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Moleculer Detection of Protozoa *Trichodina* spp. In Gourami (*Osphromenus Gourame* Lac.) Larvae with The infecting 18S rRNA Gene Marking in Exs. Residence of Banyumas, Central Java

✉**Rokhmani, Endang Ariyani Setyowati, Daniel Joko Wahyono**

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Faculty of Biology, Universitas Jenderal Soedirman, Indonesia

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Abstract

Protozoa species of *Trichodina* spp. may be found in most hatchery centers in Banyumas, Purbalingga, and Banjarnegara. However, the determination of *Trichodina* spp. types is still based on its body's morphological variations, not yet molecular. A research has been conducted to identify molekuler of the *Trichodina* spp. with the infecting 18S rRNA gene marking on the gourami larvae in Exs. Residence of Banyumas, Central Java. The research used a survey method with the samples of gourami. Amplification of 18S rRNA gene from *Trichodina heterodentata* was Performed using PCR technique. Primer used is Forward primer (5 '-AAC CTG GTT GAT CCT GCC ATG-3') and Reverse primer (5 '-TGA TCC TTC TGC AGG TTC ACC TAC-3') which produces a 600 pb amplicon of DNA. Molecular research can be a complementary identification of organisms morphologically. Amplification of the partial 18S rRNA gene may be used to identify *Trichodina* specifically. Gourami larvae taken from the hatchery centers in Banyumas, Purbalingga, and Banjarnegara. The results show that the detected percentage of *Trichodina heterodentata* genes with the infecting 18S rRNA gene marking on the gourami larvae in Central Java taken from the hatchery centers in Banyumas, Purbalingga and Banjarnegara are respectively 10%, 10%, and 45%. This research provides a benefit in mapping the presence of protozoa pathogen of *Trichodina* spp. in gourami hatcheries in the Former Exs. Residence of Banyumas, Central Java

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✉ Correspondence Author:
Jl. Dr. Soeparno 63, Purwokerto, Banyumas 53122, Central Java, Indonesia
E-mail: rokhmanitatiek@gmail.com

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INTRODUCTION

Gourami (*Osphronemus gourami Lacepede*) is one fish commodity of important fresh water fisheries and has been widely cultivated in many regions in Indonesia. The above notion occurs because gourami fish has a rather high price and a great opportunity in the market. Central Java Province is a gourami larvae center that annual production increases from 94.6 tons in 2011 to 109.7 tons in 2012 (Fisheries and Marine Office in Central Java, 2013).The above production has made gourami cultivation a very promising cultivation business. However, there are many factors affecting the growth of gourami, such as body size and parasite infection. Nuryanto et al. (2017) reported that there were genetic variations in the body size of gourami. Rokhmani *et al.* (2017) reported that the prevalence of *Trichodina* sp. infection can be as high as 100% in Bantul, Jogjakarta.

On parasite infecting the gourami larvae in all regions of gourami larvae centers is *Trichodina* sp. which mostly has a characteristic of pathogen, a protozoan parasite which has a cilia as its ectoparasite to infect fish skins and gills, for bothfresh water and salt water fish. This parasite multiplies by splitting itself, a process that takes place within the host body, swims freely well, able to detach itself from the host, and the body is supported by a correlated rigid disc ring called *chitinoid* (Durborow, 2003). Fish can also feel stressful and gets hyperplasia on its scale and receives damage on its gill structure. The *Trichodina heterodentata* species infecting gouramilarvae in the hatchery centers in Banyumas, Purbalingga and Banjarnegara, is identified based on its body measurement or morphometrics variation (Rokhmani *et al.*, 2015) that *Trichodina* sp. species found in gourami larvae in Banyumas, Banjarnegara and Purbalingga are *Trichodina nobilis*, *Trichodina reticulata. Tichodina acuta*, *Trichodina heterodentata*, *Trichodina magna*, *Trichodina pediculus*, and *Trichodina nigra.* A research conducted by Rokhmani *et al*. (2017) also found that *Trichodina heterodentata* species infects gourami larvae in the hatchery centers in several cities in Java Island.

Determination of the above species organism was conducted by measuring the variation of its body morphology. There are still a lot of shortcomings and mistake in measuring organism using morphology variation. Determination of certain species develops into an important notion for the sake of diagnoses and controlling effort. Polymerase Chain Reaction (PCR) is a method to diagnose a certain microorganism validity determination as an extension to control.

Several methods to identify protozoa species are polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), sequencing from ribosomal DNA (rDNA), internal transcribed spacers (ITS-1 and ITS-2), 5.8S rDNA (riboprinting), and mtDNA ox2 gene markers. Detection determination molecularly of a certain species based on its gen content is still rarely conducted. The previous studies for example still use the 18S r RNA gene marking. A marking is the smallest unit of a reduction result from methylation or RNA cutting. Other smallest units are r RNA 5.8S, r RNA 28S. Determination detection of *Trichodina* spp. in Indonesia is still rare or yet to be implemented. This is because determination of DNA (Deoxyribo Nucleotide Acid) has an important role or contribution.

DNA amplification in vitro. The 18S rRNA gene is often used for phylogenetic studies because it has a conserved area, and the nucleotide base data of the 18S rRNA gene allows it to be used to show the genealogical closeness to the kinship of a particular organism species. DNA is a fundamental genetic material which controls the characteristics of living creatures, expressed in polipeptida form, even though not all of it is protein (may be expressed as RNA that has catalytic reaction). Based on above explanations, we suggest an inquiry if there is *Trichodina heterodentata* with molecular detection on 18S rRNA gene marking which infects gourami larvae in local regions, such as those in the Former Residence of Banyumas, Central Java. The objective of this research is to detect the molecular Gene *Trichodina heterodentata* with 18S rRNA gene marking which infects gourami larvae in local regions, such as Residence Banyumas, Central Java.

METHODS

The initial research uses a survey method, while the data are collected using a random sampling. *Trichodina* spp. was obtained from the isolation and identification with method preparations of organ ranges on gourami larvae. Gourami larvae centers in: Kutasari Village, Baturaden Sub-District, Banyumas Regency; Luwung Village, Rakit Sub-District, Banjarnegara Regency: Kutasari Village, Kutasari Sub-District, Purbalingga Regency. The second phaseis in addition to the molecular determination to figure out the genetic features of *Trichodina* spp. in various centers of gourami larvae cultivations, for exs Resident, Banyumas, Central Java.

The DNA Extraction

The isolated *Trichodina* spp. from a larvae center in Banyumas, Purbalingga, and Banjarnegara is stored in a cup as well as the NaCl fluid. Before making an observation on genetical variation and genetical distance, isolation of *Trichodina* spp. is conducted on DNAgenome isolation using an alkaline lysis method. Usinga sterile pipete, 10 individual isolates of *Trichodina* spp. From organ preparation the gourami larvae cultivation centers level 1, insert them into ependorf tube containing 180 µl STE. Next, crush isolate*Trichodina* spp. using asterile pipette. Add 20 µl proteinase K and 200 µl buffer (bufferlysis) to the sample. Next, at the vortex, evenly mix and incubate at 70 °C for 10 minutes with box heater, add 200 µl etanol 100% to each sample and revortext denaturalize the proteinase K. Aspirate the solution mixture with a pipette and insert into *Dneasy spincolumn tube* and then centrifuge at 8,000 rpm for 1 minute. Remove the solution passing the spincolum and transfer to a new 2 ml tube and then wash with 500 µl buffer AW2, then centrifuge at 16,000 rpm for 3 minutes to dry the Dneasy membrane. Re-remove the solution and pipe collection. Put Dnea sycolumn in sterilized micro centrifuge and add 200 µl buffer AE with a pipette directly on the membrane. Centrifuge the sample for 1 minuteat 8,000rpm. Remove the Dneasy spincolumn and keep the solution at-20 °Celcius for the next test. Amplify the resulted DNA withthe RAPD technique. Amplify the DNA fragment with the RAPD technique. The total volume for PCR was 10ul consisting of 5ul go taqgreen PCR mastermix 2x; 2.25ul DNA; 0.25ul prime and 2.5ul water of nuclease free. The DNAis amplified using PCR Thermal Cycler BOECO.

The primer determination on molecular parasite identification of *Trichodina* spp., uses the gene from *Trichodina heterodentata* was used is Forward primer (5 '-AAC CTG GTT GAT CCT GCC ATG-3') and Reverse primer (5 '-TGA TCC TTC TGC AGG TTC ACC TAC-3') expected bp 600 (Tang et al., 2013)

On electrophoresis phase, a preparation of agarose gel is weighed as needed. The agarose concentration used is 1%. Using the hotplate, dilute the agarose until boiling; let it for 25 minutes until the temperature decreases to 50°C, then cast in the agarose tray equipped with a comb to form a gel well. After cooling down the agarose, lift the tray comb up and insert the gel into electrophoresis which is already contained with TAE 1x as electrophoresis buffer. Immerse the gel from electrophoresis in ethidium bromide (with concentration of 1mg/ml). Next, wash the gel with aquadest for 10-15 minutes. Visualize the DNAon UV transilluminator and take some Figures. Document the visualization results using a digital camera. The profile of eachprimer's DNAfragment patterns is positive and negative separately based on the presence of fragments using the binary score. Positive and negative indicate DNA isolation visualized on the agarose gel yielded 19 DNA bands of 250 - 500 bp, the entire DNA band indicates the presence of *Trichodina heterodentata*

The collected data are then descriptively analyzed based on the presence of DNA fragments generated from the primer of each locus. The qualitative RAPD fragmented patternis then converted into the qualitative binary.

RESULT AND DISCUSSION

Cultivation of gourami larvae on larvaean I, lasts for 1 - 2 months with seeds obtained between 3 - 4 cm. At this age, less than perfect or not yet established an optimal immune system so it is still susceptible to infections parasitic disease.. Disease caused by *Trichodina* sp. called Trichodiniasis, which infect many at larval age and small fish or larvae (Martins et al., 2010). Variations of morphological characters Trichodina sp. indicated by differences in form variation, variation in the number and size of some body parameters (Windarto et al., 2013).

Trichodina sp. which are found in each fish body have different morphometric characters. Morphological differences are known through morphometric and meristic characters such as: body diameter, diameter of denticle ring, adhesive disc diameter, membrane width and number of dents (Dana et al., 2002). The morphometric character is the reference for determining Trichodina sp. in general is measuring the diameter of the body, diameter of the ring dentikel, adhesive disc diameter, membrane width and the number of dents. (Basson & Van As, 1994). It also looks at the morphology of the denticles, also can be based on blade shape, ray shape, apex blade type, blade apophysis, blade connection.

Even though it is mostly found on the fish mucus, *Trichodina* sp. is also found in other fish's defense structure of all fish in all age and size. Zhao and Tang (2011), reports that *Trichodina* sp. are mostly found on the fish mucus, the epithelium tissues and blood are the best nutrition for this parasite. The infection of the *Trichodina* sp. were mostly intheir gills, especially when they were still in juvenile stage, since this stage is the best environment for the parasite to grow. The body's

defense structure is complemented by scales, skin and mucus products. This suggests that the type of organisms that willing to live together is only a few. Morphometric variation of *Trichodina* spp. to be observed is, for example, the varying forms of the denticle, which are the proximal, tapered, curved and accumulated forms of denticles. The straight-tapered denticle is in *Trichodina heterodentata* and *Trichodina nobilis*. The form of a straight but slightly collected denticle is *Trichodina*acuta, while the shape of the denticle of *Trichodina magna* is curved. The specificity of in *Trichodina magna* is more curved than in *Trichodina acuta*, *Trichodina heterodentata* and *Trichodina nobilis*. Denticle in *Trichodina heterodentata* has a tapered but thicker shape, while the blade connection on *Trichodina nobilis* is narrower and its apophysis blade is tapered.

The results of a research the types of *Trichodina* sp. found in gourami larvae with morfhometric variation in Banyumas, Banjarnegara, and Purbalingga are *Trichodina nobilis*, *Trichodina reticulata, Tichodina acuta*, *Trichodina heterodentata*, *Trichodina magna*, *Trichodina pediculus*, and *Trichodina nigra* (Rokhmani *et al.*, 2015). The conducted by Rokhmani., *et. al.* (2017) find that there is *Trichodina heterodentata* in gourami larvae in several cities in Java Island. Determination of the above species is based on descriptions or variation of its body morphometrics. In general, the morphology of *Trichodina* sp. shows several ray forms and varying forms of denticles. *Trichodina heterodentata* has *blade apex* end with a blunt/dull and *denticle ray* shape which is tapered straight but thicker.

Trichodina spp has the characteristics of adhesive disc diameter of 38-82 µm; denticle ring diameter of 23-51 µm; and number of denticles of 20-30. *Trichodina heterodentata* has the characteristics of body diameter 71-106 µm; denticle ring 26-37 µm; diameter; adhesive disc 47-63 µm; number of denticles 20-27; with membrane width of 2.7 µm. The data according (Rokhmani, 2015 ; Woo ,1995) *Trichodina heterodentata* has the characteristics of adhesive disc diameter of 38-82 µm; denticle ring diameter of 23-51 µm; and number of denticles of 20-30. *Trichodina heterodentata* has the characteristics of body diameter 71-106 µm; denticle ring 26-37 µm; diameter; adhesive disc 47-63 µm; number of denticles 20-27; with membrane width of 2.7 µm (Rokhmani, 2015). However, according in Woo (1995), The morphometric characteristic of *T.heterodentata* in gourami larvae. T. heterodentata is a type of cosmopolitan Trichodina, *Trichodina heterodentata* was first identified by Martins, et.al., (2010).

Sample from each hatchery center, the researcher takes 20 samples from Banyumas, Purbalingga, and Banjarnegara. The samples are then analyzed with primer Gene 18S rRNA from *T.heterodentata* with advance primer 50-AAC CTG GTT GAT CCT GCC AGT-30 and reverse primer 50-TGA TCC TTC TGC AGG TTC ACCTAC-30 (Tang, Fa-Hui , 2012). Based on the samples taken from each gourami hatchery centerin Banyumas, Purbalingga, and Banjarnegara, the treatment is analyzed in percentage that the *T.heterodentata* is respectively at primer bp 600 each 10%, 10% and 20% (Table 1)

Table 1. The Percentage of *Trichodina heterodentata* Detectionwith the infecting 18S rRNA Gene Marking on the Gourami hatchery Exs. Residence Banyumas Central Java

Sample	Each sample		Positive Result	(%)	
Banyumas	2	0	2	1	0
Purbalingga	2	0	2	1	0
Banjarnegara	2	0	9	4	5

The Table 1., sample positive percent ofprotozoa*Trichodina heterodenta -ta*respectivelyresulted from the samples in Banyumas, Banjarnegara, and Purbalingga by 10%, 45%,and 10%. The samples in Banjarnegarapositive percent of protozoa*Trichodina heterodentata*, by 45% showed a high, is according the intensity of *Trichodina* sp. on gouramy fish larvae pond cultivation Village District Rakit Banjarnegara showed a high number. The high intensity of Trichodina sp. can be affected by several factors. According to Rustikawati (2004), the high intensity of *Trichodina* sp. in fish larvae is suspected because of the pool water conditions that support for the life of the ectoparasites. High intensity values can also be affected by high fish density in ponds. High density can cause fish to experience stress. In ponds with high fish density, fish will rub against each other, so that ectoparasitic infection will occur quickly. The average density of fish in ponds studied is 21-24. The high intensity of *Trichodina* sp. on the fish seed according because this parasite can breed quickly and always move actively.

The result of electrophoresis sample can be seen on the following illustration, enlarged at 2,000 bp from a selected sample. At 2 sample analyzes squenzing BNJ 2 and BNJ 4 , because over 600 bp. The result of electrophoresis sample can be seen on the following illustration, enlarged at 2,000bp from a selected sample (Figure 1).

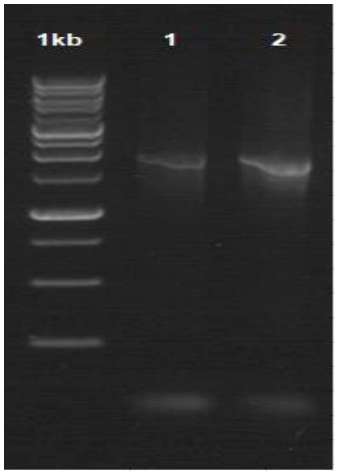

Figure 1. The Result of PCR Amplification of positive sample T. *Heterodendata* at 2,000 bps

The results of Figure 1 show a low rise of *T. heterodentate* concentration over 600 bps from sample. The qualitative RAPD fragmented patternis then converted into the qualitative binary, in sample BNJ 2 (Banjarnegara) bp 680 primer is descriptive TGCAGTCTAA GTACACACGG CCGGTACAGT GAAACTGCGA ATGGCTCATT AAATCAGTTA TGGTTCCTTT GATCGCTCTC ACGTTACTTG GATAACTGTG GCAATTCTAG AGCTAATACA TGCCAACGAG CGCTGACCTC CGGGGATGCG TGCATTTATC AGACCCAAAA CCCATGCGGG GTGCCTCTCG GGGTGCCCCG GCCGCTTTGG TGACTCTAGA TAACCTCGAG CCGATCGCTG GCCCTCGTGG CGGCGACGTC TCATTCGAAT GTCTGCCCTA TCAACTTTCG ATGGTACTTT ATGTGCCTAC CATGGTGACC ACGGGTAACG GGGAATCAGG GTTCGATTCC GGAGAGGGAG CCTGAGAAAC GGCTACCACA TCCAAGGAAG GCAGCAGGCG CGCAAATTAC CCACTCCCGA CTCGGGGAGG TAGTGACGAA AAATAACAAT ACAGGACTCT TTCGAGGCCC TGTAATTGGA ATGAGTACAC TTTAAATCCT TTAACGAGGA TCAATTGGAG GGCAAGTCTG GTGCCAGCAG CCGCGGTAAT TCCAGCTCCA ATAGCGTATC TTAAAGTTGC TGCAGTTAAA AAGCTCGTAG TTGGATCTCG GGATCGAGCT GACGGTCCGC CGCGAGGCGA GCTACCGTCT GTCCCAGCCC CTGCCTCTCG GCGCCCCCTC.

Sample BNJ 4 (Banjarnegara) is descriptive Assembly of 2 sequences 1728 bp

TAAGTACACA CGGCCGGTAC AGTGAAACTG CGAATGGCTC ATTAAATCAG TTATGGTTCC TTTGATCGCT CTCACGTTAC TTGGATAACT GTGGCAATTC TAGAGCTAAT ACATGCCAAC GAGCGCTGAC CTCCGGGGAT GCGTGCATTT ATCAGACCCA AAACCCATGC GGGGTGCCTC TCGGGGTGCC CCGGCCGCTT TGGTGACTCT AGATAACCTC GAGCCGATCG CTGGCCCTCG TGGCGGCGAC GTCTCATTCG AATGTCTGCC CTATCAACTT TCGATGGTAC TTTATGTGCC

TACCATGGTG ACCACGGGTA ACGGGGAATC AGGGTTCGAT TCCGGAGAGG GAGCCTGAGA AACGGCTACC ACATCCAAGG AAGGCAGCAG GCGCGCAAAT TACCCACTCC CGACTCGGGG AGGTAGTGAC GAAAAATAAC AATACAGGAC TCTTTCGAGG CCCTGTAATT GGAATGAGTA CACTTTAAAT CCTTTAACGA GGATCAATTG GAGGGCAAGT CTGGTGCCAG CAGCCGCGGT AATTCCAGCT CCAATAGCGT ATCTTAAAGT TGCTGCAGTT AAAAAGCTCG TAGTTGGATC TCGGGATCGA GCTGACGGTC CGCCGCGAGG CGAGCTACCG TCTGTCCCAG CCCCTGCCTC TCGGCGCCCC CTCGATGCTC TTAGCTGAGT GTCCCGCGGG GTCCGAAGCG TTTACTTTGA AAAAATTAGA GTGTTCAAAG CAGGCCCGGT CGCCTGAATA CCGCAGCTAG GAATAATGGA ATAGGACTCC GGTTCTATTT TGTGGGTTTT CTCTCTGAAC TGGGGCCATG ATTAAGAGGG

Next description sequencing is *Trichodina heterodentata* small subunit Ribosome RNA gene complete sequence, *Trichodina reticulata* small subunit Ribosome RNA gene complete sequence, and *Trichodina nobilis* 18S Ribosome RNA gene partial sequence internal transcribel spacer 1 complete sequencing and 5,8 Ribosome RNA gene partial sequenc. The result squencing according the research Martins, et.al.,(2012)

Incidence and prevalence Trichodiniasis on gourami larvae in Exs. Recident Banyumas Central Jawa, is high incidence and prevalence. On the research Utami, S. T. (2015), that has been done, showed that 100 samples of gouramy fish seeds were all infected by *Trichodina* sp. *Trichodina* sp. found on the outside of the body of gouramy fish seeds. In the body of gouramy fish, *Trichodina* sp. can adhere to adhesion (pressure from the outside), and consume cell fluid in the mucus or contained in the epidermis. *Trichodina* sp. also take organic particles and bacteria when attached to the host body.

The benefits and contribution of reserch for This research provides a benefit in mapping the presence of protozoa pathogen of *Trichodi-*

na spp. in gourami hatcheries in the Former and early control disease in Exs. Residence of Banyumas, Central Java.

CONCLUSION

The detection of protozoa identification in this research with primer *Trichodina heterodentata* resulted from the samples in Banyumas, Banjarnegara and Purbalingga shows positive percentage of containing protozoa respectively by 10%, 45%, and 10%. The above percentage shows that *Trichodina heterodentata* is also found in those three Regency. Thus, this research recommends that there should be a follow-up research patogenisitas *Trichodina* spp. conducted early control disease on regions in Central Java.

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