IDENTIFICATION OF *Clinostomum complanatum* (DIGENEA: CLINOSTOMIDAE) INFECTING *Anabas testudineus* AND *Trichogaster trichopterus* USING POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM

IDENTIFIKASI *Clinostomum complanatum* (DIGENEA: CLINOSTOMIDAE) PADA *Anabas testudineus* Dan *Trichogaster trichopterus* DENGAN MENGGUNAKAN *POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM*

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ABSTRAK

Penelitian ini bertujuan untuk mengetahui *Clinostomum complanatum* (Digenea: Clinostomidae) yang menginfeksi ikan air tawar di Yogyakarta dan Riau, Indonesia secara molekuler. Metasekaria *Clinostomum complanatum* diperoleh dari ikan betok (*Anabas testudineus*) asal sungai Progo, Yogyakarta dan ikan sepat (*Trichogaster trichopterus)* asal sungai Sail, Riau. Metaserkaria *Clinostomum complanatum* yang ditemukan di insang dan organ visceral ikan, diambil dengan menggunakan jarum dan diawetkan dalam etanol absolut. Pemeriksaan molekuler dilakukan dengan metode *polymerase chain reaction*. Hasil amplifikasi rDNA metaserkaria *Clinostomum complanatum* pada *internal transcribed spacer region* menunjukkan band yang jelas pada 1.300 bp. Hasil RFLP dengan enzim restriksi *Alu* I menunjukkan restriksisitas yang berbeda pada kelima cacing, namun dengan enzim *Rsa* I menghasilkan *single band* (1300 bp). Esimpulan penelitian adalah bahwa terdapat variasi intraspesifik antara *Clinostomum* sp. dari Indonesia.

Kata kunci: *Clinostomum complanatum*, molekuler, *Anabas testudineus*, *Trichogaster trichopterus*

ABSTRACT

The aim of study was to molecularly determine *Clinostomum complanatum* (Digenea: Clinostomidae) from freshwater fish in Yogyakarta and Riau, Indonesia. The metacercariae of the organism were obtained from climbing gouramy (*Anabas testudineus*) from Progo river, Yogyakarta and climbing perch (*Trichogaster trichopterus*) from Sail river in Riau, Indonesia. The metacercariae of *Clinostomum complanatum* found in the gills and visceral organs were aseptically taken using needle and preserved in absolute ethanol. Molecular analysis was carried out by a polymerase chain reaction. The amplification of ribosomal deoxyribo nucleid acid of metacercariae in the internal transcribed spacer region had a sharp band of 1300 bp. Digestion with the *Alu*I restriction enzyme showed different restriction sites in all five of *Clinostomum* sp. Using *Rsa*I enzyme the result showed single band (1300 bp). Therefore, it is concluded that there were intraspecific variation among the *Clinostomum* sp. from Indonesia.

Key words: *Clinostomum complanatum, molecular, Anabas testudineus*, *Trichogaster trichopetrus*

INTRODUCTION

The adult trematodes from Clinostomidae families are parasites of throat and oesophagus of piscivorous birds belong to the Ardeidae, Acciptridae, Laridae, Fregatidae and Phalacrocoracidae families (Yamaguti, 1958). The metacercariae stadium develop in fish and could be pathogenic to cichlid fingerling (Paperna, 1996), and few other freshwater fish species (Lo *et al.*, 1981). *Clinostomum complanatum* (Rudolphi, 1819) is found in Europe, Africa, and Asia (Japan, Thailand, Philippines). *Clinostomum marginatum* found in North America are similar the morphological characteristics to *Clinostomum complanatum*. The *Clinostomum* species cause disease in fish and *laryngo-pharingitis* in humans (Chung *et al.*, 1995). *Clinostomum complanatum* has been examined and classified based on its morphological characteristics and the differences in the ribosomal deoxyribonucleid acid to distinguish the species (Dzikowski *et al.*, 2004).

Aohagi *et al.*, (1992 and 1993) reported that many freshwater fish species were found to be second intermediate hosts of *Clinostomum sp*. In Korea, there are three species found in freshwater fish as a second intermediate hosts of *Acheilognathus koreensis*, *Rodeus uyekii* and *Sqalidus gracilis majimae* (Lo *et al.*, 1987). However, in Indonesia, they are found in climbing gouramy (*Anabas testudineus*) and climbing perch (*Trichogaster trichopterus*).

Specific identification of *Clinostomum complanatum* based on the morphological characteristics such as ratio of oral-ventral sucker, ceca, uterus, testis and excretory pore have been reported by Dias *et al.* (2003); Matthews and Cribb (1998). Proper identification of *Clinostomum* is very important in order to understand more about the molecular characteristics of the parasite. Identification of a species is very complex, especially in determining intra-and interspecific and variability of morphological characters (Samadi *et al.* 2000). Because of the difficulty in identifying the species, observations was conducted by molecular study to get more accurate result. One methode to identify a species at a molecular level is by using polymerase chain reaction-restriction fragment length polymorphism based on the amplification of the deoxyribonucleid acid in certain regions, and by using appropriate restriction enzymes.

MATERIALS AND METHODS

A number of five metacercarias of *Clinostomum sp.* was taken from climbing gouramy (*Anabas testudineus*) from Yogyakarta and climbing perch (*Trichogaster trichopterus*) from Riau. Excysted *Clinostomum* sp. in gill and visceral organ were aseptically taken using needle and preserved in absolute ethanol. The genom of individual worm was extracted, amplified and enzymatic digested. The metacercariae of *Clinostomum sp.* was inserted into the ependorf tube, 90 µl of ATL was added and homogenised. An ammount of 15 µl proteinase K was added, stirred well and incubated at 56 °C for 1.5 hours, and mixed by vortexing for 15 seconds. A volume of 100 µl of A1 buffer and 100 µl absolute ethanol were added to the mixture. The supernatant was transfered into a spin collumn tube, centrifuged for 1 minute at 8,000 xg and the supernatant was decanted. The spin collumn pellet was transfered to a

new tube, 250 µl buffer AW2 was added, and centrifuged for 3 minute at 13,000 xg and the supernatant was decanted. The pellet was transfered to a new spin collumn tube, 100 µl AE1 was added and incubated at room temperature for 1 minute and centrifuged for 1 minute at 8,000 xg. The results of DNA stock were stored at - 20 °C.

Deoxyribonucleidacid amplification was performed by adding 3 µl of DNA and 18 µL Intron PCR Master Mix to a microtube, mixed homogeneously. A volume of 2 µl primers which consisted of forward primer (5'-GTA GGT GAA CCT GCG GAA GGA TC-3') and reverse primer (5'-CCT TGT TTT TAG CTT CGC CTC TTC-3') was added. The 25 µl final concentration was centrifuged for 30 seconds. The PCR program for rDNA amplification consisted of: initial denaturation at 95 °C for 5 minutes, annealing at 60 °C for 5 minutes, extension at 72 °C for 1 minute 20 seconds for 26 cycles, final extension at 72 °C for 5 minutes. The final temperature used was 4 °C. The PCR results were stained with ethidium bromide, separated by electrophoresis in a 1.5% agarose gel for 30 minutes at 100 volts, visualized, and observed by UV transilluminator and documented.

The DNA extract was checked by electrophoresis using 1,5% agarose gel. The gel was formed, firsthy, by weighing0,40 gr agarose, dissolved into 40 ml of 1x TAE buffer and boiled on microwaves using 100 ml sample size bottles. The solution was let cool in a waterbath of 60 °C for 10 minutes, added with 5 ml ethidium bromide and mixed well. Agarose was poured to a gel tank that has been fit with comb. After 20 minutes, the agarose solution would solidify to gel form. The comb was taken off so that 8 or 12 wells was formed. Tris Acetat EDTA buffer was poured into molds. The hole was then filled with the PCR product.

Five µl of PCR product was mixed with 5 µl of loading buffer on parafilm, after which 10 µl solution was added into each wells of the agarose gel. The last well was filled with 5 µl of molecular marker. Electrophoresis was performed at 100 V for 30-40 minutes. The gel was lifted from the gel tank. Finally the DNA band was observed and visualized under UV transilluminator. The results is then documented.

Restriction fragment length polymorphism was performed using *Rsa*I on agarose gel and *Alu*I restriction enzymes with silver staining on Polyacrylamide gel. The reaction was carried out with 8 µl reagents consisted of a series of 4 µl PCR product, 2 µl enzyme, and 2 µl buffer. The mixture of reaction result was incubated for 24 hours (*Rsa*I Enzyme) at 37 °C. All results were electrophoresed on 1.5% agarose gel in 1x TAE buffer. Electrophoresis was performed at 100 V and 20 minutes. The electrophoresis results were visualized on UV transilluminator and documented with a digital camera.

RESULTS AND DISCUSSION

The polymerase chain reaction results showed that in the internal transcribed spacer regions (ITS1) showed the same band at 1300 bp (Figure 1). The success of a PCR process is determined by the amount of the DNA produced (Adlard, 1993). According to Dzikowski *et al.* (2004), the PCR results for the *Clinostomum complanatum* using specific primers the same as the ones used here and 1% agarose obtained a 1230 bp bands. A molecular study on trematode digenea of *Eurytrema* resulted a

band of 1500 bp in the ITS1 (Mirza, 2001).

Restriction fragment length polymorphism test using the *Rsa*I enzyme showed the same band (1300 bp) in five worms from Yogyakarta and Riau, Indonesia (Figure 2). The *Rsa*I enzyme can not be used to cut all samples because there is no specific nucleotide at the restriction site (Lee *et al.,* 2009). According to Marcilla *et al.* (2002), the difference in the results obtained with specific restriction enzymes is due to different temperatures for each enzyme to react optimally.

The result of the digestion with *Alu*I enzyme in worms from Yogyakarta (sample 1 - 4) showed 5 different sites, namely 219, 334, 458 and 478 and 809 bp, whereas worms from Riau (sample 5) showed 6 restriction sites, 219, 334, 458, 478, 809 and 879 bp (Figure 3). These results indicate that *Alu*I can cut DNA in ITS region. There must be differences in ITS1 of *Clinostomum* sp. Restriction fragment length polymorphism occurs when the site of the detected fragments vary between individuals. Each fragment size is considered as an allele that can be used for genetic analysis. The RFLP analysis using restriction enzymes cut DNA single strand or double strand at specific nucleotides, known as restriction sites. In general, the allele with the correct restriction site will show two or more visible bands on the gel. The number of bands that appeared shows genotype of samples, for example on restriction mapping (Anonim, 2011). It is suggested that by using *Alu*I restriction enzyme, the DNA genome has more than one site of cutting, but there is no restriction site using *Rsa*I enzyme. Based on the sequence differences, a PCR-RFLP was established for the unequivocal delineation of the *Fasciola* sp. (Trematoda, Digenea) from China using restriction endonuclease *Hsp*92II and *Rca*I (Huang *et al.,* 2004). Blair (1993) reported that there was no intraspecific variation in the rRNA of *Fasciola hepatica* and *Fasciola gigantica* originated from different countries using *Rca*I restriction enzymes. Therefore, it can be concluded that there was an intraspecific variation in all five *Clinostomum sp.* under study.

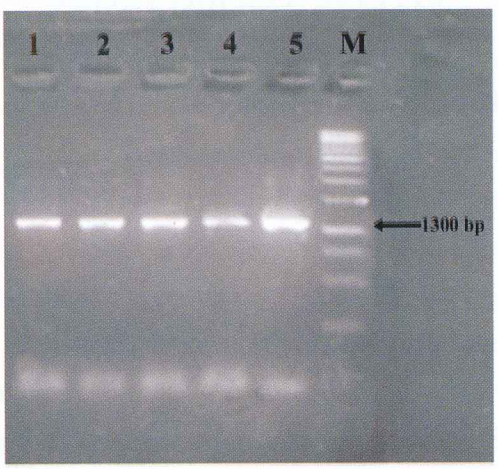

Figure 1. The amplified *Clinostomum* sp. in ITS1 region. Lanes 1- 4: worms from Yogyakarta, 5: from Riau, M: Marker.

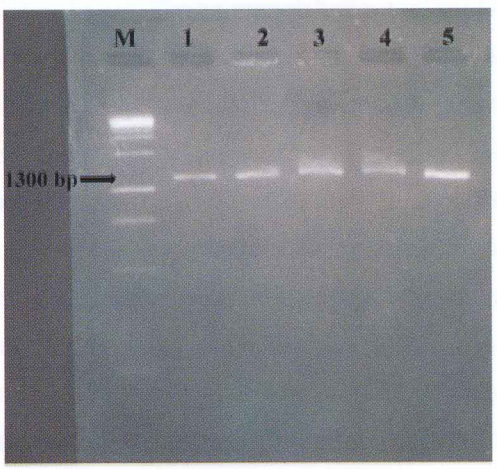

Figure 2. The result of digestion with *Rsa*I restriction enzyme for *Clinostomum sp.* in ITS1 region. Lanes 1 - 4: worms from Yogyakarta, 5: from Riau, M: Marker.

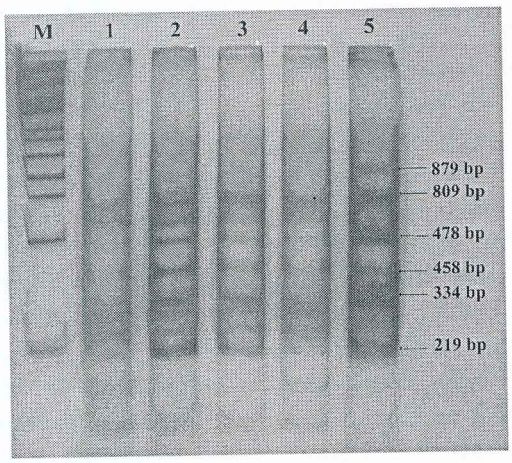

Figure 3. The result of digestion with *Alu*I restriction enzyme for *Clinostomum sp.* in ITS1 region. Lanes 1 - 4: worms from Yogyakarta, 5: from Riau, M: Marker.

PCR results on ITS1 region containing a clear band at 1300 bp. The Restriction Fragment Length Polymorphism results with *Alu*I restriction enzyme showed the different restriction site in five worm, but the *Rsa*I enzyme showed the single band. It can be concluded that there was intraspecific variation in five *Clinostomum sp.* worms from Indonesia.

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