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Comparison of the 12S rRNA and COI Regions of Mitochondrial DNA for eDNA Detection of Alligator Gar (*Atractosteus spatula*)

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ABSTRACT
Alligator gar (*Atractosteus spatula*) has been introduced to many places outside its native range. The fish is considered as an invasive alien species that needs to be eradicated upon encounter in Indonesia due to its aggressive behavior. Despite existing regulations in Indonesia, the fish is still bred and widely distributed as a pet fish which increases the potential of being released into native waters. Non-invasive early detection of alligator gar is important as part of management efforts. This experiment was conducted to compare the 12S rRNA and COI regions of mitochondrial DNA for detection and estimation of alligator gar. Water samples (250 mL) were obtained from mesocosms containing one and three fish. The environmental DNA (eDNA) was filtered and extracted from the samples before amplified using quantitative PCR (qPCR). The eDNA concentrations amplified using 12S primers were higher than COI primers with 49.2×10^6–14.2×10^9 average copies/sample. There was negligible correlation between fish biomass and eDNA copy numbers amplified using 12S primers, but there was significant correlation ($y = 330.6x - 1175, R^2 = 0.3356,$ $p < 0.05$) between fish biomass and eDNA copy numbers amplified using COI primers. Thus, targeted qPCR using specific primers is more effective for detection and estimation of alligator gar in nature.

Keywords: *Atractosteus spatula, Detection, eDNA, Invasive, qPCR*

1. INTRODUCTION

Alligator gar (*Atractosteus spatula*) has been found as an introduced species outside its native range in Turkmenistan [1], Iran [2], Iraq [3], India [4], Malaysia [5], and Indonesia. Alligator gar has been encountered in Jakarta (2007) [6], in Aceh (2011) [7], and in Bali and East Java (2019) [8]. Alien species introduced into a habitat may become invasive due to having better colonization and competition capabilities than native species. Invasive alien species (IAS) can disturb the balance of selective pressures and threaten the biodiversity of native species [9].

In Indonesia, alligator gar is categorized as IAS due to its ability to grow into a large size, carnivorous and aggressive nature, and poisonous eggs [6, 10]. There are bans put on trading and keeping alligator gars based on Law Number 31 of 2004 which was amended to Law Number 45 of 2009 [11]. The statute is also complemented by Regulation of the Minister of Marine Affairs and Fisheries of Republic of Indonesia Number 41 of 2014 on Import Prohibition of Hazardous Fish species into the Territory of Republic of Indonesia [12, 13]. Despite the law, it is still bred and widely distributed as a pet fish, thereby increasing the potential of being released into Indonesian native waters [6]. Early detection of alligator gar is important to determine

whether eradication should be carried out when prevention has failed [14].

Environmental DNA (eDNA) is DNA found in the environment that may be obtained from feces, mucus, skin, or gametes. Instead of search and capture method, target species can be detected using water samples and eDNA method. Therefore, it offers a non-invasive and relatively more efficient approach to monitor a species [15, 22].

Among current technologies used to detect eDNA, quantitative polymerase chain reaction (qPCR) is more accessible with effective cost [16]. It is important to use species-specific primers in PCR to avoid amplifying the DNA of other species besides the target [17]. Results from previous studies of eDNA assessment using quantitative PCR (qPCR) have also showed relationship between the amount of eDNA in water samples and biomass that was important for species conservation and population management [18, 19, 20].

Alligator gar has been successfully detected using eDNA method. Previous study using a primer pair that was designed for metabarcoding of vertebrate eDNA targeting the hypervariable region of mitochondrial 12S rRNA gene and V5 region [10]. Another successful detection has also been reported using qPCR with primers targeting the cytochrome oxidase I (COI) gene in alligator gar [21]. Both amplified targets resulted in a high amount of DNA. The probability of detection and specificity are also high. However, there has been no comparison of the two regions for alligator gar detection. The eDNA method also has not been used to estimate alligator gar biomass. This experiment was conducted to (1) compare the amplification copy numbers from using the universal vertebrate 12S rRNA primer pair and the COI primer pair and (2) understand the relationship between alligator gar biomass and eDNA amount.

2. METHODS

2.1. Mesocosm Experiment

Mesocosm experiment was conducted in the greenhouse at Department of Biology Universitas Indonesia, Depok. Juvenile alligator gars were placed within six aerated containers (35×50×25 cm, filled with 23 L of water) with three out of six each contained one fish and the rest each contained three fish. Three other containers were prepared with no fish to serve as negative controls. The containers were sterilized with bleach, rinsed, and dried before each use. The fish were left within the mesocosms for three days before weighed and given rest in a larger and more stable environment for four days. During three weeks, a 250-mL water sample was collected with sterilized bottles in three turns from each mesocosms once every three days after the fish were put inside. Each fish was given smaller live fish as food every two days before put into the containers and went through a fast while inside the container. Water in the containers is replaced after each cycle.

2.2. Filtration and eDNA Extraction

The process of filtration and eDNA extraction followed the protocol used by Nur et al. (2020). Water samples were filtered to collect eDNA using cellulose nitrate filter membrane with 0.45 μm pore size [Ahlstrom] and stored within Longmire buffer. The filtration apparatus was sterilized using bleach and rinsed before filtrating each sample. Extraction of the filtered eDNA was performed with a conventional method of phenol-chloroform-isoamyl alcohol (PCI) modified by Nur et al. (2020). The filter membranes were moved into CTAB (cetyltrimethylammonium bromide) buffer and homogenized using tungsten beads [QIAGEN] before β-mercaptoethanol was added [10].

2.3. Quantitative Polymerase Chain Rreaction (qPCR)

Extracted DNA was quantified in the presence of SYBR-green using real-time PCR with Rotor-Gene Q Thermocycler [Qiagen]. Two sets of primers were used in the experiment. The first set of primers named ecoPrimer had been designed for metabarcoding of vertebrates and was used to amplify the hypervariable region of mitochondrial 12S rRNA gene and V5 region with a product length of 73–110 bp. The ecoPrimer sequences were 5'-ACTGGGATTAGATACCCC-3' and 5'-TAGAACAGGCTCCTCTAG-3' as forward and reverse primer respectively [10]. The second set of primers named AspCOI had been designed by Farley et al. (2018) to be a species-specific primer set used to amplify the cytochrome oxidase I (COI) gene in alligator gar (*Atractosteus spatula*) with a product length of 159 bp. The AspCOI primers were AspCOI428F 5'-TAAAACCACCCGCAGCTTCC-3' and AspCOI587R 5'-CCTGCAGGGTCAAAGAA GGT-3' with F letter designated for forward primer and R letter for reverse primer [21].

PCR amplification was performed in triplicate with initial denaturation at 95°C for 3 minutes followed by 40 cycles of denaturation at 95°C for 3 seconds and annealing at 53.4°C for 30 seconds. Each qPCR reaction had 20 μL final volume consisted of 10 μL KAPA (Sigma Aldrich) 2X SYBR FAST qPCR Master Mix, 0.4 μL of each primer (10 μM), 8.2 μL nuclease free water, and 1 μL DNA. Each run was performed with a no-template reaction that served as a negative control. A 4-point standard curve for ecoPrimer pair was created using 2X dilution series of DNA extracted from the fin tissue of alligator gar ranging from 2–2.5×10^{-2}

ng/reaction (R^2 = 0.99, E = 103%). Another 7-point standard curve for AspCOI pair was created using 5X dilution series of synthetic gBlocks® Gene Fragment (Integrated DNA Technologies, IA, USA) containing AspCOI primer sequences ranging from 4.0×10^{-1}–2.5×10^{-5} ng/reaction (R^2 = 0.99, E = 92%). The efficiency was calculated according to the MIQE Guidelines [25]. Replicates showing positive amplification were considered as successful quantification. Replicates that did not show positive amplification were given copy number value of 0 for analysis.

2.4. Statistical Analyses

The data was analyzed using the Shapiro-Wilk Goodness of Fit Test to test the normality of the distribution. The eDNA concentrations used were obtained by averaging the quantified copy number of technical replicates. The eDNA copy number was given $\log_{10}$ transformation when the normality assumption was not satisfied. The relationship between fish biomass and eDNA copy number per 1 L was examined using bivariate scatterplots and regression only when significant correlation was found. The tests were performed using GraphPad Prism version 8.0.1 (GraphPad Software, USA).

3. RESULTS AND DISCUSSION

No negative sample showed significant amplification which indicated success in limiting contamination during the experiment. The amplified eDNA concentration highly varied for every positive amplification. For every DNA sample collected from containers with alligator gar, all replicate showed positive amplification with ecoPrimer primers while at least one replicate showed positive amplification with AspCOI primers.

Comparison between samples amplified using ecoPrimer resulted in significantly higher concentration than samples amplified using AspCOI, ranging from 1.2×10^7–3.5×10^9 copies/replicate or 49.2×10^6–14.2×10^9 average copies/sample (Figure 1). The eDNA copies for three fish tend to be higher in average compared to one fish with both AspCOI and ecoPrimer. The individuals used in each week were different and therefore the week was not considered as a variable, but the average biomass was higher for the following week.

Negligible correlation ($p > 0.05$) was found between fish biomass and eDNA copy numbers amplified using ecoPrimer primers, while significant correlation($p < 0.05$) was found between fish biomass and eDNA copy numbers amplified using AspCOI primers. The bivariate scatterplot and regression showed a positive relationship between fish biomass and eDNA copy numbers amplified using AspCOI primers ($y = 330.6x - 1175, R^2 = 0.3356,\ p < 0.05$; Figure 2 , although the R-square value indicated only a weak relation.

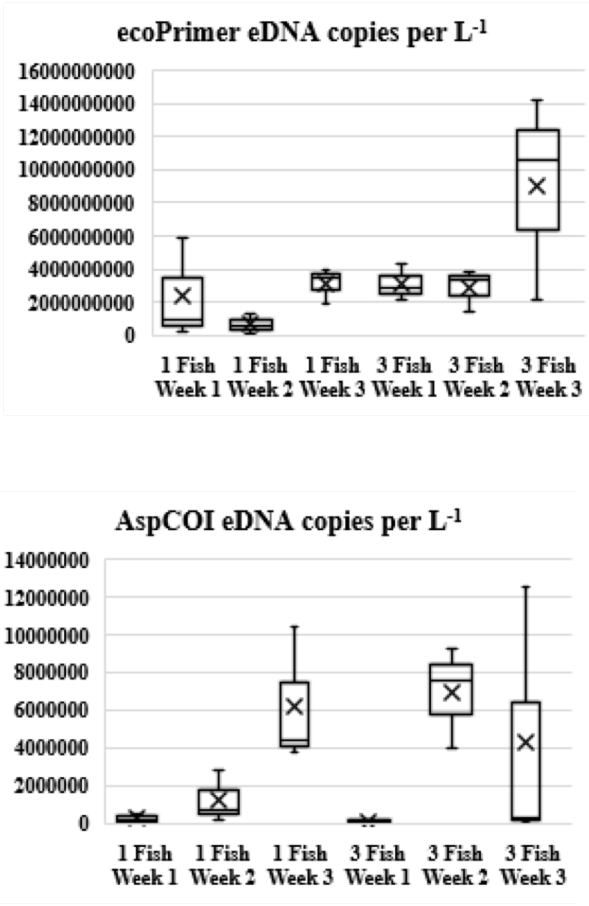

Figure 1. Result of eDNA copies with AspCOI and ecoPrimer with one and three fish within three weeks. The box plot lines from below respectively show the 25th quartile, median, and 75th quartiles. The × shows the average value. Whiskers show the 1.5 times the interquartile range.

The high variation of eDNA amount and weak correlation for AspCOI was possibly influenced by factors other than biomass. Although conducted at the same place, there was no strict control of the environmental factors such as temperature, pH, and UV exposure on the different days in three weeks. Environmental factors such as temperature, pH, and UV exposure can affect eDNA degradation [22]. Various findings with diverse organisms reported that higher temperature generally correlates with higher decay rate. Some reported higher decay rate in more acidic environment, but there was also an opposite result. There was generally no impact on decay rate with sunlight, but there was also finding with higher decay rate with more UV exposure [28].

The containers used in the experiment had the same size and filled with similar water volume. This can be another factor affecting the result as the shedding rate can be driven higher with more organism density [28].

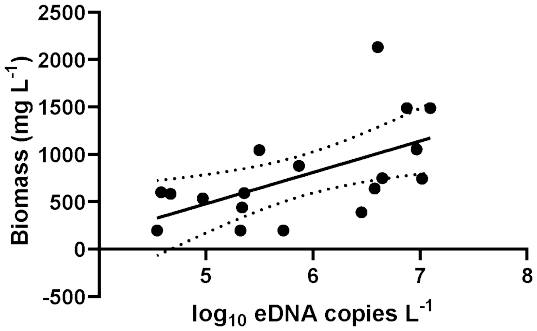

Figure 2. Relationship between eDNA copies (AspCOI) and biomass of alligator gar per 1 L. The regression line showed a significant trend (p, 0.05). Dotted lines represent 95% confidence bands of the best-fit line.

Other factors such as the juvenile life stage, behavior, stress, and metabolism and physiological activity of alligator gar can also affect eDNA release [22]. The effect of alligator gar life stage on eDNA release has not been studied, but an experiment with bluegill sunfish (*Lepomis macrochirus*) reported slightly higher eDNA release rate for juvenile stage compared to adult [23]. Some alligator gars in kept during experiment were observed to show more movements to catch bigger live prey. The alligator gars used in the experiment also appeared to be aggressive at times against individuals of the same species which were relatively smaller in size. Both observations supported that the behavior of alligator gars during the experiment can affect eDNA release. This finding is also in line with how alligator gar lives in solitary, has aggressive behavior, and cannibal towards smaller individuals of the same species [6, 24].

The use of ecoPrimer and AspCOI primer sets brought out different amplification result and correlation with fish biomass. The ecoPrimer set were also used in some preceding experiments but the primer pairs were not designed specific for alligator gar and would also amplify eDNA of vertebrates other than the target species. Although the alligator gars were fed before they were put inside the containers, it is possible that the feed eDNA was still detected if it was carried along into the containers and remained in water for three days. It accounted for a high chance of false positive and false high concentration result for the experiment. The ecoPrimer set can be used for experiments in strictly controlled environment free from vertebrates other than the target species, but it is not recommended for experiments using samples from nature. While the AspCOI primers which were designed specifically for alligator gar can also amplify the DNA of the Cuban gar [21], both species are not native in Indonesia and false positive for Indonesian nature samples would be a less concern.

The problem of varying result and accuracy may be reduced by using ddPCR [26]. Capo et al. (2020) also found that temperature was not a significant factor while using ddPCR for eDNA estimation [27]. However, ddPCR can cost more funds and time compared to qPCR [27].

In summary, targeted qPCR method can offer a more efficient routine in early detection and monitoring of alligator gar as an invasive alien species. Specific primers are needed for alligator gar when eDNA from other species is present in a sample. There was a positive correlation between alligator gar biomass and eDNA copy number using qPCR method with species-specific primers. Although there is positive correlation using qPCR, estimating alligator gar biomass is not yet recommended due to the possibility that other factors may affect the eDNA amount of alligator gar in water samples. The behavior of alligator gar might have greatly affected the amount of eDNA and needs careful consideration in future experiments.

AUTHORS' CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection, lab experiment and analysis were performed by Patricia Agustin and Andi Eko Maryanto. The first draft of the manuscript was written by Patricia Agustin and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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