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Development of multiplex PCR assay for species-specific detection and identification of *Saprolegnia parasitica*

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ABSTRACT

Saprolegnia parasitica is the most important pathogen under the genus, *Saprolegnia* which causes devastating oomycete diseases in freshwater fish. At present, the most common molecular method for identification of *Saprolegnia* species is sequencing of ribosomal DNA internal transcribed spacer (rDNA-ITS) region. In this study, a highly sensitive multiplex PCR targeting rDNA-ITS region and a hypothetical protein gene was developed using two sets of primer pair. In this PCR, two amplicons of different size of 750 bp and 365 bp are produced only in case of *S. parasitica* while other *Saprolegnia* species had single amplicon. This protocol could also differentiate *Saprolegnia* species from other fungus based on the size of rDNA-ITS region. The protocol does not require sequencing and can identify *S. parasitica* in a single reaction. Therefore, the multiplex PCR developed in this study may prove to be an easier, faster and cheaper molecular method for identification of *S. parasitica.*

1. Introduction

Saprolegniasis is a disease characterized by visible white or gray patches of filamentous mycelium on skin and fins. In severe cases, the hyphae invade epidermal tissues, muscles and blood vessels and ultimately leading to death of the infected fish due to osmoregulatory failure [1–4]. It is the most devastating oomycete diseases of freshwater fish caused by species of the genus, *Saprolegnia* which comprises 23–24 species [1,5–7]. *Saprolegnia* infection in fish can cause huge losses in terms of millions of dollars to the aquaculture industry annually [1,8]. Their infection is not only confined to the cultured fish but also linked to worldwide decline in wild fish as well as amphibian populations [8]. Earlier, *Saprolegnia* infections were controlled by using malachite green, but its use in aquaculture has been banned since 2002 due to its carcinogenic and toxicological effects and this has resulted in its re-emergence particularly *S. parasitica* as economically important fish pathogen, especially for catfish, salmon and trout species [1,9].

The genus *Saprolegnia,* contains pathogens of aquaculture importance although they are often considered as opportunistic pathogen that is saprotrophic and necrotrophic [1,3,10]. However, some strains particularly *S. parasitica* are highly virulent and can cause primary infections [11–13]. Therefore, it is essential to demarcate those pathogenic species from the non-pathogenic one. For identification of isolated species, several techniques like microscopic examination of mycelia, sexual reproductive structures and oospores, had been the main method until development of molecular techniques. Species identification, based on microscopic examination of the sexual structures is difficult, as these structures do not generally form in lesions nor usually found in fresh water [14,15] and hence require taxonomic expertise to delineate different species. Moreover, many isolates fail to produce sexual structures *in vitro* or develop after a long period of culture [14]. At present, the most common molecular technique for identification of *Saprolegnia* species is sequencing of the internal transcribed spacer (ITS) region. However, sequencing facility is not available in many of the laboratories and it takes time in outsourcing. Therefore, a molecular tool that can rapidly and accurately detect and identify *S. parasitica*, the most destructive pathogen among *Saprolegnia* species, will be highly beneficial. Among the molecular techniques, multiplex PCR is a powerful tool for identification of viruses, bacteria, fungi/parasites [16]. This form of PCR uses two or more primer pairs to amplify more than one gene in a single reaction [17]. Thus the expenses, effort and time are less in multiplex PCR as compared to multiple reactions required in uniplex PCRs [18]. Further, an internal amplification control included in multiplex PCR indicates the quality and quantity of the template and

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also helps to rule out the false negative results [19,20]. Keeping this in mind, the present study was carried out to develop a sequencing free molecular assay based on multiplexing of primers for identification of *S. parasitica*.

2. Materials and methods

2.1. Oomycetes culture and isolation of genomic DNA

A total of 40 isolates belonging to different genus of oomycete and fungus were used in the study. All the species used in this study were isolated from diseased farmed fishes and water and maintained in Molecular Biochemistry Laboratory of ICAR-DCFR, Bhimtal. Oomycetes and other fungus were cultured in potato dextrose agar (PDA) containing 500 mg/L of ampicillin and 200 mg/L of chloramphenicol at 20 ± 1 °C till pure isolates were achieved [21,22]. For isolation of genomic DNA, a small amount of agar with advancing edges of the growing colony was excised and inoculated in 5 ml of potato dextrose broth (PDB) for around 5–7 days at 20 ± 1 °C. Mycelium from the broth culture was used for isolation of genomic DNA using commercial kit (Quick-DNA™ Fungal/Bacterial Miniprep Kit, Zymo Research). Concentration of the DNA was determined by Nanodrop sphectrophotometer (Thermo Scientific) and kept at −20 °C until further use.

2.2. PCR amplification and sequencing

Genomic DNA of fungal and oomycete isolates was used as template for amplification of ITS region and hypothetical protein gene using published primers [23,24]. The primers, ITS1/ITS4 (ITS1: 5′-TCCG TAGGTGAACCTGCGG-3′, ITS4: 5′- TCCTCCGCTTATTGATATGC-3′) and Puf112/Puf310 (Puf112: 5′- GTTGTACCATAGCCACTGTATC-3′ and Puf310: 5′- CATCTCGATGCTGTTCTTGC-3′) were obtained from Eurofins Genomics India Pvt. Ltd. Bangalore. The PCR reaction mixture consisted of 10 µl of 2 X PCR master mix (NEB), 1 µl of template DNA (more than 100 ng), 0.5 µl (5 pmol) of each primers, 8 µl of nuclease free water in a 20 µl volume. Amplifications were performed in an Eppendorf Mastercycler with initial denaturation at 95 °C for 2 min. The amplification cycle for ITS region consisted of 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min and final extension 72 °C for 10 min. The amplification cycle for the hypothetical protein gene consisted of 35 cycles of denaturation at 95 °C for 20 s, annealing at 58 °C to 66 °C (gradient) for 20 s, extension at 72 °C for 30 s and final extension at 72 °C for 5 min. The PCR products were then gel electrophoresed in 1.5% agarose stained with ethidium bromide with a 100 bp DNA ladder (Thermo scientific) to determine its size. Gel was visualized in a gel documentation system (Molecular Imager® Gel Doc™ XR System, Biorad). The PCR products were cleaned up using commercial kit (Wizard® SV Gel and PCR Clean-Up System, Promega) and sequenced at Agrigenome Labs Pvt. Ltd., India.

2.3. Evaluation of sensitivity and specificity

The PCR protocol using primer pair, Puf112/Puf310 was tested for its sensitivity with two fold dilutions of genomic DNA from *S. parasitica*. Similarly, ITS1/ITS4 primer was also tested using two fold serial dilution of genomic DNA starting from 20 ng to 10 pg to confirm its amplification at low concentration of template. The Puf112/Puf310 primers were also evaluated for its specificity towards *S. parasitica* as compared to other *Saprolegnia* species and other fungus. All the tests were repeated at least three times to validate the result.

2.4. Multiplex PCR using ITS1/ITS4 and Puf112/310 primers

Different combination of primer concentrations of ITS1/ITS4 and Puf112/310 were tested in the PCR. The reaction mixture contained 10 µl of 2X PCR master mix, ITS and PUF primers, 2 µl of template (more than 100 ng) and nuclease free water to make up to 20 µl. Similarly, different annealing temperatures varying from 55 to 62 °C was evaluated to determine the suitable annealing temperature for the primer pairs. The amplification steps consisted of initial denaturation temperature of 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. Concentrations of the primers, template and different annealing temperatures are presented in Table 1. The protocol was evaluated to determine its sensitivity using two fold serial dilutions of *S. parasitica* genomic DNA. The protocol was also evaluated against 40 different isolates of *Saprolegnia* and other fungus species which are listed in Table 2. Further, to validate the specificity, sensitivity and inhibition, simulation study was carried out by adding *S. parasitica* in fish pond water followed by extraction of genomic DNA using 10-fold serial dilution method. The extracted DNA was tested by the multiplex PCR to determine its suitability to detect *S. parasitica* in pond water samples.

3. Results

3.1. Culture and isolation of genomic DNA

Saprolegnia isolates showed radial growth with white hyphae extending towards the edges of the PDA plates. At 20 ± 1 °C, the hyphal growth of all the isolates was clearly visible by 72 h of culture. Some of the fungal contaminants also showed white cottony like growth which required expert eye to differentiate from *Saprolegnia* species. For molecular identification, extraction of genomic DNA from the isolates was performed using commercial kit. DNA concentration was found to vary from 100 to 170 ng/µl with the ratio of A260/280, 1.95.

3.2. PCR amplification and sequencing

In all the isolates, amplification of rDNA-ITS region using ITS1 and ITS4 produced a single band of PCR product in gel electrophoresis. In the case of *Saprolegnia* species, the product was found to be of 750 bp approximately whereas other fungus had products of smaller size (Fig. 1). With Puf112 and Puf310 primers, there was a single amplicon of 365 bp in all the tested annealing temperatures. The product band was bright at 58 °C, 60 °C and 62 °C but less intense at 64 °C and 66 °C and hence 62 °C was selected for further amplification. Amplification was observed only in the case of *S. parasitica* indicating the specificity of the primer. Amplicons from two isolates showing bright bands in gel electrophoresis were sequenced to confirm its locus and submitted to NCBI GenBank (Accession numbers-OK492200 and OK492201).

In alignment using MEGA X, the sequences were found to have high similarity with a part of *S. parasitica* hypothetical protein gene (Gene ID: 24,137,098) of the genomic scaffold, NW_012156556 [8]. The nucleotide sequences, OK492200 and OK492201 were found to have initiation codon of the coding region along with introns. As compared to the

Table 1
Amplification conditions followed in the multiplex PCR for identification of *S. parasitica*.

Reaction mixture		Quantity
2X PCR master mix		10 µl
ITS1/ITS4: Puf112/Puf310		5 pmol : 5 pmol, 10 pmol : 10 pmol and 5 pmol : 10 pmol
Saprolegnia genomic DNA		More than 100 ng
Nuclease free water		To make up the volume to 20 µl
Amplification program		
Initial denaturation		95 °C for 2 min
35 cycles of	Denaturation	95 °C for 30 s
	Annealing	55, 56, 59, 60 and 62 °C for 30 s
	Extension	72 °C for 1 min
Final extension		72 °C for 10 min

Table 2
Evaluation of the multiplex PCR for identification of *Saprolegnia parasitica* among different isolates.

Sl. No.	Name	Accession number	*Source*	Amplicon size of rDNA-ITS region (~bp)	Amplicon of hypothetical protein gene (~365 bp)
1	*S. parasitica*	MK163535	*Tor putitora*	750	Present
2	*S. australis*	MT912582	*Oncorhynchus mykiss*	750	–
3	*S.parasitica*	MT912580	*Oncorhynchus mykiss*	750	Present
4	*S.parasitica*	MT912581	*Oncorhynchus mykiss*	750	Present
5	*S.parasitica*	MT912584	*Schizothorax richardsonii*	750	Present
6	*S.parasitica*	MT912585	*Schizothorax richardsonii*	750	Present
7	*S.parasitica*	MT912586	*Schizothorax richardsonii*	750	Present
8	*S.parasitica*	MT912589	Water	750	Present
9	*Alternaria species*	MT912588	*Schizothorax richardsonii*	650	–
10	*Achlya species*	MT908192	*Tor putitora*	750	–
11	*Achlya species*	MT908847	*Tor putitora*	750	–
12	*Achlya species*	MT908917	Fish tank water	750	–
13	*Didymella species*	MT908911	*Tor putitora*	550	–
14	*Emmia species*	MT912577	*Tor putitora*	650	–
15	*Fusarium species*	MT907512	*Tor putitora*	550	–
16	*Fusarium species*	MT912583	*Oncorhynchus mykiss*	550	–
17	*Mucor species*	MT912587	*Schizothorax richardsonii*	650	–
18	*Phoma species*	MT912578	*Tor putitora*	550	–
19	*Schizophyllum species*	MT908224	Water	650	–
20	*Schizophyllum species*	MT909559	Water	650	–
21	*S. diclina*	OK448358	*Oncorhynchus mykiss*	750	–
22	*S. parasitica*	OK448359	*Oncorhynchus mykiss*	750	Present
23	*S. diclina*	OK448360	*Oncorhynchus mykiss*	750	–
24	*S. aenigmatica*	OK448361	*Oncorhynchus mykiss*	750	–
25	*S. parasitica*	OK448362	*Oncorhynchus mykiss*	750	Present
26	*S. parasitica*	OK448363	*Oncorhynchus mykiss*	750	Present
27	*S. parasitica*	OK448364	*Oncorhynchus mykiss*	750	Present
28	*S. parasitica*	OK448365	*Oncorhynchus mykiss*	750	Present
29	*S. diclina*	OK448366	*Oncorhynchus mykiss*	750	–
30	*S. diclina*	OK448367	*Oncorhynchus mykiss*	750	–
31	*S. diclina*	OK448368	*Oncorhynchus mykiss*	750	–
32	*S. parasitica*	OK448369	*Oncorhynchus mykiss*	750	Present
33	*S. asterphora*	OK448370	*Oncorhynchus mykiss*	750	–
34	*S. diclina*	OK448371	*Oncorhynchus mykiss*	750	–
35	*S. diclina*	OK448372	*Oncorhynchus mykiss*	750	–
36	*S. parasitica*	OK448373	*Oncorhynchus mykiss*	750	Present
37	*S. parasitica*	OK448374	*Oncorhynchus mykiss*	750	Present
38	*S. parasitica*	OK448375	*Oncorhynchus mykiss*	750	Present
39	*S. diclina*	OK448376	*Oncorhynchus mykiss*	750	–
40	*S. diclina*	OK448377	*Oncorhynchus mykiss*	750	–

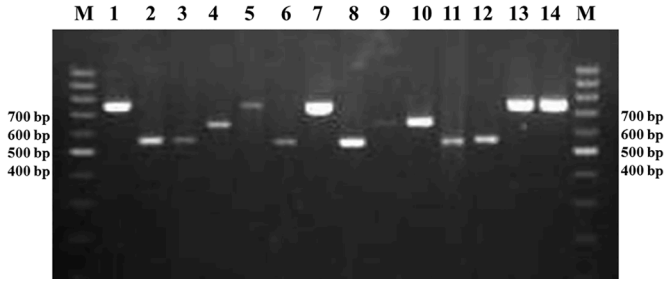

Fig. 1. rDNA-ITS region amplified with ITS1/ITS4 primers. M: 100 bp DNA ladder. A: Lane 1, 5,7, 13 and 14- amplicons of different *Saprolegnia* species (~750 bp). Lane 2 and 3- *Fusarium equiseti,* lane 4- *Schizophyllum commune,* lane 6- *Didymella heteroderae,* lane 8-*Emmia lacerate,* lane 9 and lane 10-*Schizophyllum species*, lane 11-*Phoma* species, lane 12- *Fusarium oxysporum*. The fungal contaminants produced an amplicon size of ITS region varying from ~550 to 650 bp.

reference sequence, OK492200 differed by one nucleotide whereas OK492201 had differences of two nucleotides in the exons. The combined exons translated into 54 amino acid sequences which were 100% identical to N-terminal side of the hypothetical protein (XP_012209840) of NW_012156556 (supplementary file).

3.3. Sensitivity and specificity of the PCR protocol

Both the PCR protocol using ITS1/ITS4 and Puf112/Puf310 primers were found to be highly sensitive. The limit of detection for Puf112/Puf310 primer was found to be 8 pg of genomic DNA. Amplification of rDNA ITS region was observed in all the tested concentration of genomic DNA. Similar results were observed in repeated experiments. There was no amplification of the targeted hypothetical protein gene in the fungal isolates. Among the *Saprolegnia* species, amplification of hypothetical protein gene was observed only in the case of *S. parasitica* (Fig. 2).

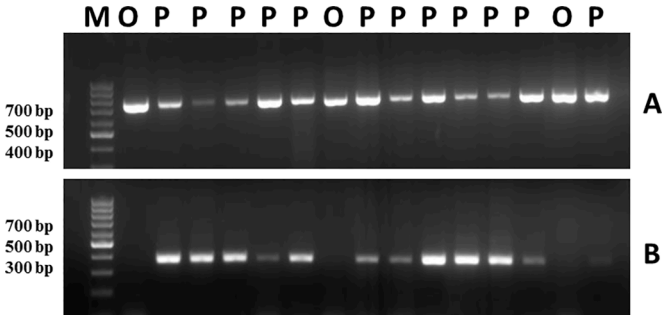

Fig. 2. PCR amplification of rDNA ITS region (A) and hypothetical protein gene (B) of different *Saprolegnia* species. M: 100 bp DNA ladder. A-All the samples had amplicons of rDNA-ITS region at ~750 bp. B-*Saprolegnia parasitica* (P) produced amplicons at ~365 bp but no amplification in other (O) *Saprolegnia* species.

3.4. Multiplex PCR for easy identification of S. parasitica

The optimum annealing temperature for both the primer sets in the multiplex PCR was found to be 59 °C. Two amplification products of approximate size at 750 bp for nrDNA ITS region and another at 365 bp for the hypothetical protein gene were observed. When the primers were used at equal concentration, amplification of nrDNA ITS region was much higher than that of the hypothetical protein gene. When ITS1/ITS4 primers were used at 5 pmol and Puf112/310 at 10 pmol each, the resulting yield of the amplified products was more or less similar. The protocol was found to be highly sensitive with detection limit up to 16 pg of genomic DNA. It was also found to detect *S. parasitica* specifically among other *Saprolegnia* species (Fig. 3). In simulation assay using fish pond water, the PCR protocol could detect up to 50 pg of genomic DNA (Fig. 4).

4. Discussion

Until the advent of molecular technique, microscopic examination of mycelia, sexual reproductive structures and oospores had been the main method for identification of *Saprolegnia* species [10]. Species identification, based on morphological features is often complicated as many isolates do not generally form these structures in lesions or fail to develop *in vitro* [14,15]. During isolation of *Saprolegnia* species from the site of infection or water, some fungal contaminants may also be present. Some of the fungal species encountered were of *Didymella, Phoma, Schizophyllum* and *Emmia* which also produced white colonies [25–28]. Hence identification of *Saprolegnia* species based on culture morphology may be difficult for untrained eyes. Therefore, more accurate way to identify *Saprolegnia* species is through molecular approach.

The most commonly used molecular technique for identification of *Saprolegnia* species is the sequencing of ITS region located between 18S rRNA gene and 28S rRNA gene present in the chromosome [29]. Eukaryotic rDNA ITS region is widely used for taxonomy and molecular phylogeny due to its small size, high copy number and conserved flanking sequences [30]. PCR amplification of *Saprolegnia* rDNA-ITS region which consists of partial 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and partial 28S rRNA gene using ITS1 and ITS4 primers resulted to product of 750 bp approximately. Liu et al. [31] also found the size of ITS region in different species of *Saprolegnia* to be around 700 bp. Successful amplification of rDNA-ITS region was observed even at a very low concentration of genomic DNA indicating its high copy number.

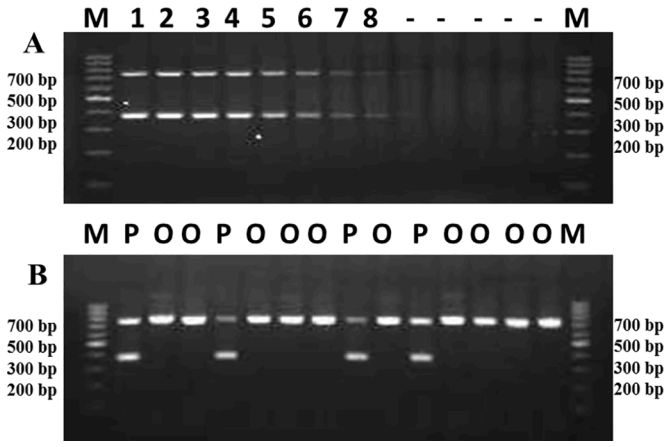

Fig. 3. Multiplex PCR using ITS1/ITS4 and Puf112/Puf310 primer pairs. M: 100 bp DNA ladder. A-Determination of detection limit of the protocol using two fold dilution (1 ng onwards) of genomic DNA. Amplification was observed up to 0.016 ng in lane 8. B- Evaluation of the protocol against different *Saprolegnia* species. Two bands of PCR product in *S. parasitica* (P) and single band in other *Saprolegnia* species (O).

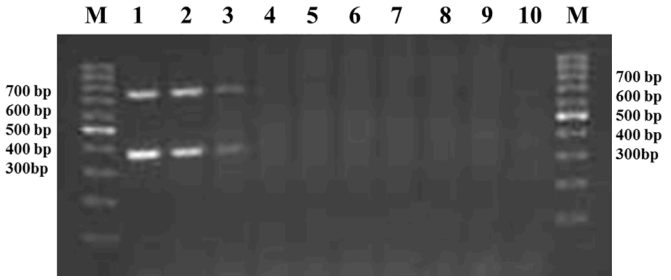

Fig. 4. Multiplex PCR using genomic DNA from *Saprolegnia parasitica* simulated fish pond water. The amplification was observed up to 50 pg of genomic DNA in lane 3. M: 100 bp marker. Lane 10: negative control without template.

Similar size of PCR product was also observed for *Achlya* species that belong to family *Saprolegniaceae,* and order Saprolegniales. Interestingly, the fungal contaminants had smaller PCR product of ITS region thus enabling to differentiate from *Saprolegnia* species based on the amplicon size. However, identification of *Saprolegnia* at species level requires sequencing of the ITS region [32]. Recently, Ghosh et al. [33] developed a loop-mediated isothermal amplification (LAMP) for the detection of *Saprolegnia* species. The developed protocol targeted the ITS region and the cytochrome C oxidase subunit 1 (CoxI) gene and was shown to be specific only to *Saprolegnia* genus [33].

Earlier, a PCR protocol using puf112 and puf310 markers targeting hypothetical protein gene was reported to specifically identify *S. parasitica* [24]. This hypothetical protein contains Pumilio-family or Puf RNA binding domain at the C-terminal. Puf locus is highly conserved at C-terminal RNA-binding domain but the variable N-terminal region was used for developing *Saprolegnia*-specific markers [24]. The PCR protocol was also found to be highly sensitive with detection limit as low as 8 pg of genomic DNA. However, the yield may vary depending upon the copy number of the particular gene as expression of Puf protein in *S. parasitica* differs with the stages of its life cycle [34]. This may be the reason for different yield of PCR product by puf112/ puf310 primers irrespective of template concentration in the samples.

These two PCR reactions with ITS1/ITS4 and Puf112/Puf310 primers target unique sites resulting into different product size. So, if the two reactions are combined in a single run, considerable effort and consumables may be saved. Considering this, a multiplex PCR using the primer pairs, ITS1/ITS4 and Puf112/Puf310 was developed. Multiplex PCR is a fast molecular technique that simultaneously amplifies different targets enabling to detect multiple pathogens in a single reaction [35–37]. The technique has been used in diagnosis of various viral, bacterial, fungal and parasitic diseases [38]. In the study, the protocol was optimized to amplify both the rDNA-ITS region and hypothetical protein gene with similar efficiency and also devoid of nonspecific products. The amplicons were visible as two distinct bands at ~750 bp and ~365 bp. In multiplex PCR, the amplicon sizes should be different enough to produce distinct bands in gel electrophoresis [39]. In this multiplex assay, the amplicon of ITS region served as internal amplification control and the amplicon resulting from other primer pair helps in species identification of *S. parasitica*. Internal control is important in multiplex PCR as it helps to rule out the false negative results. If the amplicons of internal control is detected, non-appearance of the species specific amplicon can be more confidently interpreted as absence of the target gene rather than failure of the PCR reaction. In the case of bacteria, universal primers targeting 16 s rDNA serve as a good choice for internal amplification control [20]. In the multiplex PCR for detection and identification of different yeast strains, universal primers, ITS1 and ITS2 have been used in combination with ITS2 and ITS3 primers [40]. Similarly, we have also used the universal primer pair ITS1/ITS4 as internal control in the multiplex PCR. This primer pair could successfully detect the ITS region in all the tested fungus and produced amplicon of different size ranging from 550 to 650 bp approximately

whereas it was around 750 bp in case of *Saprolegnia*. The other primer pair, Puf112/310 showed positive reaction only in the case of *S. parasitica*. Thus, *Saprolegnia* species could be differentiated from the fungal contaminants based on the size of rDNA-ITS region and simultaneously identify *S. parasitica* by the presence of amplicon at ~365 bp in a single test run. The protocol was also found to be highly sensitive having a low detection limit and hence may be applied in less concentration of genomic DNA. Further, the protocol was also suitable to detect *S. parasitica* in fish pond water as observed in simulation assay. The detection limit in *S. parasitica* simulated pond water was higher which may be due to presence of genomic DNA of other microbes. As species identification could be achieved in a single PCR reaction without sequencing, there was substantial reduction in time, effort and cost in this protocol.

5. Conclusion

Sequencing of rDNA-ITS region is the most common method for identification of *Saprolegnia* species. As sequencer is not a common facility in many of the laboratories, sequencing free molecular method for species identification specially *S. parasitica* would be beneficial. In the study, a multiplex PCR was developed using two different primer pairs. Based on the amplicon size resulting from the first primer pair, *Saprolegnia* species can be differentiated from other fungal contaminants and the PCR product resulting from the second primer pair specifies *S. parasitica*. Therefore, this multiplex PCR may prove to be an easier, faster and cheaper method for molecular identification of *S. parasitica*. The protocol was found to be sensitive and specific and may also be suitable for molecular screening of large number of *Saprolegnia* isolates.

Ethical approval statement

No experimental fish and no genetically engineered organisms were used in this study. All the protocols and methods used in this study were approved by Institutional Research Committee of ICAR-Directorate of Coldwater Fisheries Research, Bhimtal.

Author's contributions

KVC and DT conceptualised and designed the experiments. KVC wrote the manuscript and DT edited. KVC, DT, VP, SB did the molecular works. SB also contributed in literature search. KVC, DT and RST collected the isolates. KVC and VP cultured and maintained the isolates.

Declaration of Competing Interests

The authors declare no conflict of interest with respect to publication of this article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2022.e00758.

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