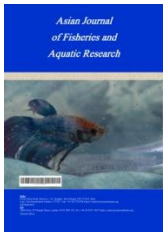

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# Internal Transcribed Spacer (ITS) Region as Molecular Marker for Parasite Identification in Aquaculture: A Review

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.*

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## ABSTRACT

Molecular technologies may be quicker and more accurate than the culture, serology, and histology procedures that have historically been employed to identify fish parasites. By using molecular diagnostic methods, parasites can be found in asymptomatic fish, allowing disease outbreaks to be stopped. This review discusses the use of Internal Transcribed Spacer (ITS) markers to detect parasite in aquaculture and their potential applications are discussed. It was found that it is a more reliable marker and species identification in combination with other DNA markers. ITS region are highly polymorphic non - coding region with sufficient taxonomic units. Thus, sequences can be separated at the species level. though ribosomal ITS as a universal barcode marker for parasite identification is still hampered by few limitations, the ITS will remain as the key choice for parasite identification. It remains the most important option for parasite identification. Many studies have been carried out using ITS markers for parasites identification such as cestode, nematodes, *Pseudoterranova* spp., Anisakis type I, Diplostomum, Gyrodactylids *Hysterothylacium* sp, Glochidia larvae. This identification of parasites are taken from the species Rainbow trout (*Oncorhynchus mykiss*), Argentinesea bass, (*Acanthistius patachonicus*), and other samples from various places.

*Keywords: ITS (Internal Transcribed Spacer); molecular marker; parasite; aquaculture.*

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## 1. INTRODUCTION

Aquaculture plays an important role in providing animal protein for the global community, and Indonesia is no exception [1]. Therefore, fisheries are currently in great demand by various groups and the demand for its products continues to increase. However, in the production of fisheries, there are many obstacles, including parasitic attacks. This parasite can cause significant production losses by interfering with fish health. The high pathological effect of this parasite can cause death in the host [2].

This is partly because the risk factors associated with aquaculture production in infectious aquatic environments are analyzed and the reasons for the absence of zoonotic parasites in trout aquaculture are pinpointed. Based on the analysis recommendations for management practices securing parasite freedom in cultured fish are given [3]. One of them based on research revealed that the life cycle of *P. cattani* mainly involves demersal and benthic organisms, with preferences marked by the surrounding environment [4].

Fish disease management is a major concern for aquaculture activities. The ability to quickly identify the presence of pathogenic organisms in fish would have significant economic benefits. An additional benefit will also be obtained if concentrations of infectious organisms can be determined in fish or the environment so that changes in the abundance of these pathogenic organisms can be monitored. The development of a system that can evaluate fish parasites and disease carriers in an introduced area will also assist in the development of aquaculture management programs [5].

In controlling parasites, it is still hampered due to difficulties in identifying them. Most parasitic eggs have very similar morphological characteristics, so identification down to the species level is not always possible with a microscope [6]. The fact that certain species of parasite infect more successfully than other species of the same genus is also true in the case of treatment where some strains show sensitivity to certain drugs while others are resistant to the same drug. Therefore, studying and identifying organisms molecularly is important to know their taxonomic status, whether the species are distant or close relatives [2].

For example parasites species *Hysterothylacium amoyense* was the most common species (prevalence 47.2%, mean intensity 14.9 ± 17.1). *Hysterothylacium thalassini* and *R. lophii* were reported in Japanese freshwater fish for the first time. Two distinct genotypes of A. typica (3.4% overall prevalence; mean intensity 1.7 ± 0.9) were discovered in the South China Sea for the first time [7]. Therefore, one solution is to carry out molecular identification which makes it possible to detect the unique species-specific nucleotide sequences from the internal transcribed spacer (ITS) for a particular species [6].

“The development of molecular techniques for taxonomic analysis of parasites has led to great improvements in their precise identification and factuality. These molecular techniques, in particular the use of molecular markers, have been used by researchers to identify and validate parasites. Although, improvements in marker detection systems in particular rDNA elements such as 18S, ITS, and 28S used in parasites, among others, monogeneans have allowed great progress to be made in recent years in various countries” [8].

The ITS (Internal Transcribed Spacer) region is widely used as a molecular marker for taxonomy and phylogenetic analysis [9]. The advantages of ITS over other non-coding regions are: [1] Multiple copies of rRNA genes; [2] Can be used for PCR amplification with multiple universal primers for different organisms; [3] The size of the fragment is not long to facilitate sequencing; and [4] high variation at the generic and species level due to the presence of nucleotide polymorphisms or insertions/deletions in sequences [10,11]. So this journal was compiled to review the application of the ITS region as a molecular marker for the identification of parasites in aquaculture.

## 2. WHAT IS ITS?

“One of the phylogenetic studies with a molecular approach is to use DNA characters from target organisms. DNA characters are known to be more consistent in establishing phylogenetic relationships than morphological characters” [12]. One of the molecular characteristics that can be used is the nuclear genome. The nuclear genome or nucleus that is often used to conclude a phylogenetic is ribosomal DNA called rDNA. rDNA is a region of the genome that codes for ribosomal RNA [13].

The highly conserved coding DNA region (18S, 28S rDNA) is a major evolutionary region that is often used for comparison at the species and related genera levels. Each rDNA unit in a set of chromosomes has a coding region of 18S, 5.8S, and 28S flanking ITS1 and ITS2 [14]. The 18S rDNA gene, along with the two ITS regions and the 5.8S rDNA gene, has a total length of 2600 bp, apart from the 28S rDNA gene which has a length of 3300 bp. Molecular markers that can be used for taxonomic and phylogenetic studies at the species level for Zingiber are molecular markers ITS [15].

ITS in the 18S-28S nuclear rDNA region is the main focus for use in phylogenetic reconstruction. This is because the ITS region has a high level of variation compared to other regions in the small subunit and large subunit rDNA [14]. Baldwin [16] added, that the ITS region is small, which is approximately 700 base pairs, and has many copies in the core genome. This character causes the ITS region to be easily isolated, amplified, and analyzed. “Provides extensive phylogenetic information in prokaryotes and eukaryotes, collectively known as ribosomal DNA (rDNA), consisting of coding regions (18S, 5.8S, and 28S) and non-coding regions 2 internal transcription spacers (ITS-1, ITS-2) and one non-transcriptional spacer (NTS)” [17].

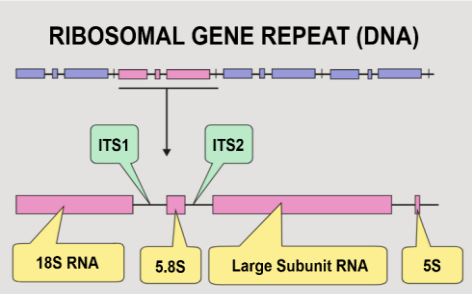


**Fig. 1. Ribosomal subunits and internal transcribed spacer region [18]**

The rDNA units can be seen in Fig. 1. In the section between the 18S region and the 5.8S region, there are several hundred base pairs of DNA called ITS1 and between the 5.8S and 28S regions, there are ITS2 regions. The ITS region has advantages compared to other target molecular regions, including having a high level of sensitivity because it has about 100 replicates in the genome. The ITS region also has a high rate of evolution and is present in all eukaryotic rDNA genes [19].

## 3. MOLECULAR IDENTIFICATION OF FISH PARASITES WITH ITS

Diseases of fish are one of the most common problems in aquaculture and export-import businesses. Diseases do not only attack humans but also attack livestock and plants, as well as fish that live in water. attack diseases caused by parasites, fungi, viruses, and those caused by some kind of chemical substance in the form of pollution so that the fish's organs are damaged and disease arises [20].

Parasitism in fish is an ecological coexistence between two organisms in close contact. Some parasites form a balance of host-parasite relationships to become encysted or dormant until conditions are favorable for reproduction [21]. Fish encounter many disease agents, including parasites, in their environment. Parasites are mechanically and functionally harmful to living things and also have an exploitative effect on preying, eating host nutrients, and interfering with respiration. This parasite reduces demand in the market and causes huge economic losses, weakens and even kills fish by taking advantage of their food [22].

Some of the parasites that are often found attacking fish in aquaculture are Platyhelminthes of the Trematoda class, both from the Monogenea and Digenea subclasses. Platyhelminthes is one of the largest which includes a group of parasites commonly found on the gills, skin and fins of fish, lower aquatic invertebrates and some other amphibian vertebrates [8].

Digenean genera that affect mainly humans and livestock are best studied to date based on immunology, epidemiology and other medical fields [23]. Recently, efforts have been made toward understanding the origin, diversification and phylogenetic interrelationships. The other genus Hysterothylacium has a cosmopolitan and circumpolar distribution and is mainly found in temperate and cold waters [24].

Another case study is the discovery of the parasite *Anisakis* sp. the prevalence of infection was generally higher in bigger fish than in smaller ones [25]. Other parasites are *Diplostomum* spp. sampled in Spain: D. spathaceum with only one haplotype shared with adult isolates from central and northern Europe. No specific geographic pattern of the distribution

**Table 1. ITS Primary Applications on the Identification of Various Eukaryotic Parasites**

| No. | Host | Origin | Parasites | ITS Primer | References |
|---|---|---|---|---|---|
| 1. | Rainbow trout (*Oncorhynchus mykiss*) | Denmark | Cestode | ITS1-5.8SITS2 (forward primer-BD1 (5′ -GTC GTAACA AGG TTT CCG TA-3′ ) and reverse primer-BD2 (5′-TAT GCT TAA ATT CAG CGG GT-3′ )) | [30] |
| 2. | Rainbow trout (*Oncorhynchus mykiss*) | Denmark | Nematodes | NC5 (5′ -GTA GGT GAA CCT GCG GAA GGA TCA TT-3′ ) and NC2 (5′ -TTA GTT TCT TTT CCT CCG CT-3′ ) primers | [30] |
| 3. | Argentinesea bass, (*Acanthistius patachonicus*) - Argentine sand-perch, Pseudopercis semifasciata - flounder, Paralichthyspatagonicus | Argentine coast | Nematodes | NC5 (5_-GTAGGTGAACCT GCGGAAGGATCATT-3_) and NC2(5_-TTAGTTTCTTTTC CTCCGCT-3_) | [31] |
| 4. | Sea lion *O. flavescen*s | Antarctic | *Pseudoterranova* spp. | primersNC5 (5-GTAGGTGAACCTGCGGAAGGATCATT) and NC2(5-TTAGTTTCTTTTCCTCCGCT-3), | [31] |
| 5. | Threadfin bream *Nemipterus japonicus* | China | Ascaridoid nematodes | A (forward: 5'-GTC GAA TTC GTA GGT GAA CCT GCG GAA GGA TCA-3′) and B (reverse: 5'-GCC GGA TCC GAA TCC TGG TTA GTT TCT TTT CCT-3′) | [32] |
| 6. | *Katsuwonus pelamis, Auxis thazard* | Southern Makassar Strait, Indonesia | Anisakis type I | NC5 (5´-GTAGGTGAACCTGCGGAAGGATCATT-3´) and NC2 (5´-TTAGTTTCTTTTCCTCCGCT-3´) | [25] |
| 7. | Pseudochondrostoma willkommi and Salmo trutta | Different regions in Spain | Diplostomum | D1 (forward: 5′-AGG AAT TCC TGG TAA GTG CAA G-3′) and D2 (reverse: 5′-CGT TAC TGA GGG AAT CCT GGT-3′) | [26] |
| 8. | Tilapia | Mexico and Kenya | Gyrodactylids | ITS1-fm (5'-TAG AGG AAG TAC AAG TCG -3') and ITS2-rm (5'-GCT YGA ATC GAG GTC AGG AC-3′) internal primers ITSR3A (5′-GAG CCG AGT GAT CCACC-3′) and ITS4.5 (5′-CAT CGG TCT CTC GAACG-3′) | [33] |

| No. | Host | Origin | Parasites | ITS Primer | References |
|---|---|---|---|---|---|
| 9. | *Lophius piscatorius, Zeus faber, Engraulis encrasicolus, Merluccius merluccius, Mullus barbatus, Trachurus trachurus, Trachinus draco, Trigla lyra, Conger conger, Phycis phycis,* and *Pagellus erythrinus.* | Southern Mediterranean Sea (Southern Italy) | *Hysterothylacium* sp. | NC5 primer (forward: 50-GTA GGT GAA CCT GCG GAA GGA TCA TT- 30) and the NC2 primer (reverse: 50-TTA GTT TCT TTT CCT CCG CT-30) | [24] |
| 10. | *Salmo trutta* | Northand Central European freshwater | Glochidia larvae | Primer sequences were ITS-1-F (for-ward): AGACTGGGTTGCGGAGGT; and ITS-1-R (reverse): CGAGTGATCCACCGCTTAGA. | [28] |

of the novel haplotypes was found. This first molecular exploration of the diversity of *Diplostomum* spp. in southern Europe indicates much lower species richness compared with the northern regions of Europe [26].

In addition, the application contains parasites found in morpho-molecular characterization of *Gyrodactylus* parasites of farmed tilapia and their spillover to native fishes in Mexico [27]. Apart from that phylum, there are also mussels (Unionoida) and parasitic glochidia larvae from host fish gills: a molecular key to the North and Central European species with identification that can be applied to infection rates as low as three glochidia per gill arch to inhibit parasite growth early in life and prevent early spread [28].

Molecular markers are increasingly being used and are highly relevant to fish diseases. The complete genome sequencing of the pathogen enables major advances in studying the biological properties of the parasite and improves diagnosis for the control of pathogenic parasites. Using nucleic acids as targets, and new methods for analyzing polymorphisms in these nucleic acids, can improve the sensitivity, specificity, and speed of diagnosis and can examine the relationship between the genotype and phenotype of various pathogens. Advances in these techniques aid epidemiological studies as well as identify the cause of disease outbreaks or the presence of pathogens. Therefore, molecular biology can become a routine tool in the search for better methods of diagnosis and control of fish pathogens and the epidemiology of infectious fish diseases. The ITS region is one area that can be used as a molecular marker that can be used for this [5].

Many studies on the identification of parasites using the ITS region have been carried out. Several ITS primers are designed to analyze the region but there are some obstacles. Processing high-throughput data for community analysis involves a series of steps from high quality filtering to taxonomic mapping, with dedicated computational tools for each step. For bacteria and archaea in particular there is a wide range of freely accessible, standalone bioinformatics applications for all aspects of analysis. So we need software packages that combine many applications to help researchers automate the processing of data [29].

The primer sequences that have been used to identify various parasites on various hosts can be seen in Table 1.

## 4. LIMITATIONS OF ITS IN AQUACULTURE

However, it is generally accepted that ITS copies within the genome can be highly homogeneous due to coordinated evolution [34,35]. Importantly, there are observed a low level of ambiguity in sequence data. Nevertheless, the limitations mentioned here also apply to mitochondrial markers [36].

Inappropriate homogenization of rDNA repeats can be compared with heteroplasmy of different mitochondrial haplotypes in a single organism [37,38], and rDNA pseudogenes are the nuclear copies of mitochondrial genes [39]. Therefore, one should be aware of this complication with either ribosomal or mitochondrial markers, although they are not common enough to seriously affect their broad applicability [38].

Moreover, “there are several nucleotide sequence databases in the world. GenBank is one of the most important and the most influential nucleotide sequence database that can be accessed publicly. As GenBank is constructed by direct submissions from individual laboratories, some of the sequence data may be unreliable due to incorrect identification of the organism from where the submitted sequence originates” [40].

However, “as dedicated databases are built in various locations, initial morphological identification at the genus level must be completed before any gene can be applied. For this reason, ribosomal ITS as a universal barcode marker for fungi is still hampered by several limitations” [41]. Moreover, ITS remains the primary choice for identification.

## 5. CONCLUSION AND PROSPECT

The multiple rRNA gene copies, suitability for PCR amplification with numerous universal primers for various organisms, average sequence length for sorting, and high degree of variation at the generic and species levels due to frequent nucleotide polymorphisms or insertions/deletions in sequences are just a few benefits of ITS markers. So it is effective to be applied to detect parasites in fish. Many studies have been carried out using ITS markers, including Rainbow trout (*Oncorhynchus mykiss*), Argentinesea bass, (*Acanthistius patachonicus*), and other samples from various places.

The ITS region is a highly polymorphic noncoding region with enough taxonomic units to segregate sequences at the species level. Although ribosomal ITS as a universal barcode marker is still hampered by several limitations, ITS remains a major option for parasite identification in aquaculture in future.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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