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The Internal Transcribed Spacer (ribosomal DNA) of Fish Protozoan, *Cryptocaryon irritans*, Isolated from Cultured Seabass, *Lates calcarifer*, in Penang Waters

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Abstract

Sequence of the first internal transcribed spacer region (ITS-I) of rDNA of fish protozoan, *Cryptocaryon irritans*, isolated from local seabass was important in order to determine the intraspecific variation with the 16 isolates of *C. irritans* from Australia, Israel and the USA. A total of 321 bases of ribosomal DNA (rDNA) nucleotide sequences were determined, including 138 bases from 18S region, 170 bases from ITS-I region and 13 bases from 5.8S region. There was one variable base between isolates in the 18S region and 13 variables in the ITS-I region. The dendrogram clearly demonstrated that 16 *C. irritans* isolates tested could be divided into three distinctive groups: a) USA, b) Israel and Heron Island, and c) Australia. Comparative studies showed that the rDNA from *C. irritans* isolates from Penang have more similarities with the strain from the Laboratory at Moreton Bay, Australia as compared to the strains from USA, Israel and Heron Island.

Introduction

Aquaculture is developing rapidly in Malaysia and the 2005 production was valued at RM 1,196 million. However there is a rise in dis-

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ease incidence following the increase in aquaculture activities. Fish protozoan, *Cryptocaryon irritans*, a ciliated protozoan that gives white spot symptoms at the body and gills in marine fish was identified as the main problem in hatchery (Ali 1987; Hussin 1987; Sufian 1998). It is also known as white spot disease and can cause death especially for fry and fingerling (Colorni & Burgess 1997). Occurrence of cryptocaryoniasis is a significant problem in Malaysian marine fish culture systems particularly at hatchery and nursery stages. Eradication of *C. irritans* is difficult. The tomont or encysting stage is resistant to drugs while the trophonts are often buried deep in fish tissue. Serious financial losses have been incurred by farmers and hatchery operators due to high mortalities, high costs of control measures and treatments during outbreaks. Our own records indicated that massive mortality of fry and fingerlings of seabass were as high as 60 and 80% respectively in 2001 (Kua 2002).

In spite of cryptocaryoniasis that have been reported in various marine species and different locations throughout the world, there is no information on the extent of intraspecific variation between *C. irritans* isolated in Malaysia compared with other isolates. Analysis of the ribosomal DNA (rDNA) gene repeat particularly on region of the internal transcribed spacer (ITS) was reported useful in comparing the species and subspecies levels of many taxa (Hillis & Dixon 1991). The internal transcribed spacer (ITS) regions of rDNA gene repeat was selected as the regions are non-coding regions that evolve at a high rate (Li & Graur 1991). The ITS spacer regions have been useful for comparisons at the species and subspecies levels of many taxa (Adlard et al. 1993; Fritz et al. 1994; Goggin 1994). A study using the ITS spacer regions on *C. irritans* conducted by Diggles & Adlard (1997) showed that there are two major groupings from the dendrogram of the 16 *C. irritans* isolates obtained from Australia, USA and Israel. The first group was composed of the Heron Island, Israel and USA isolates while the second group consisted of the Moreton Bay and the laboratory isolates. Hence, the main purpose for this study was to compare the sequence of *C. irritans* isolate from cultured sea bass in Penang water, Malaysia with 6 *C. irritans* isolates from the Diggles & Adlard (1997) study in order to determine the extent of their variation in the first internal transcribed spacer region (ITS-1).

Materials and Methods

Source of parasite

Samples of up to 400-500 trophonts from Jelutong, Penang were cleaned of excess debris using a paintbrush and fixed with 70% ethanol before transferring into 100% ethanol.

Extraction of the parasite DNA

The DNA extraction method described by [Sambrook et al. \(1989\)](#) was used. Four hundred trophonts were placed into 250 µl extraction buffer (2.5mM Tris pH 8, 0.05mM EDTA, 2 mM NaCl) with 50 µl of 10% (w/v) sodium dodecyl sulphate (pH 7.2) and 5 µl of 10 mg·ml⁻¹ proteinase K. Trophonts were then crushed using a sterile needle and incubated at 56°C for 24 h, after which DNA was extracted using a phenol-chloroform method and precipitated with ethanol using standard procedures.

Amplification and gel electrophoresis of parasite's DNA

The DNA from the isolate was then amplified by polymerase chain reaction (PCR). Double stranded amplifications were performed using forward primer (GTTCCCCTTGAACGAGGAATTC) and reverse primer (CGCATTTTCGCTGCGTTCTTC) in 50 µl reaction volumes. These primers amplify approximately 230 bases of the 3' end of the 18 S region and the entire ITS-I region of the rDNA gene of *C. irritans* ([Diggles & Adlard 1997](#)). Thermal cycling was performed on Gene Amp PCR System 2400 Perkin Elmer thermal cycler with the following program: denaturing 95°C for 60 s, annealing 55°C for 30 s, extension 72°C for 60 s and a total of 30 cycles. At the completion of PCR, 2 µl of the amplified products were electrophoresed through a 1.5% agarose gel with a DNA molecular weight standard in the presence of ethidium bromide, and viewed under UV light.

Purification and sequencing of parasite's DNA

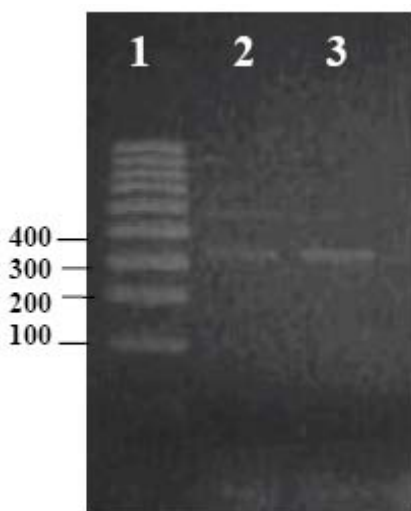
Amplified products were purified using QIAquickTM Gel Extraction Kit (QIAGEN). The purified products were then sent for custom service (BioSynTech Sdn. Bhd) for sequencing. The other six *C. irritans* isolates sequences ([Table 1](#)) compared with the present study sequence were aligned using the Clustal-W program downloaded from the internet (<http://www.clustalw.genome.ad.jp/sit.b>). Sequences were aligned using clustal w option in Bioedit software ([Kumar et al. 1994](#)) and Molecular Evolutionary Genetics Analysis (MEGA), Version 4.0 ([Tamura & Neil](#)

1993) was used to assess the levels of pair-wise nucleotides variation and to determine nucleotides substitution at overall codons position for each taxon. All phylogenetic analyses were executed using MEGA 4.0 version. The bootstrap value was calculated using NJ with 1000 replicates and according to Kimura 3 parameter.

Table 1. Origin of the six *C. irritans* isolates

Isolate	Locality collected	Date isolated	Host species	References
1. USA	Georgia, USA	1989	Poecilia hybrids	Diggles & Adlard 1997
2. Israel	Elat, Israel	1993-1994	Cultured <i>Sparus aurata</i>	
3. Heron	Heron Island, Australia	1993-1994	Wild <i>Gymnocranius audleyi</i>	
4. Moreton Bay	Moreton Bay, Australia	1994-1995	Wild <i>Acanthopagrus australis</i>	
5. Laboratory	From Moreton Bay then laboratory propagation	1993-1994	<i>Lates calcarifer</i> used for serial passage	
6. Penang	Penang, Malaysia	2000	Cultured <i>L. calcarifer</i>	Present study

Results and Discussion



A total of 321 bases of ribosomal DNA (rDNA) nucleotide sequence were determined from the cultured seabass (Fig. 1). The rDNA consisted of 138 bases from 18S region, 170 bases from ITS-I region and 13 bases from 5.8S region (Table 2). Sequence divergence between the *C. irritans* isolated from local seabass was evident at 12 positions over the amplified region (Fig. 2). In the partial 18 S region there was a single variable base at position 47, for which the encoding nucleotide was cytosine which was

Figure 1. Agarose gel electrophoresis of the products by PCR amplification using primers specific to the ITS-I region of the rDNA gene of *C. irritans* and the nucleic acids extracted from *C. irritans* trophonts as template. Lane 1: 100 bp molecular weight marker, Lanes 2 and 3: PCR product with 1 μ l of DNA as template.

similar to the Moreton Bay and Laboratory isolates. However, adenine was seen in the Israel and USA isolates and thymine from the Heron Island isolates. In the ITS-1 region there were 13 variable bases between isolates. As for the 5.8S region, no differences were obtained.

Table 2. Sequencing of rDNA of *C. irritans*

Region	Nucleotide base	Sequencing of nucleotide base (5' – 3')
18S	138	CTACCGATTTCGAGTGATCCGGTGAACCTTCTGGACTGCG CTAACACTAGTTATGTGCGGGAAGTTAAGTAAACCACTT CACTTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTA GGTGAACCTGCGGAAGGATCA
ITS-1	169	TTAACACAATTAAGATCAAACCTAAAAATTTATTCTGAT GTATTGAGATCTGATAATTTTAAATTATCAATCTCAAATT TTTACAAATTTATTTTAATAATAAATATCATTAAGTTAAT TAAATTAATAAAGAAAATTTTCAACGGTGGATATCTTG GCTCCCATAAC
5.8	13	GATGAAGAACGCA
Total	321	

Analysis of sequences with NJ (Kimura 3-parameter) showing two major groupings namely: a) isolates from Moreton Bay, the laboratory and Penang, and, b) isolates from Heron Island, Israel and USA (Fig. 3). It shows the Penang isolates clustered with the Australian isolates but clustered closely to isolates from Laboratories A and B. Ribosomal DNA sequence analysis revealed consistent genetic variations in the ITS-1 region between the *C. irritans* isolates collected from the Penang waters, Moreton Bay, Heron Island, Israel and the USA. The magnitude of sequence divergence between the Australian isolates was greater than their divergence between the Israel and USA isolates. The present comparative studies show that the rDNA from Penang isolates of *C. irritans* has more similarities with the strains from the Laboratories (A & B) and Moreton Bay, Australia as compared to the strains from USA, Israel and Heron Island.

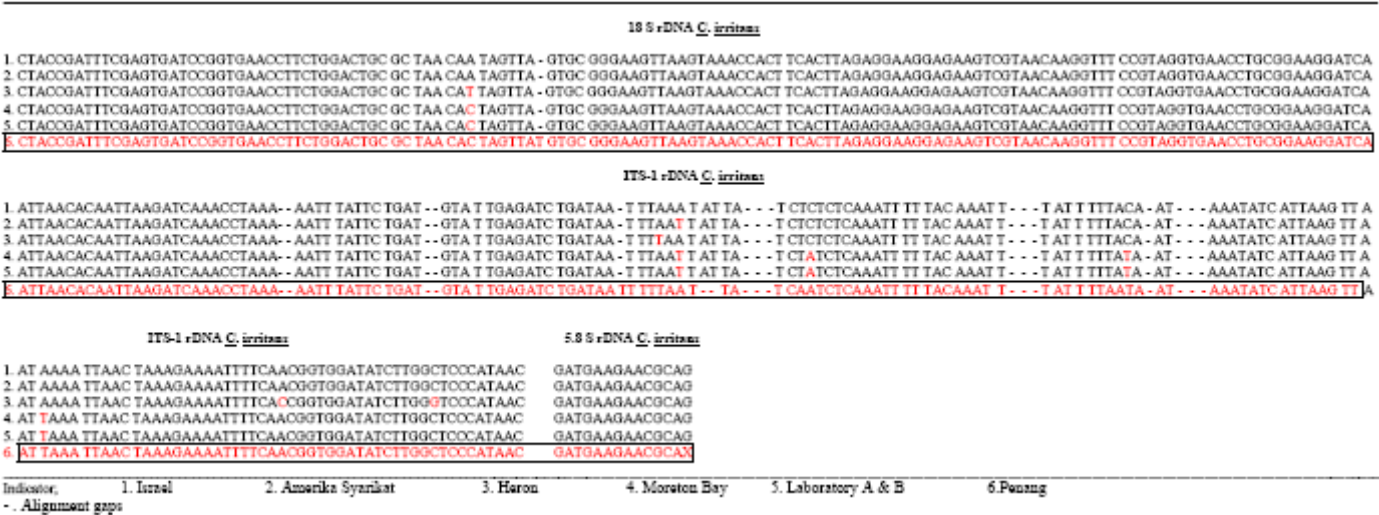


Figure 2. Comparison of the nucleotide sequence of rDNA (18S, ITS-1 dan 5.8 S) of *C. irritans* isolated from seabass in Penang waters with 5 isolates from [Diggles & Adlard \(1997\)](#) study.

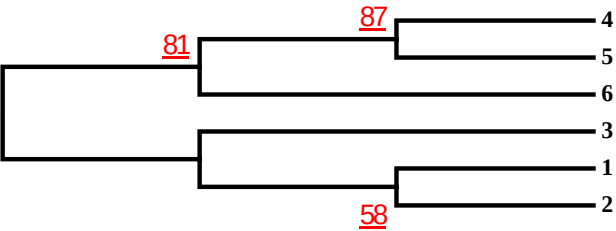


Figure 3. NJ with 1000 replicates and according to Kimura 3 parameter of the sequences of ITS-1 rDNA of *C. irritans*. Note: 1. Israel, 2. Amerika Syarikat, 3. Heron, 4. Moreton Bay, 5. Laboratories A & B, 6. Penang

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