columnare*

F. columnare specific Col-F/Col-R primers produced a distinct 675 bp amplicon in gills of all the moribund fish tested; whereas no amplification was obtained in kidney and liver from the moribund fish (Fig. 3). Infection with *F. columnare* was reconfirmed with amplification of ~1193 bp fragment from gill tissues with FvpF1/FvpR1 specific primers in the moribund fish (Fig. 4). All the tissues collected from control fish tested negative by PCR.

Sequence analysis

Phylogenetic analysis of the curated FCPG sequence (1100 bp) revealed that *F. columnare* belonged to genetic group 2 (Fig. 5).



(a) (b)

Fig. 2. Histological section of infected gills of pacu showing (a) loss of lamellar epithelium (arrow head), epithelium covering at the tip of primary gill filaments with increase in number of mucous cells (arrow), loss of cartilagenous tissue (star) and extensive haemorrhage (line); (b) inflamatory exudate (triangle) between secondary lamellae, haemorrhages (line) and lamellar eptithelial cell loss (arrow head) are visible more clearly at higher magnification (H and E).

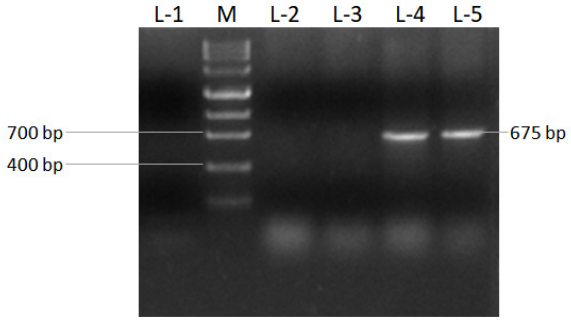


Fig. 3. Representative gel image of *F. columnare* detection by PCR in different tissues of moribund pacu using Col F and Col R primers. Lane M: Zip Ruler Express DNA ladder 2; Lane 1: No template control; Lane 2: Liver; Lane 3: Kidney; Lane 4: Gills; Lane 5: Positive control (RDC-1)

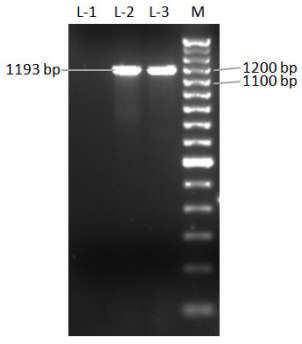


Fig. 4. Representative gel image of *F. columnare* detection by PCR in infected gills of pacu using 16s rDNA specific primers (FvpF1/ FvpR1). Lane M: 100 bp DNA ladder; Lane 1: No template control; Lane: Positive control (RDC-1); Lane 3: Gills.

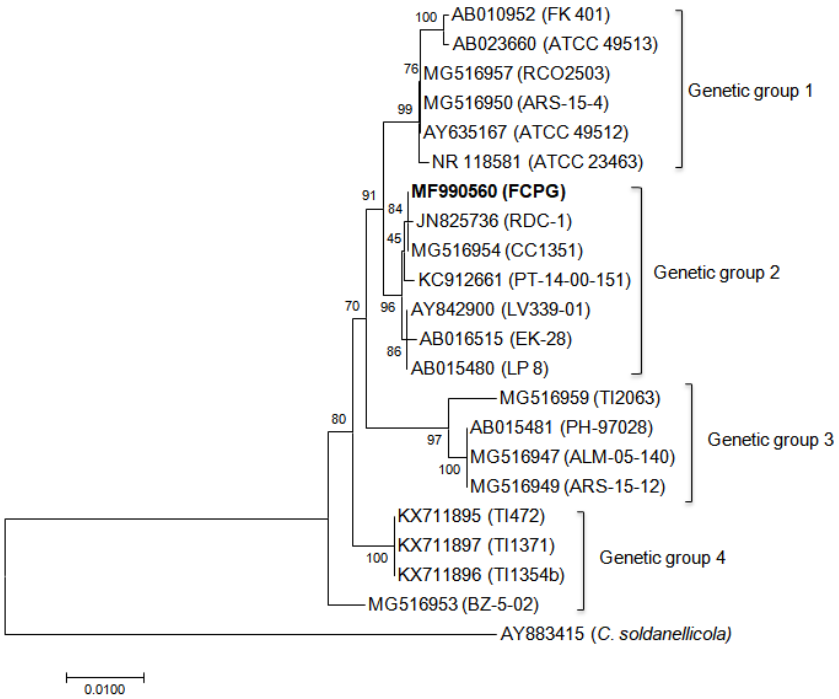


Fig. 5. Neighbour-joining phylogenetic tree based on 16S rDNA sequences, showing the position of *F. columnare* sequence (FCPG) detected from diseased pacu gills, among different genetic group of *F. columnare*. *Chryseobacterium soldanellicola* was used as a outgroup for rooting the tree. Numbers at nodes are bootstrap values from 1,000 replicates. Bar indicates substitution per nucleotide position.

Amplification of ITS region of isolated oomycete from pacu yielded a PCR product of 741 bp (Fig. 6). Sequence analysis of 741 bp (MF962873) ITS region showed maximum identity (100%) with *S. ferax* isolates from China (JN400035) and Argentina (GQ119935). Phylogenetic analysis revealed that the pacu isolate of *S. ferax* grouped with isolates of *S. ferax* (Fig. 7).

Discussion

Diseases remain a major obstacle to aquaculture, causing reduced production and significant economic losses to the farmers. Effective disease management and long-term sustainability of aquaculture require systematic documentation of antimicrobial resistance,

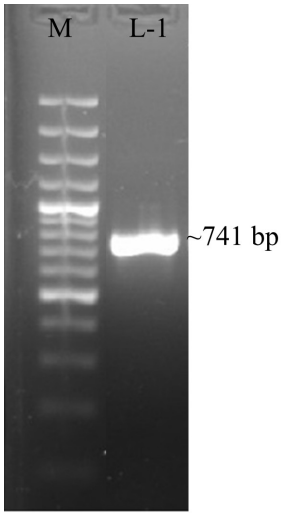


Fig. 6. Representative gel image of PCR amplification of ITS region of oomycete isolated from pacu. Lane M, Gene Ruler 100 bp plus DNA ladder; Lane 1: Oomycete isolate

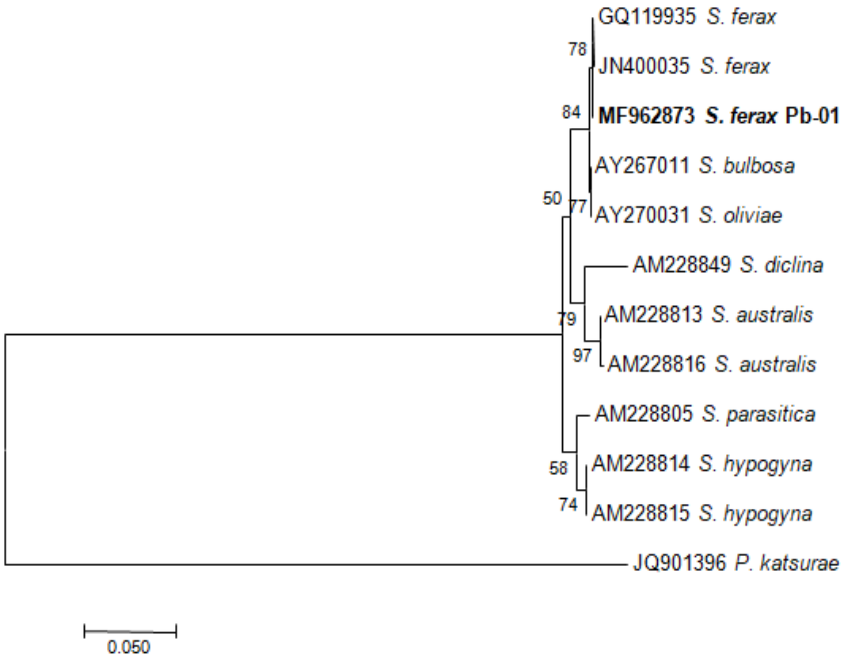


Fig. 7. Neighbour-joining phylogenetic tree based on ITS sequences, showing the position of pacu strain of *Saprolegnia ferax* (Pb-01) among different species of the genus. Accession numbers are shown along with *Saprolegnia* sp. *Phytophthora katsurae* was used as outgroup for rooting the tree. The bootstrap values (%) are shown next to the branches and scale bar represents distance values

timely pathogen detection, and prompt disease reporting. (Preena *et al.,* 2021; Kumar *et al.,* 2024; Kumar *et al.,* 2026).

In the present study, we report the simultaneous detection of *F. columnare* and *S. ferax* in farmed *P. brachypomus* during natural disease outbreak in winter season from India. The clinical signs observed in moribund pacu were similar to those reported for columnaris infection in other fish (Durborow *et al.,* 1998; Declercq *et al.,* 2013; Verma and Rathore, 2013; Sarker *et al.,* 2017; Baldissera *et al.,* 2020). Infection with *F. columnare* has been previously reported in *P. mesopotamicus* from Brazil, where the pathogen was isolated from kidney and characterised using phenotypic profiling (Pilarski *et al.,* 2008). On bacteriological examination of the diseased samples, colonies typical of *F. columnare* were observed on TYES agar streaked with gill lesions from moribund pacu. However, pure bacterium could not be isolated, so we opted for molecular detection of *F. columnare* in the tissues. Molecular detection of *F. columnare* infection using 16S rDNA gene sequencing has been recommended by Tiirola *et al.* (2002) for diagnosis of Flavobacterial diseases. Molecular confirmation of *F. columnare* infection in tissues of pacu was done using two species-specific primers [Col-F/Col-R and FvpF1/FvpR1]. However, out of three tissues tested, liver and kidney of moribund pacu did not show amplification in PCR for this pathogen indicating localised infection only in gills. This indicates that bacterial load in kidney and liver was too low or absent for detection by PCR. Reports suggest that *F. columnare* infection in fish may or may not cause septicaemia (Bernardet, 1997; Decostere *et al.,* 1999). Based on 16S rDNA sequence analysis, FCPG showed 99.6% identity to our *F. columnare* strain, RDC-1 isolated from *Catla catla* (Verma and Rathore, 2013) and belonged to genetic group 2. This sequence also showed >99% homology to other published sequences of genetic group 2 of *F. columnare*. In our study, FvpF1/FvpR1 primer set (Bader *et al.,* 2003) was used for amplification of 16S rDNA gene and its sequence was phylogenetically analysed for identification of genetic group. Our results show that phylogenetic sequence analysis of PCR product generated by this primer set has the capability to identify different intra-specific genetic groups of *F. columnare* strains. LaFrentz *et al.* (2014) described intragenomic heterogeneity in the 1250 bp fragment of 16S rRNA of *F. columnare* for genomovar assignment by RFLP using *HaeIII* enzyme. They described five genomovars of *F. columnare* namely I, II, II-B, I/II and III along with their expected restriction patterns. However, recent findings indicate that *F. columnare* isolates should be assigned to the four genetic groups rather than genomovars in order to facilitate a standard nomenclature across the scientific community (LaFrentz *et al.,* 2018).

It has been well documented that the *F. columnare* strains exhibit varying degree of virulence capacities and host specificities. Isolates belonging to genetic group 2 are known to be highly virulent such as strains ALG-02-36 and ALG-00-530 as reported in channel catfish (Shoemaker *et al.,* 2008; 2018), strain BE in goldfish (Ravindra *et al.,* 2019); and RDC-1 in *C. catla* (Verma and Rathore, 2014). In our findings, *F. columnare* detected in pacu gills belonged to genetic group 2 and could be responsible for extensive damage in gill tissues as observed in histopathology. Degradation of gill tissues could be due to extracellular proteases, which are known to be potential virulence factors secreted by *F. columnare* (Li *et al.,* 2017). Recent findings show that pathogenesis of columnaris disease in gill tissue is associated with the inhibition of creatine kinase and pyruvate kinase by the bacterium in silver catfish (Baldissera *et al.,* 2020). Considering this, it is suggested that *F. columnare* could be responsible for degradation of gill tissues of diseased pacu.

In this study, clinical signs also revealed cotton wool-like growth on the body surface in moribund fish, which is indicative of oomycete infection reported in various freshwater cultured fish (Rowland and Ingram, 1991; Hussein *et al.,* 2001). The oomycete was isolated from skin/fin lesions and identified as *S. ferax* using phylogenetic analysis based on ITS region. ITS region has been used for molecular phylogenetic analysis of *Saprolegnia* spp., as morphology based identification does not resolve taxonomic positions of closely related species of the genus (Ke *et al.,* 2009; Sandoval-Sierra *et al.,* 2014). The first Indian record of *S. ferax* was described by Wani *et al.* (2017). The Indian *S. ferax* isolate has been redescribed based on morphotaxonomy and phylogeny. *S. ferax*, belongs to genus *Saprolegnia*, order Saprolegniales having both sexual and asexual morphs. The isolates may exhibit morphological variations of zoosporangium with dispersed encysted zoospores, and variation in shape and size of antheridia (Singh *et al.,* 2018). Several species of *Saprolegnia* are known to affect fish and amphibians. Previously, *S. ferax* has been isolated from infected cruician carp and zebra fish (Ke *et al.,* 2009); infected eggs of yellow catfish (Cao *et al.,* 2012); and diseased Atlantic salmon, chinook salmon, rainbow trout (Sandoval-Sierra *et al.,* 2014). Experimental infection of *S. ferax* has been reported from Nile tilapia (Zahran and Risha, 2013). In addition, many of the fish species, namely *Salmo* sp., *Salvelinus* sp., and *Oncorhynchus* sp. are common carriers of *Saprolegnia*, including *S. ferax* (Richards and Pickering, 1978; Wood and Willoughby, 1986). Rainbow trout has been demonstrated to be a vector for transfer of *S. ferax* to cultured amphibians resulting in massive embryo mortalities (Kiesecker *et al.,* 2001). Another species of pacu, *P. mesopotamicus* has been used as experimental model to demonstrate the pathogenicity of oomycete, *S. aenigmatica* under immunosuppression and skin abrasion (Carraschi *et al.,* 2018). However, there are no reports of natural infection of *S. ferax* in pacu. This study provides first record of detection of *S. ferax* in cultured *P. brachypomus* during natural infection. Oomycetes are saprophytic opportunist pathogens, which infect stressed fish (Pickering and Willoughby, 1982). Low water temperatures play an important role in inducing mortalities as it causes host immunosuppression and also favours release of zoospores from *Saprolegnia* sp. (Bly *et al.,* 1993). The motile zoospores can enter the host through damaged epithelium of the skin and/or gills caused by injury or bacterial infection (Roberts, 2001). In the present study, the mortality was observed in pacu during winter when the water temperature was 20±1°C. These environmental conditions are known to favour the growth of *F. columnare* as well as *Saprolegnia* sp., but are stressful for pacu as it is a warm water fish. Therefore, it is assumed that fish became susceptible to both the pathogens and their cumulative effect resulted in higher mortality. This hypothesis could have been validated by single or simultaneous experimental challenge with both the pathogens in susceptible fish. However, the inability to sub-culture the *F. columnare* isolate from the primary plate proved to be a major limitation in validating the co-infection model. We tried several selective media for the sub-culture, but the isolate failed to grow indicating its fastidious nature. Several researchers have had trouble in isolation and culture of *F. columnare* from clinical samples (Farmer, 2004), either due to natural microbial fauna overgrowth or due to growth of antagonistic bacteria (Rintamäki-Kinnunen *et al.,* 1997; Tiirola *et al.,* 2002).

Two or more genetically different pathogens can cause infection, where each pathogen can cause harm to the host (Cox, 2001; Kotob *et al.,* 2017). Simultaneous infection of multiple pathogens in fish is quite common in natural environment, as evidenced by number of publications on co-infection involving *F. columnare* (Bandilla *et al.,* 2006; Dong *et al.,* 2015a, b; Zhang *et al.,* 2015) and *Saprolegnia* sp. (Egusa and Nishikawa, 1965; Johnsen, 1978). The interactions involving multiple pathogens can have an important impact on the development and severity of the diseases and should be considered during the planning of therapy and vaccination. Further research is needed to improve the understanding on the interactions between *F. columnare* and *S. ferax*, and how they interact with the immune response of the fish.

In conclusion, this study reports simultaneous detection of two important pathogens *F. columnare* and *S. ferax* from diseased pacu. This finding provide valuable insight into natural disease outbreaks in cultured pacu fish and highlight the susceptibility of pacu to co-infections under culture conditions.

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