Methods to maximize environmental DNA (eDNA) for detection the presence of Alligator Gar (*Atractosteus spatula*)

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Abstract. Introduction of alligator gar (*Atractosteus spatula*) into a new range of its distribution has been reported in several freshwater ecosystems. The presence of alligator gar in Indonesia has been recorded in Jakarta during big flood in 2007. The concern about introducing this species is because of its predatory behavior and can impact the trophic system in freshwater ecosystem in its non-native range. Here, we demonstrated two methods, conventional and kit method, to maximize eDNA concentration in addition to detect the presence of alligator gar in freshwater ecosystem. We used mesocosm study to mimic the real condition of freshwater system in tropic region. We found that both methods can extract eDNA from water samples. The kit showed better yield of eDNA with average concentration 62.28 ± 22.27 ng/g than conventional method with average concentration 19.35 ± 9.89 ng/g. Another advantage of the kit method was better purity of eDNA, with average purity 1.92 ± 0.05 compared to conventional method with average purity 2.23 ± 0.42. The better concentration and purity of eDNA could be advantageous for further analyses such as PCR and DNA sequencing.

Keywords: Alligator gar, environmental DNA, eDNA preservation, longmire buffer, sodium acetic buffer.

1. Introduction

The introduction of alien species to its non-native range may cause problems. In many cases, alien species are invasive. Alien species are usually being invasive due to its predatory behavior. The predatory behavior may out-compete the native species, disrupt the trophic system, and may cause biodiversity loss in the introduced ecosystem [1].

One case of the introduction of invasive alien species that has occurred in Indonesia is the introduction of alligator gar (*Atractosteus spatula*) in freshwater ecosystem. Alligator gar presence was found during the Jakarta flood in 2007 [2, 3]. Alligator gar is one kind of gars (order Lepisosteiformes) originated from northern to central America, especially in the United States. Its former distribution covers the Mississippi River-basin to Illinois, Ohio, Missouri, and Gulf Coastal rivers from western Florida to Mexico. Alligator gar is carnivorous fish that prey on appropriate size of prey such as fish, turtle, bird, waterfowl, and crab. It is known to be the largest freshwater fish. The maximum size reported of adult alligator gar is a nearly 3 m and 179 kg specimen. This massive size and predatory behavior make alligator gar has almost no natural predator, becoming invasive species to its non-native range,
and may cause ecological catastrophe. Alligator gar has been prohibited to be introduced to Indonesia by the Ministry of Maritime Affairs and Fisheries since 1982, but it is to be known that this fish introduced Indonesia in the late 1990s [4, 5].

Molecular techniques such as environmental DNA (eDNA) applications have been applied recently in biodiversity and ecological studies to overcome problems usually encountered by direct surveys in the field [6]. For instance, it might be difficult to detect elusive species or species with low abundance in a freshwater ecosystem by direct survey [7-9]. eDNA remains in the environment can be extracted from water and be applied to detect the elusive species. This principle also allows eDNA possible to be applied for the detection of invasive species such as alligator gar to prevent further problematic ecological and biodiversity issues [10].

The mesocosm experiment is commonly used in freshwater eDNA-research to simplify the experiment condition due to the uncontrollable conditions of the real environment. By using the mesocosm-experiment, we can limit external factors such as temperature, pH, light intensity, number of individuals in mesocosm, and any others that may influence the dependent variable. By limiting the external factors or setting it up into any certain condition, we may study more about how particular condition may influence eDNA presence. A mesocosm experiment is commonly set up with similar conditions to the real environment, so the experiment can represent the effects of environmental conditions to the eDNA in the field [10, 11].

eDNA extraction may be carried out using a similar method that is commonly used to extract non-environmental DNA. There are two main methods, the conventional and kit method. The conventional methods are protocols to extract DNA using standard chemical reagents such as phenol, chloroform, ethanol, salts, chelating agent, and is carried out more ‘manually’, while kit is more standardized protocols and chemical reagents developed by manufacturer. Sometimes manufacturers also provide special instrument for typical product of kit that may help the extraction process becomes more ‘automatic’. Here we demonstrated two methods, conventional and kit method, to maximize eDNA detection from alligator gar using mesocosm experiment.

2. Materials and method

We use a mesocosm-experiment that mimics tropic-freshwater environment. The mesocosms were stored in the greenhouse that belongs to the Department of Biology Universitas Indonesia, Depok. We put an alligator gar in mesocosm as eDNA source. Alligator gar was used to directly represent invasive species that found in Indonesian freshwater ecosystem. The alligator gar we use is a gar that was found in the lake in Universitas Indonesia area. The gar is about 20 cm long and treated in a mesocosm with aerator. Water from the mesocosms was collected for a month in 4 turns, once a week.

2.1. Water collection and filtration

Water collection was done by aspirating 200 mL water from the mesocosm using a peristaltic pump. Aspirated water then was collected into a sterilized bottle. Collected water then filtered by cellulose nitrate (CN) filter membrane with 0.45 μm pore size [Sartorius Stedim]. Filtration was performed to trap eDNA into a filter membrane. Filtration was carried out using a vacuum pump [Wigens] and filtration apparatus [Nalgene]. Filtration apparatus was sterilized using bleach, distilled water, and 70 % alcohol before usage. Collected water was homogenized by shaking the collection bottle before the filtration process. This step was performed to prevent debris that may contain eDNA remains at the bottom of the bottle [6]. The filter membrane was preserved in microcentrifuge tube with Longmire buffer at room temperature until before the eDNA extraction was performed [12, 13].

2.2. eDNA extraction

eDNA extraction was performed using the conventional and kit method. We used phenol-chloroform-isoamyl alcohol, hereafter PCI for the conventional method and FastDNA™ SPIN Kit for Soil for kit method. PCI has been commonly used for DNA purification in the conventional method of DNA
extraction. This reagent is commonly used due to the potential to reduce cost per sample, so it will be advantageous for extracting DNA in large amounts of sample. FastDNA™ SPIN Kit for Soil is DNA extraction kit manufactured by MP Biomedicals. This kit is manufactured for extracting DNA from environmental materials such as soil, water, and biofilm [14].

2.2.1. eDNA Extraction Using PCI. The protocol was adapted from Renshaw et al. [14]. The main procedures of this protocol are double purification with chloroform and precipitation with ethanol and NaCl [15, 16]. The preserved filters containing eDNA were moved to CTAB (cetyltrimethylammonium bromide) buffer [12]. We made a modification by crushing the filter membrane into pieces using tungsten beads [QIAGEN]. 2-mercaptoethanol was added to the crushed filter membrane in CTAB and was incubated at 65 °C (10 min.). PCI (one phase, 25:24:1) with equal volume to CTAB was added to each sample and was vortexed. Samples were centrifuged at 13,000 rpm (5 mins). Aqueous layer was transferred to a fresh tube and was added by chloroform-isoamyl alcohol (24:1). Samples were centrifuged at 13,000 rpm (5 mins), and the aqueous layer was transferred to fresh tube. Samples were added with 100 % ice-cold ethanol, NaCl 5 M, and were precipitated overnight at -20 °C. Precipitated samples were centrifuged at 13,000 rpm for 10 minutes, and supernatant was decanted. Pelleted DNA was dried using heat block at 45 °C until no visible liquid remained. Pelleted DNA then was rehydrated with TE buffer (low EDTA).

2.2.2. eDNA extraction using FastDNA™ SPIN kit for soil. The filter membranes containing DNA were cut into 2 mm x 2 mm pieces before the DNA extraction. The pieces were then moved to Lysing Matrix Tube provided by kit. DNA extraction was performed by following guidelines from the manufacturer.

2.3. Gel electrophoresis and DNA quantification
Gel electrophoresis was performed to verify that genomic DNA was successfully isolated from the filter membrane using both the conventional and kit method. Electrophoresis was performed by running samples on 0.8 % agarose (100 V) for 30 minutes and was visualized by Gel Doc™ [Bio-Rad]. DNA quantification using Nanodrop spectrophotometer [Biodrop] was performed to measure DNA concentration and purity based on A260/280 ratio.

2.4. DNA amplification
Polymerase chain reaction (PCR) was performed to amplify the hypervariable region of 12s rRNA gene including V5 region with 73–110 bp PCR product [17]. We used ecoPrimer, primer pair (forward 5’-ACTGGATTAGATACCCC-3’ and reverse 5’-TAGAACAGGCTCCTCTTAG-3’) that designated for metabarcoding of vertebrate eDNA. PCR component of 25 μL total volume for each reaction contained 12.5 μL DreamTaq Green PCR MasterMix [Thermofisher], 1.5 μL of each forward and reverse primer, 4.5 μL Nuclease-Free Water [Thermofisher] and 5 μL template DNA. PCR was started with an initial denaturation at 95 °C (3 mins), followed by 30 cycles of denaturation at 95 °C (30 s), annealing at 53.4 °C (30 s) and elongation at 72 °C (1 min), and terminated by a final extension at 72 °C (10 mins).

3. Results and discussion
Genomic eDNA was successfully extracted from water using both PCI and FastDNA™ SPIN Kit for Soil. This result was verified by visualization of gel electrophoresis from both methods. The visualized gel shows bands above the top band of DNA Ladder 1 kb (10,000 bp-sized) (figure 1 and figure 2). It is corresponding to genomic DNA because the size of isolated DNA is higher than 10,000 bp [18]. We only show eDNA visualization from the 4th water collection for the conventional method, because only samples from the 4th water collections were successfully visualized. The lane that consists of the corresponding sample to this experiment is ‘1L (7)’. DNA bands from both PCI and kit show smear. It may occur due to DNA degradation, since eDNA may come from extracellular DNA.
Figure 1. Genomic eDNA visualization (PCI). 1: number of alligator gar in the mesocosm; L: Longmire buffer, (7): days of preservation period, M: DNA Ladder 1 kb.

Figure 2. Genomic eDNA visualization (kit). 1) DNA Ladder, 2) Positive control, 3) 1st sample, 4) 2nd sample, 5) 3rd sample and 6) 4th sample.

It might be exposed by environmental conditions that accelerate DNA degradation rate such as acidic pH, UV radiation, microbial and even endonuclease activities [19-21]. Quantification was performed to measure eDNA concentration and purity from all samples and to verify if samples from the conventional method that were unsuccessfully be visualized were caused by unsuccessful DNA extraction. The quantification-result shows that all samples contain eDNA. This indicates that both methods have successfully extracted eDNA from water. The kit method yields higher DNA concentration for each sample and yields more than three times higher average concentration compared to the conventional method. The kit method also gives a better purity compared to the conventional method. Isolate purity is scored by the absorbance ratio of UV wavelength at 260 nm by 280 nm (A_260/280). DNA-isolate generally be called ‘pure’ if the ratio ranges from 1.8 to 2.0 [22].

Higher eDNA concentration yielded by FastDNA™ SPIN Kit for Soil might be caused by the optimized and suitability of the product for extracting DNA from environmental samples such as soil and water. On the other hand, conventional methods of DNA extraction such as the usage of phenol and chloroform or any other conventional protocols are known to produce higher DNA yield. We also extracted eDNA from water using PCI (the experiment has no correspondence with this study) and we got a significantly higher concentration of DNA up to 590 ng/µL. However, we got also very low
concentration up to 1 ng/μL. There was such an inconsistency of DNA yield range, and the difference between the lowest and the highest concentration is significant. Conventional DNA extraction using phenol and chloroform is not specific for extracting DNA from certain types of samples and may be used to extract DNA from various types of samples. On the other hand, this unspecified property may not be suitable or optimal to extract DNA from other typical sample and produces more inconsistent results although sometimes it gives better DNA yield.

FastDNA™ SPIN Kit for Soil contains Lysing Matrix E, a mixture of ceramic and silica particles designed to efficiently lyse all soil organisms such as bacterial spores, yeast, algae, and fungi. These particles make the sample be more homogenized, maximize cell lysis and may produce higher DNA yields. Actually, the usage of tungsten beads to homogenize filter membranes has similar principles to Lysing Matrix E from FastDNA™ SPIN Kit for Soil. The principle of both treatments is homogenization by bead-beating. But, the tungsten beads we used to homogenize our filter membranes were bigger than Lysing Matrix E particles. It may cause the samples were not efficiently homogenized as samples that were treated with Lysing Matrix E.

The lysis process with Lysing Matrix E particles is carried out with the presence of MT buffer and sodium phosphate buffer. Both buffers work to allow extraction with minimal risk of RNA contamination. FastDNA™ SPIN Kit for Soil also contains SPIN column that may optimize DNA purification as the DNA extraction using kit produces better purity and with 1.8 to 2.0 absorbance ratio. Although the main role of PCI is purifying the DNA, PCI excess may have a role as contaminant in DNA-isolate as it produces poor DNA isolate quality in this study and inhibits further application such as PCR and DNA sequencing. Because of the optimization by the manufacture and specified properties of kit component to typical sample type, this study shows that kit method yields higher concentration of DNA with better purity [23].

DNA amplification was successfully performed for both methods. Verification was carried out by gel electrophoresis. Samples were running on 1.5 % agarose gel (70–100 V) for 30 minutes. Each sample from the conventional method and the positive control were successfully amplified, but the 1st and the 3rd PCR products showed a very thin band. All bands appeared between the two-lower band of DNA Ladder 100 bp. This indicates that PCR products are sized between 100–200 bp. It is corresponding to the 73–110 bp PCR product of 12s rRNA-V5 targeted area [17]. This result showed that DNA isolated with poor quality of purity still can be amplified. Not all samples from the kit were successfully amplified. There were no bands on the gel for the 2nd and the 3rd sample (table 1).

Performing DNA extraction using many samples may be problematic because the kit method is quite expensive. FastDNA™ SPIN Kit for Soil for 50 preps costs USD 312.25 (nearly IDR 4.6 million per 10th of July 2019), meanwhile phenol-chloroform-isoamyl alcohol (25:24:1 for 100 mL) costs SGD 185.00 (IDR 1.92 million per 10th of July 2019). If at least 100 samples are going to be extracted, two sets of FastDNA™ SPIN Kit for Soil for 50 preps are needed, meanwhile only a bottle of 100 mL phenol-chloroform-isoamyl alcohol (25:24:1) needed. With that calculation, it costs IDR 92,000.00/reaction if FastDNA™ SPIN Kit for Soil is used, compared to IDR 19,200.00/reaction

| Water collection | DNA concentration (ng/μL) | DNA purity (A260/280) |
|------------------|--------------------------|-----------------------|
|                  | Kit                      | PCI                   |
| 1st              | 80.89                    | 27.80                 | 1.94     | 2.17     |
| 2nd              | 42.37                    | 15.90                 | 1.88     | 2.30     |
| 3rd              | 82.22                    | 6.91                  | 1.88     | 1.77     |
| 4th              | 43.63                    | 26.78                 | 1.98     | 2.74     |
| Average          | 62.28                    | 19.35                 | 1.92     | 2.23     |

Table 1. Concentration and purity of extracted eDNA.
Figure 3. Visualization of PCR product from samples extracted with PCI.
M: DNA Ladder 100 bp [Smobio]; (+): positive control; number: sampling turn. Corresponding samples are marked by the rectangles.

Figure 4. Visualization of PCR product from samples extracted with kit.
1) DNA Ladder 1 kb; 2) negative control; 3) positive control; 4) 1st sample; 5) 2nd sample; 6) 3rd sample; 7) 4th sample.

if phenol-chloroform-isoamyl alcohol is used. For such of case, it may cost almost five times more expensive if kit method is preferred. So, conventional method is still more affordable for extracting DNA with a big number of samples.

Not all samples from the kit were successfully amplified. There were no bands that appeared on the gel for the 2nd sample. Successfully amplified samples showed that all the bands appear below the lowest band of DNA Ladder 1 kb (250 bp-sized) (figure 3 and figure 4). It indicates that the PCR products are sized under 250 bp. It corresponds to the 73–110 bp PCR product of 12s rRNA-V5 targeted area. There was no band on the negative control lane, indicating that there was no contamination or non-specific amplification [24].

4. Conclusion
The kit method in this study gave a better yield of eDNA average-concentration (62 ng/µL) than the conventional method (19.35 ng/µL). The kit method also gave better purity of eDNA (1.92) compared to the conventional method (2.23). The better concentration and purity of eDNA could be advantageous for further analyses such as DNA sequencing.
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