

Detection Molecular of Putative 18S rRNA Gen Protozoa *Trichodina* sp. Infected Larvae Gurami (*Osphronemus gouramy* L) in Balai Benih Ikan Kutasari Purbalingga Central Java

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Abstract

Trichodina spp. are ectoparasitic pathogens of ciliata group that commonly infect both freshwater and marine fish, including gouramy fish. As a result of infection of *Trichodina* spp. this will lead to inhibition of fish growth and decreased fish production, resulting in low fish selling value. The rate of occurrence of *Trichodina* spp. that infects gurami can reach 100%. Research has been conducted to determine which one *Trichodina* spp. Protozoa that infects the gouramy seeds of BBI (Fish Seed Center) Kutasari Purbalingga following detection of 18S RNA gene. Gene detection method used in this research is Polymerase Chain Reaction (PCR) is a technique of DNA synthesis and amplification in vitro. This research is done following these methods: (1) sampling of Gurami fish with purposive sampling which obtained from BBI Kutasari Purbalingga, (2) isolation of *Trichodina* spp., (3). Preparation of *Trichodina* spp. sample and its identification, and (4). Molecular character observation following detection of 18S rRNA gene. This study obtained 10% percentage of detection of 18S rRNA genes of the species of *Trichodina paraheterodontata* that infect on the gouramy fish of Purbalingga. The percentage rate of detection of these genes is low when compared with the results of the detection of 18S rRNA *Trichodina paraheterodontata* gene that infects gouramy fish in Banjarnegara.

Keywords: *Trichodina* spp., *T. paraheterodontata*, Gurami Fish, Gen 18s RNA.

INTRODUCTION

Trichodina spp. is a pathogenic ectoparasite from the ciliate group that commonly attacks freshwater and marine fish (Margolis & Arthur, 1979). This ectoparasite is \pm 50 nm in size, round in shape, with a bell-shaped lateral side, has a denticle ring for attachment and has cilia around its body. Ectoparasites are known to cause mortality of goldfish (*Cyprinus carpio*), Mujair fish (*Oreochromis mossambicus*) in South Africa (Van *et al.*, 1984; Dana *et al.*, 2002), Rainbow trout (*Salmo gairdneri*) and Salmon (*Salmo salar*) by attacking or infecting the skin and gills of fish in the UK (Mc Ardle, 1984 in Dana *et al.*, 2002). One type of fish is often infected by *Trichodina* spp. is gouramy. The disadvantages of ectoparasite infection are not as big as those caused by infection with other organisms such as viruses and bacteria, but ectoparasite infection can be one of the predisposing factors for infection with more dangerous pathogenic organisms (Rokhmani, 2009).

Trichodina paraheterodontata was first identified by Duncan (1977), and has been known to attack Mujair fish (*Oreochromis mossambicus*) (Duncan 1977; Van As & Basson 1986; Bondad-Reantaso & Arthur 1989), Tilapia (*Tilapia rontoli*), Sepat (*Trichogaster tricopterus*) (Bondad-Reantaso & Arthur 1989), Tilapia (*Oreochromis niloticus*) (Natawidada *et al.*, 1986) and Goldfish (*Cyprinus carpio*) (Basson *et al.*, 1983; Van As *et al.*, 1984; Albaladejo & Arthur 1989).

Gouramy fish (*Osphronemus gouramy* Lacepede) is a type of freshwater fish from the Osphronemidae family and one of the types of freshwater cultivated fish that is widely developed by farmers. Gouramy as a cultivated fish has been chosen by many farmers because it is able to reproduce naturally and is relatively easy in its cultivation. Meanwhile, as a consumption fish, gouramy is quite popular because of its delicious and savory taste. Market demand for gouramy continues to increase at a high enough price. Economically, the selling price of gouramy is relatively stable (Tim Lentera, 2003). Purbalingga is one of the districts that has gouramy production as a regional commodity. This is evidence that this district is one of the centers for gouramy cultivation in the province of Central Java.

Morphological characters are commonly used for taxonomic studies of a species because different variations between individuals often indicate different species (Darwin, 1854; Pilsbry, 1916; Permadi, 2014). (Syoc, 2010) noted that Darwin saw species that varied morphologically as variations within a species to encourage speciation. Investigations down to the genetic level can help in distinguishing 2 species that cannot be resolved morphologically. Morphological parameters of *Trichodina* spp. including body diameter, denticle ring diameter, adhesive disc diameter, number of denticles and membrane width of *Trichodina* spp. The problem that arises is the large number of biased

species (cryptic species) and this problem can be solved using molecular characters, however to identify an organism using molecular techniques has not been widely used. Several molecular techniques have been developed to trace the presence of a specific DNA sequence from a particular organism, for example the use of the 18S rRNA gene sequence to determine the relationship of an organism in general morphological features.

The 18S rRNA gene is often used for phylogenetic studies because it has conserved areas. The unique and conserved region can be used to characterize the organism concerned, so that it becomes the signature sequence of an organism. Likewise, the data on the nucleotide base of the 18S rRNA gene allows it to be used in constructing a phylogenetic tree that shows the ancestry and kinship of an organism (Suwanto, 2011). Molecular research can complement the morphological identification of organisms. Partial amplification of the 18S rRNA gene can be used to specifically identify *Trichodina paraheterodonta* (Tang *et al.*, 2013).

Research Problems are what are the morphological characters of *Trichodina* spp. morphometrically isolated from gouramy at BBI Kutasari Purbalingga and What is that infected gouramy larvae at BBI Kutasari Purbalingga using 18S RNA gene detection. The objectives to detect the *Trichodina* spp. 18S RNA gene. which infected larvae gouramy. Benefit providing information on the results of the detection of the putative 18S RNA gene of *Trichodina* sp. which infected larvae gouramy

METHODS

The tools used in this study were sample containers, tissue, gloves, masks, dissection kits, micropipettes, Erlenmeyer, digital scales, autoclaves, refrigerators, freezers, PCR machines (PQLAB), electrophoresis kits, slide glass, vortex, microscopes, Cavity slide, centrifuge and water bath, camera. Tool specifications are in Appendix The materials used in this study were *Trichodina* sp. from Gouramy fish taken from BBI Kutasari Purbalingga, NLS (Nuclei Lysis Solution), Rnase Solution, PPS (Protein Precipitation Solution), Isopropanol, DNA Rehydration Solution, 70% alcohol, TBE 1x, Agarose, EtBr, distilled water, gloves, Promega Kit, Master mix, PCR mix (Primer F, Primer R, template DNA, ddH₂O), Microcrystal tips rack, Microcrystal tips 0.5-10ul, Yellow tips 200 ul, Blue tips 1000 ul, Microtube 1.5 ml.

The implementation of this research was carried out from May to July 2017. Sampling of the research will be carried out at the Kutasari Purbalingga Fish Seed Center. Research observations will be carried out at the Parasitology Laboratory and the Genetics and Molecular Laboratory, Faculty of Biology, Jenderal Soedirman University.

Sampling Technique

This research will be conducted using a survey method with purposive sampling technique. The

gouramy which was taken from BBI Kutasari Purbalingga was put into plastic container then taken to the Laboratory of Parasitology, isolated and taken ectoparasite species *Trichodina* spp. only, whose characteristics are known by reference. Sampling was done once. Samples were taken in genomic DNA isolation at the Molecular Laboratory of the Faculty of Biology, Jenderal Soedirman University and carried out DNA extraction and PCR. *Trichodina* was isolated from the caudal fin of Gurami (*Osphronemus goramy*) to obtain its DNA through the extraction method, DNA amplification using PCR (Polymerase Chain Reaction) techniques using universal primers. Then, DNA Visualization uses electrophoresis techniques.

Molecular Identification

Genomic DNA Isolation of *Trichodina* sp. DNA *Trichodina* sp. extracted from the gouramy fin tissue. DNA extraction method with working steps:

1. Pellets (*Trichodina* sp.) are added 600 µl of Nuclei lysis Solution into tube 1.5 (homogeneous for 10 seconds)
2. 3 µl of RNase was added to the tissue which had been given Nuclei Lysis solution after that it was homogenized
3. Incubate in a water bath at 37 ° C for 5-10 minutes
4. Added 200 µl of Protein Precipitation Solution
5. Vortex for 10 seconds
6. Incubate in the refrigerator at -20 ° C for 5 minutes
7. Centrifugation at 4 ° C at 13,000 rpm for 4 minutes
8. The supernata at the top was taken and then transferred into a new 1.5 tube
9. Add 600µl of isopropanol to the tube that has been moved (homogeneous by means of back and forth)
10. Centrifugation 13,000 for 1 minute at 4 ° C
11. Supernata at the top are removed
12. The supernata below are added with 600 µl of 70% Ethanol (homogenized)
13. Centrifuge at 4 ° C 13,000 rpm for 1 minute
14. Ethanol removed and dried for 30 minutes
15. Add 100 µl of DNA Rehydration solution then incubate at 20 ° C (over night)

Amplification of the 18S rRNA gene using PCR technique

The amplification of the 18S rRNA gene from *Trichodina paraheterodonta* was carried out using PCR techniques. The primers used were Forward primers (5'-AAC CTG GTT GAT CCT GCC ATG-3') and Reverse primers (5'-TGA TCC TTC TGC AGG TTC ACC TAC-3') which produced 600 bp DNA amplicons (Tang *et al.*, 2013). The gene amplification procedure uses the PCR technique with the following steps:

1. Preparation of PCR mixture (PCR mix) in a volume of 15 µl in an eppendorf tube (1.5 ml) with the composition in Table 1.

Table 1. PCR mixture composition

No.	Metrials	Volume (µl)
1	Master Mix	7.5
2	Forward Primer (15 µM)	0.25
3	Reverse Primer (15 µM)	0.25
4	ddH ₂ O	4
5	Template DNA	3
	Total	15

* Does not take concentration measurements.

- Amplification was carried out on a Thermocycler (PeqLab) machine with PCR conditions used to amplify 35 cycles of 18S rRNA genes, as follows: pre-denaturation stage at 95°C for 5 minutes, denaturation at 95°C for 1 minute, annealing at 61°C for 1 minute, extension at 72°C for 1 minute, and post-extension at 72°C for 5 minutes (Folmer, 1994).
- Visualization of DNA amplicons using agarose gel electrophoresis technique (1%) resulted in DNA amplicons measuring 600 bp.

Data Analyses

Positivity of the DNA amplicon of 18S rRNA gene from the study sample

RESULT AND DISCUS

The morphology of the body parts of *Trichodina paraheterodontata* shows several features and varying shapes of denticles, namely having a blunt blade apex tip and a straight tapered but thicker denticle ray shape. *Trichodina paraheterodontata* research results were found in gouramy seeds on the tail fin. According to Basson & Van As (2006), *Trichodina paraheterodontata* is found mostly on the skin surface and is rarely found in gills. Meanwhile, research by Rokhmani *et al.* (2015) on the type of *Trichodina* sp. The gouramy seeds in Banyumas, Banjarnegara and Purbalingga are *Trichodina* sp. those found were *Trichodina nobilis*, *Trichodina reticulata*, *Tichodina acuta*, *Trichodina paraheterodontata*, *Trichodina magna*, *Trichodina pediculus*, and *Trichodina nigra* (Rokhmani *et al.*, 2015).

Trichodina spp. are spherical and supported by a rigid ring of interconnected discs called chitinoids (Durborow, 2003). The ability to infect *Trichodina* spp., In fish seeds is related to the structure and morphology of the denticle ring of

Trichodina spp. (Kabata, 1985). The structure and morphology of the denticle rings are the morphometric characters of *Trichodina* spp. which can be used to determine the type of *Trichodina* spp. One of the morphometric size characters of *Trichodina* spp. in the form of a denticle tip. The denticle tips of *Trichodina* spp. There are two types, namely sharp and blunt (Windarto *et al.*, 2013).

Morphological characteristics of *Trichodina* spp. In gouramy seeds, there are known differences in the characteristics of the size of the body diameter, denticle ring diameter, adhesive disc diameter, number of denticles and membrane width. Types of *Trichodina* spp. Research results can be identified based on Duncan (1977); Padua *et al.*, (2012); Lom (1961) in Basson (2010); Albaladejo & Arthur (1989) in Dana *et al.*, (2002); Van & Basson (1986) in Woo (2006). According to Duncan (1977); Padua *et al.* (2012), *Trichodina paraheterodontata* has a characteristic body diameter of 71-106 µm; denticle ring diameter 26-37 µm; adhesive disc diameter 47-63 µm; the number of denticles 20-27; membrane width 2.7 µm. Meanwhile, according to Van and Basson (1986) in Woo (2006), *Trichodina paraheterodontata* has a characteristic adhesive disc diameter of 38-82 µm; denticle ring diameter 23-51 µm; the number of denticles 20-30. According to Albaladejo & Arthur (1989) in Dana *et al.*, (2002), the type of *Trichodina paraheterodontata* has a characteristic body diameter of 53.0-71.5 µm (57.9 µm ± 3.7); denticle ring diameter 24.5-41.2 µm (35 µm ± 4.2); adhesive disc diameter 44.0 - 61.0 (52.1 ± 5.4); the number of denticles 20-24 (21.8 ± 1.2) and a membrane width of 4.0 -6.5 µm (5.5 µm ± 0.6).

The results Amplification of the 18S RNA gene in *Trichodina* sp. from Purbalingga was done using the PCR method. The interpretation can be seen in Figures 1 to 4. Each band on the gel indicates the presence of *Trichodina paraheterodontata*.

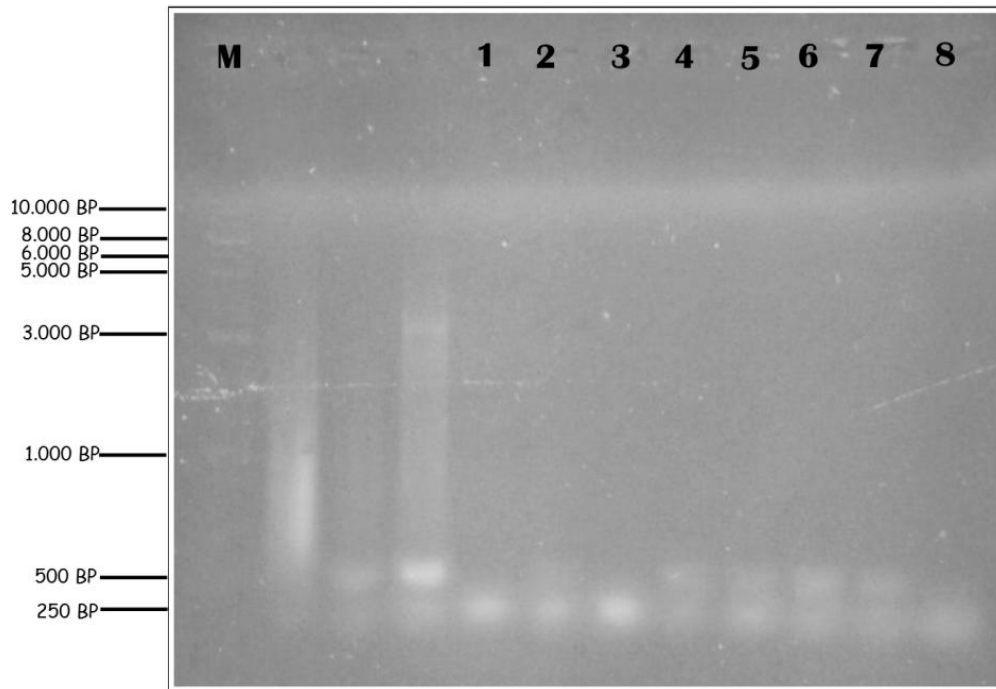


Figure 1. Results of DNA extraction from *Trichodina* spp. on Gouramy fish at BBI Kutasari Purbalingga.
Note : (M = Marka, 1 = Purbalingga 1, 2 = Purbalingga 2, 3 = Purbalingga 3, 4 = Purbalingga 4, 5 = Purbalingga 5, 6 = Purbalingga 6, 7 = Purbalingga 7, 8 = Purbalingga 8).

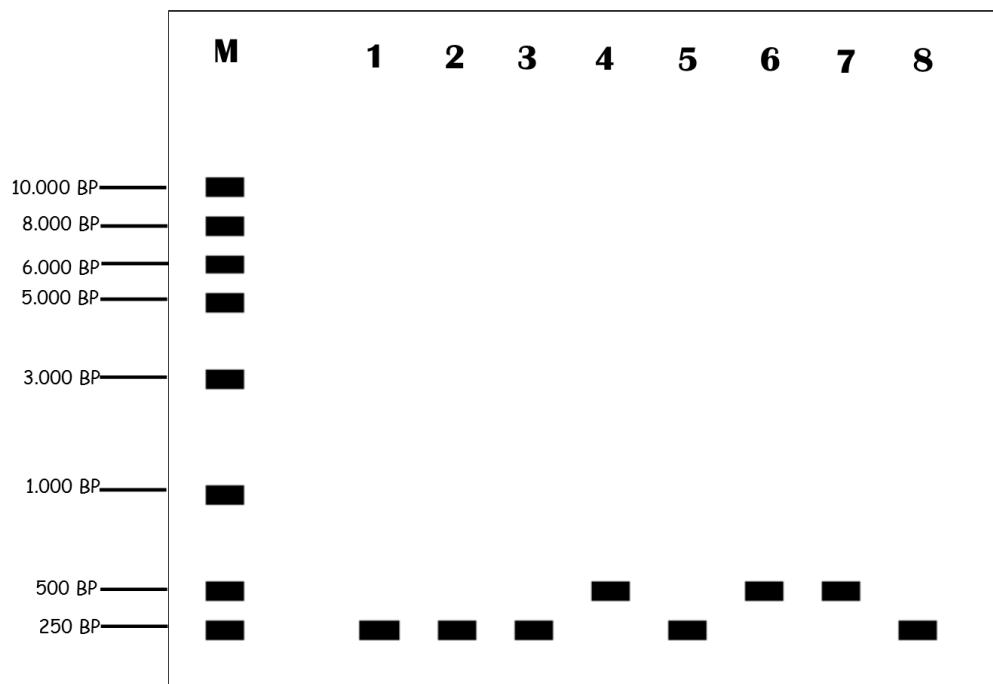


Figure 2. Interpretation of the extracted DNA from *Trichodina* spp. on Gouramy fish at BBI Kutasari Purbalingga.
Note : (M = Marka, 1 = Purbalingga 1, 2 = Purbalingga 2, 3 = Purbalingga 3, 4 = Purbalingga 4, 5 = Purbalingga 5, 6 = Purbalingga 6, 7 = Purbalingga 7, 8 = Purbalingga 8).

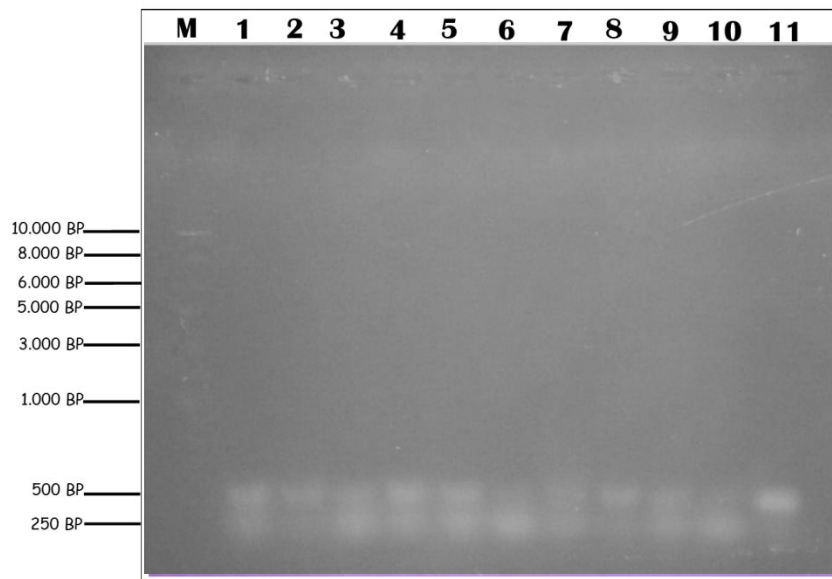


Figure 3. Results of DNA extraction from *Trichodina* spp. on Gouramy fish at BBI Kutasari Purbalingga.
Notes : (M = Marka, 1 = Purbalingga 1, 2 = Purbalingga 2, 3 = Purbalingga 3, 4 = Purbalingga 4, 5 = Purbalingga 5, 6 = Purbalingga 6, 7 = Purbalingga 7, 8 = Purbalingga 8, 9 = Purbalingga 9, 10 = Purbalingga 10).

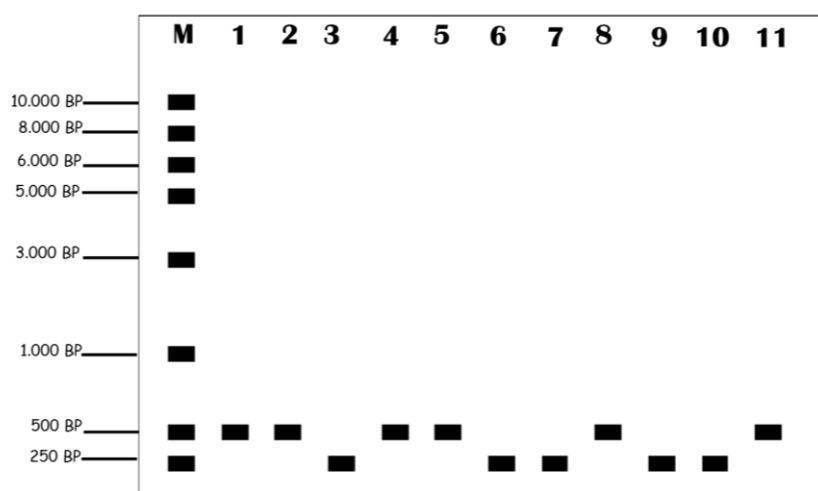


Figure 4. Interpretation of the extracted DNA from *Trichodina* spp. on Gouramy fish at BBI Kutasari Purbalingga.

(M = Marka, 1 = Purbalingga 1, 2 = Purbalingga 2, 3 = Purbalingga 3, 4 = Purbalingga 4, 5 = Purbalingga 5, 6 = Purbalingga 6, 7 = Purbalingga 7, 8 = Purbalingga 8, 9 = Purbalingga 9, 10 = Purbalingga 10).

The results of DNA isolation visualized on agarose gels in Figures 1 and 4 produced 19 DNA bands measuring 250 - 500 bp, all DNA bands showed the presence of *Trichodina paraheterodentata* in gouramy taken from BBI Kutasari Purbalingga. The entire DNA band is contaminated (smear), appears due to the presence of RNA contaminants or nuclease. The intact DNA is characterized by the absence of an electrophoretic DNA smear. This is important because in the PCR process, the intact DNA will provide relatively more accurate results (Syafaruddin & Tri, 2011).

Detection of the 18S rRNA gene in *Trichodina* spp. 10% *Trichodina paraheterodentata* is found in gouramy seeds belonging to BBI Strongasari Purbalingga. This result is low, when compared to the research of Rokhmani (2017) which detected the 18S rRNA gene in gouramy seeds belonging to Banjarnegara Rakit fish farmers, which was 45%. Based on the environmental conditions between the two sampling locations, BBI Kutasari Purbalingga is quite good, while in the Rakit Banjarnegara fish farmer the environment is less or bad enough. Observations on the maintenance of gouramy seeds at BBI Kutasari Purbalingga show

that the aquatic environment is good enough so that it does not support parasites to breed. According to Syafaruddin & Tri (2011), *Trichodina* spp. can reproduce rapidly, has a wide spread, and is a parasite commonly found in freshwater fish and can infect various types of fish. Incidence of infection with *Trichodina* sp. The host is influenced by water quality and poor pond maintenance techniques, namely high stocking density and calm, inundated and non-flowing ponds. The high stocking density will cause the fish to come into contact with each other so that parasites will be easily transmitted to other fish.

CONCLUSION

Based on the results and discussion that has been described, it can be concluded that, The morphological description of *Trichodina* spp. identified was *Trichodina paraheterodontata* and the detection percentage of the 18S rRNA gene for the protozoan species *Trichodina paraheterodontata* that infected the gouramy seeds from Purbalingga was 10%. The percentage of detection of this gene was low when compared to the results of the detection of the 18S rRNA *Trichodina paraheterodontata* gene that infected Banjarnegara gouramy seeds (45%).

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