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Research Article

Molecular detection of a soil nematode (*Panagrellus redivivus*) in artificial culture media

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ABSTRACT

Nematodes are thought to be troublesome organisms to identify due to few apparent taxonomic characteristics with overlapping morphometric and morphological similarity among the species. Molecular based techniques are more sensitive and reliable than microscopic morphological study to identify the species and subspecies avoiding similar ambiguity. Therefore, this study was undertaken to apply PCR analysis in identifying a soil nematode, *Panagrellus redivivus* cultured in potato, ceral and oatmeal media so as to show the feasibility of those artificial media as suitable for *P. redivivus* production. To do so, the starter of *P. redivivus* was cultured in those artificial media prepared in association with baker's yeast. The *P. redivivus* grown in each media were heated and centrifuged to isolate the DNA which were finally amplified with Pan28s (F/R) and Pan28s NES(F/R) primers retrieved from 28s ribosomal RNA gene of the species. A fragment of 1131-bp for Pan28s (F/R) primer in case of first step PCR and a 1050-bp band for Pan28s NES (F/R) in case of 2nd step PCR (nested) confirmed the species. By identifying the species at molecular level in those media, the study concluded that the three media were suitable for artificial culture of *P. redivivus* species to be used as a potential alternative live feed in prawn hatcheries in Bangladesh

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INTRODUCTION

In Bangladesh, brine shrimp or *Artemia* nauplii is conveniently used in hatcheries as live food for the nutrition and growth of prawn, *Macrobrachium rosenbergii* larva (Islam *et al.,* 2019). However, it has some prominent negative aspects viz. variable cost and availability, unpredictable hatching rate, competitive nature, varying growth and nutritional quality (Sautter *et al.,* 2007; Biedenbach *et al.,* 1989). Therefore, some other live foods such as copepods, rotifers, daphnia, moina and nematodes have also been suggested as potential live organisms for their use in larval nutrition in full or partial replacement of *Artemia* (Couto *et al.,* 2018; Lavens and Sorgeloos, 1996). In this regard, application of a soil nematode, *Panagrellus redivivus* can be an alternative of *Artemia* as a starter feed in the hatcheries (*Couto et al., 2018).*

The *P. redivivus* is a tiny nematode of about 0.05 to 2.0 mm in length and 0.05 mm in diameter which make it suitable food item for fish larvae, especially those with a small mouth. Several studies reported that the free-living nematode *P. redivivus* is a suitable first feed of fish larvae (Kahan and Appel, 1975; Kahan *et al.,* 1980) containing 400-620 mg/g protein, 150-200 mg/g lipid and 310 mg/g carbohydrate (Biedenbach *et al.,* 1989; Watanabe and Kiron, 1994). Investigations suggested using the free living nematodes as larval feed for various penaeid shrimps (Kumlu and Fletcher, 1997; Wilkenfeld *et al.* 1984). Likewise, its suitable size and high nutritional values have rendered the species to be a valuable food item for prawn larval rearing in hatchery which could be produced large quantities using suitable culture media (Ricci *et al*., 2003).

Prior to mass production, proper identification of the nematode species in an artificial media is essential to find out the suitability of the media for *P. redivivus* culture. An attempt had been initiated in Bangladesh as the first time by the author (Mostafa *et al.,* 2016) for mass production of the nematode using different artificial culture media where the species was identified based on its morphological structure by microscopic examination which might have some unavoidable ambiguity. The ambiguity might be arisen due to its tine size, few apparent taxonomic characteristics with overlapping morphometric and morphological similarity with others (Handoo, 2002). Microscopic identification is

also considered unreliable because it demands considerable skills, effort and labor (OEPP/EPPO, 2004). To circumvent the obstacle, DNA-based molecular diagnosis by PCR offers an alternative sensitive method for the identification of nematodes, including *P. redivivus* (Jones *et al*., 1997; Powers *et al*., 1997; Mulholland *et al.,* 1996; Bulman and Marshall, 1997; Fullaondo *et al*., 1999). Therefore, the present study was aimed to predict the feasibility of culturing *P. redivivus* species artificially in boiled potato media, cereal media and oatmeal media by identifying the species at molecular level through application of PCR technology.

MATERIALS AND METHODS

Study location

The study was conducted at Biochemistry and Molecular Genetics (BMG) laboratory in Fisheries and Marine Resource Technology (FMRT) Discipline of Khulna University for a period of six (6) months during June, 2014 to November, 2014.

Starter production and collection

The starter of *P. redivivus* was produced from four rotten potatoes by placing it under the soil at about 10-15 cm depth near a lake located in the University area. The soil site was chosen based on the soil characteristics such as pH, moisture, temperature and depth suitable of the species for habitat (Al-imran, 2013; Mostafa *et al.,* 2016). Following checking of the potatoes at two days interval for 7-14 days, the rotten potatoes were collected from the soil and immediately taken to the laboratory of FMRT Discipline. The starter grown in the rotten potatoes were then separated through a soft wash. Prior to culture in artificial media, the starter were morphologically examined under a high resolution light microscope (Model: Carl Zeiss Micro imaging GmbH) as described by Stock and Nadler (2006) and Kumlu and Fletcher (1997) (Fig. 1).

Fig. 1: The soil nematode, *P. redivivus* morphologically identified under a light microscopic.

Artificial culture media preparation and culture of the starter

Three artificial culture media were prepared with three culture based media namely boiled potato media, cereal media and oatmeal media in association with baker's yeast in each media as described by Ricci *et al*. (2003) and Mostafa *et al.* (2016). Briefly, ten gram (10gm) of each media was mixed into 20 ml of warm water in three different plastic containers to get paste forms. Then the mixers were cooled in room temperature. After cooling, a pinch of baker's yeast was spread over the each different paste (Hechler, 1970). Finally, the mixtures were incubated at 37 ^{0}C temperature for 24 hours.

After incubation, a tiny amount of the collected starter described in the section 3.2 was placed on the three different culture media for growing. The plastic containers with culture media and *P. redivivus* cysts were then placed in incubator (Model: VS-8480SL) at 26^{0}C temperature (Mostafa *et al*., 2016). After 7-10 days of incubation, the growing *P. redivivus* started to climb up the container wall which indicated that they were ready to harvest. The *P. redivivus* was harvested from the inner wall of the container with a sterile scarp of a blade or scalpel and preserved in 10% formaldehyde following two times gentle wash with sterile distilled water (Al-imran, 2013).

Identification by PCR

DNA extraction

Simple boiling-centrifugation method was followed for the extraction of DNA by a modification of Ausubel *et al.* (1987). Briefly, about 20-25 mg of young culture *P. redivivus* sample containing low amount of liquid from each media was taken and placed into a 1.5ml sterile micro-centrifuge tube. About 5-10 mg pellet was collected from the tube following a low speed centrifugation and discarding the supernatant. Then, 250µl of sterile distilled water was added in the tube containing pellet and the tube was heated to ~100°C in a dry bath for 15-20 minutes. After cooling, the tube was centrifuged at high speed for producing a clear supernatant which were transferred carefully to a new sterile tube. DNA precipitation and washing were done by successive use of 100% ethanol and 75% ethanol into the tube. Finally, DNA pellets were solubilized with 50µl TE buffer (10mM Tris-HCl and 1mM EDTA). The DNA quality and quantity was measured photometrically at 260 nm wavelength (OD_{260}) and at 280 nm wavelength (OD_{280}) according to Aquadro *et al.* (1998).

Primer design

Two pairs of oligonucleotide primers (Table 1) were used for the amplification of DNA. The primers were retrieved from 28s ribosomal RNA genes of the *P. redivivus* species through BLAST search alignment (EU195986). First pair of the primers, Pan28s F and Pan28s R, was designed to serve as outer primer. Second pair of the primers, Pan28s NES F and Pan28s NES R, was designed as inner, nested primers that acted on amplified DNA resulting from the first pair of primers.

Table 1: Nucleotide sequences of primers used for the downstream applications

Primer name	Sequence (5'-3')	Length (bp)	Band size
Pan28s F	CTTCGGCTCAGCGCTCGGTTG	21	1131
Pan28s R	ATCTGCACCAGTGGCTGCTCC	21	
Pan28s NES F	TGACTCGTTGTGCCGGCACGG	21	1050
Pan28s NES R	AACCACTGCGACCCTCTTACAC	22	

Amplification by PCR and visualization of the amplified products

PCR thermal cycling conditions were executed with simple modifications according to the thumb rule of PCR amplification protocols described by Knebelsberger and Stoger (2012). In amplification, two step nested PCR-based assay was employed using 0.2 ml thin-walled PCR tubes separately using an automated thermal cycler (Bio Red, C-1000 thermo-cycler). In first step PCR, DNA of the sample was amplified separately with Pan28s (F/R) primer in a 25µl tube with the mixture of 1.5 mM $MgCl_2$, 0.8 µM of each primer, 0.2 mM dNTP, 2.5 units of Taq DNA polymerase enzyme and ~30-35ng DNA (20 times diluted). The cycling conditions were empirically determined consisted of an initial 25 sec at 95°C for the first round followed by 32 cycles of denaturation for 20 sec at 95°C, 30 sec of annealing at 58°C, and 45 sec of extension at 72°C. For nested PCR, 1µl of PCR product from 1st step PCR (10 times diluted) was amplified with Pan 28s NES (F/R) primer at 62°C annealing temperature for 32 cycles. After PCR, the amplified products were loaded into 1.5% agarose gel using a 100 bp DNA marker and electrophoresis was done for the migration of the PCR products. After electrophoresis, the gel was carefully exposed to UV trans-illuminator. The bands were analyzed by taking picture in a desktop computer.

RESULTS AND DISCUSSION

Sufficient quantity and purity of extracted DNA is very much essential for successful downstream application like PCR amplification. In this study, simple boiling-centrifugation method efficiently extracted DNA from *P. redivivus* cultured in three different artificial media. High resolution banding pattern was observed after gel electrophoresis visualization of the DNA's (Figure 2) which were found to be a concentration of above 600ng/µl and a purity of above 1.70 indicating enough quality and quantity for successful PCR operation (Boesenberg-Smith *et al.,* 2012).

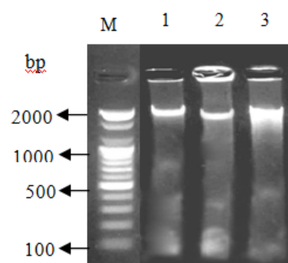

Fig. 2: DNA visualization under UV trans-illuminator after 1% agarose gel electrophoresis. Lame M: 100 bp DNA marker; Lane 1: DNA of *P. redivivus* collected from potato media; Lane 2: DNA of *P. redivivus* collected from cereal media and; Lane 3: DNA of *P. redivivus* collected from oatmeal media.

Following first PCR, a fragment of 1131-bp of band size visualized on the agarose gel (Figure 3A) indicated the successful amplification of *P. redivivus* DNA with Pan28s F/R primer that ultimately revealed the detection of the species with its ribosomal RNA (rRNA) gene. The detection was further confirmed employing 2nd step PCR with an inner fragment of nested primer Pan28s NES (F/R) where an expected band at 1050-bp position on agarose gel was visualized successfully (Figure 3B). No band was observed in the negative controls and in the reagent control amplifications (Figure 3A and B, Lane 4 and 5). An annealing temperature of 58°C for first PCR and 62°C for nested PCR were found to be optimum for amplification of the DNA's with strong band intensity or resolution.

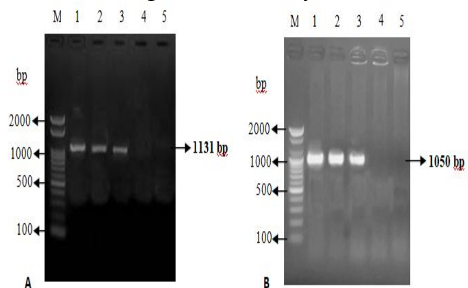

Fig. 3: Electophoretic pattern of gel electophoresis by A) first step PCR amplification using Pan28s (F/R) primer where 1131 bp of band size confirm the *P. redivivus* detection and B) nested PCR amplification of Pan28s NES (F/R) primer where 1050 bp of band size further confirm the *P. redivivus* detection. Lane M: 100 bp DNA marker; Lane 1-5 for both A and B: amplification with DNA from *P. redivivus* artificially cultured in potato media (1), cereal meal media (2) and oatmeal media (3), negative control (tilapia fish DNA) (4) and reagent control (no DNA) (5).

Once the confirmation based on PCR assay has been done, further microscopic morphological examination might not require necessarily because PCR based assay are certified as more simple, accurate and specific than the conventional microscopic one. Molecular based simple PCR technique with DNA based sequence information of *P. redivivus* genome employed in this study confirmed the presence of *P. redivivus* species in potato (boiled), cereal and oatmeal media prepared for the culture of the species artificially. In other word, the confirmation PCR assay of the study strongly revealed that the nematodes produced from the three media were *P. redivivus* species which indirectly confirmed the suitability of those media for using as artificial culture for mass production. Moreover, the outer and nested primer designed in this study as well as reagent compositions, thermal cycling conditions followed can be used further for other routine analysis of the *P. redivivus* species because of its (PCR assay) easiness, reliability and simplicity

CONCLUSION

The study found that the nematode species cultured in the boiled potato, cereal and oatmeal media were *P. redivivus* species based on PCR analysis. Accordingly, the results of this study concluded that artificial culture of *P. redivivus* would hopefully be feasible using those media. Artificial culture of *P. redivivus* in large scale using those media and application of this nematode as live feed for prawn's larval nutrition in fully/partially replacement of *Artemia* would bring a breakthrough for the prawn hatchery industry in Bangladesh.

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"Feasibility of Nematode (*Panagrellus* sp.) culture in Bangladesh and its suitability as live feed for larval rearing of giant prawn, *M. rosenbergii* (2011-14)"

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