PCR-RFLP as a detection method of allelic diversity seahorse

_Hippocampus comes_ (Cantor, 1849) from Bintan waters, Riau Island

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**Abstract.** Seahorse (_Hippocampus comes_) is a unique species which reproductive system pregnancy occur in male organisms. According to IUCN and CITES, seahorses are noted as endangered or vulnerable and Appendix II species. The aimed of this study was to identify the allelic diversity based on Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP). Samples were collected from Tanjung Berakit, Bintan waters, Riau islands. Total of 11 individuals of the species were analyzed two types of restriction enzyme, _EcoRI_ and _AluI_. This study showed that there were two types of DNA fragmentations based on restriction enzyme. _EcoRI_ enzyme was unable to cut the target DNA fragments. _AluI_ enzyme was capable of bypassing the target DNA fragment so it could be visualized by two alleles. The genetic diversity values of less than one showed that the genetic diversity of the population of seahorse species of _H. comes_ in Bintan waters was low.

**Keywords:** allele, genetic diversity, seahorse (_Hippocampus comes_)

1. Introduction

Seahorses have grouped into Pipefish, Pipehorses, and Seadragons and one of the member of family Syngnathidae. This organism has a wide range of habitat, from tropical to subtropical area. Seahorse has a unique reproductive cycle, which pregnancy occurs in male organism (Chang _et al_ 2013).

Seahorses are widely used and sold as traditional Chinese medicine and ornamental fish. Seahorses also have function in health aspect, such as increase hormonal activity, anti-tumor, anti-aging, anti-fatigue, regulation of urogenital, and reproduction (Zhang _et al_ 2003). This variations of usability make this species as one of the most important trading commodities in different countries and captured on a high scale (Zhang _et al_ 2017).
High seahorse capture causes these organisms suffer into a significant reduction, until 15-50% in the worldwide since 1990 (Vincent 1996). The decline of this species can be reduced by reducing its trade. Prevent big scale trading of seahorse are needed, because of this reason, seahorse set in the status of Appendix II by CITES, with a minimum size that can be captured and sold is 10 cm. IUCN also set seahorse as endangered or vulnerable species (Chang et al 2013).

Diversity of seahorses found in Indonesia is quite high. The types of seahorses include Hippocampus barbouri, H. bargibanti, H. comes, H. histrix, H. kelloggi, H. kuda, H. spinosissimus, and H. trimaculatus (Lourie et al 2004). Total 7 of them were found in the Bintan waters (Fianda et al 2015). This high diversity of seahorses needs to be guarded, one of the ways is formulating appropriate management measures to reduce the decline in nature's population. The first step is to start the management to develop research related to seahorses, such as molecular-based research. Molecular-based research on seahorses in Indonesia is still rare. A molecular or genetic marker is an excellent method for identifying and classifying species at a population or individual level (Carreira et al 2018). Hippocampus comes research based on molecular analysis had previously done by Nurulmala et al (2018). However, this research has not determined the population diversity based on the allele.

Allele diversity is one of the molecular research required. The diversity of Allele is a parameter used to describe the genetic diversity of a population in nature (Allendorf and Luikart 2009). One of the methods that can be used to determine the diversity of allele is restriction fragment length polymorphisms analysis of PCR-amplified fragments (PCR-RFLP). This method can amplify the target DNA, and bypass DNA fragments using the endonuclease enzyme. This cutting will be shown in smaller fragments with a base length that depends on the location of the target DNA fragments. The fragments then separated through Electrophoresis (Teletchea 2009).

This research aims to identify the diversity of allele in the population of H. comes in the Bintan waters. The diversity of allele on seahorses H. comes in the Bintan waters can be used to identify the balance of H. comes population as a basis in the management and utilization of the resource of seahorses in Indonesia.

2. Materials and methods

2.1. Materials

The main material in this research is H. comes. This seahorse is collected from the fisherman in Tanjung Berakit, Bintan, Riau Island. A total of 11 sea horses were analyzed consisting of 7 female seahorses and 4 male seahorses. Samples of the obtained seahorses have more than 10 cm size. Other materials used are alcohol 96%, distilled water, commercial kit (Qiagen Blood and Tissue), electrophoresis reagent, agarose 1.2%, buffer TAE, ddH2O, PCR reagent, and RFLP Enzymes (Alul and EcoRI). Pieces of equipment in this research are tube 1.5 mL, microtube PCR, digital scales, vortex, micro tip, micropipette, Spin GD column, freezer, pistle, incubator, centrifuge machine, UV visual machine, PCR machine, and electrophoresis horizontal machine.

2.2. Methods

2.2.1. Genetic data collection. Preparation is the first method in this research. Preparation aim is to clean the sample tissue. Seahorse samples were cut from head to tail then the abdominal and tail tissue was taken and inserted into the 1.5 mL tube. The samples then washed with aquades and dried. After that, samples then weighed 25 mg and continued into the total DNA isolation process.

2.2.2. DNA isolation and extraction. Total DNA Isolation performed to separate DNA samples from other components. Total DNA isolation was done by a manual procedure of the commercial kit Qiagen
Blood and Tissue with modifications. The total DNA isolates products are subsequently processed into the DNA quality test process.

2.2.3. **DNA quality test.** This process was tested by electrophoresis process. Electrophoresis was performed with 1.2% agarose gel and 50 mL TAE 1× buffer. Total DNA isolates were migrated to the electrophoresis chamber with 100 V voltage for 23 minutes. Total DNA visualization was done by ultraviolet (UV) machines. Good quality of total DNA then processed into the amplification phase.

2.2.4. **DNA fragment amplification and visualization of the 16S rRNA gene.** Amplification is a procedure to cut and multiply DNA fragments to fit the target gene. The stage of amplification in this research was done by PCR method which refers to Joshi and Deshpande (2010) with modifications. The kit that has been used was the MyTax commercial kit, and it was repeated 35 times. The primer used in this research was 16S rRNA primer, which is a universal primer that can be applied to some aquatic biota. Amplification has several stages with specific temperature and time. Predenaturation was performed at a temperature of 94°C for 3 minutes. The denaturation stage was performed for 45 seconds at 94°C. The annealing stage was performed for 45 seconds at 46°C. Elongation phase is done for 1 minute at 72°C. The next stage is the additional stage, the post step elongation for 5 min at 72°C. The PCR results then stored at 8°C for 10 minutes. Product of this amplification then retested with DNA quality test process.

2.2.5. **Cutting and visualization of DNA fragments.** Amplicon DNA cuts are carried out with EcoRI and AluI restriction enzymes. The DNA of the PCR process was incubated at 37°C for 4 hours. The separation of DNA fragments was done by electrophoresis and then visualized on ultraviolet machine.

2.2.6. **Genetic data analysis.** The genetic diversity parameters are used in this research data analysis. Calculated parameters consist of haplotype diversity, haplotype frequency, and heterozygosity. Mathematical equations of genetic diversity based on Suryo (1984) as follows.

\[
H = n \left(1 - \sum_{i=1}^{n} p_i^2\right) / (n-1)
\]

Notes:
H : haplotype diversity
\(p_i\) : haplotype frequency of each sample
n : number of haplotypes in a sample

\[
p_i = \frac{n_i}{N}
\]

Notes:
\(p_i\) : haplotype frequency of each sample
\(n_i\) : frequency of the allele
N : total samples in one population

\[
He = 1 - \sum_{i}^{n} p_i
\]

Notes:
He : heterozygosity
\(p_i\) : frequency of the allele
3. Result and discussion

3.1. Biology of seahorse H. comes (Cantor 1849)

H. comes has yellow and black colour. The color of this type of seahorse can change according to the environment, or when going through the spawning period. This yellow and black color forms a line and interchangeable with each other (Darmawan et al. 2015). seahorse type of H. comes displayed in figure 1.

The reproductive system of seahorses is unique, which pregnancy occurs in male organisms. The pregnancy cycle of this male sea horse occurs after a sea horse receives an egg from the female. The eggs that have been received in the curling pockets are then fertilized and can develop in optimal habitat conditions (Chang et al. 2013).

![Figure 1](image-url)  
**Figure 1** Hippocampus comes (a) male (b) female (personal documentation).

According to the IUCN (2013) Classification of the H. comes are as follows.  
Kingdom: Animalia  
Phylum: Chordata  
Class: Actinopterygii  
Order: Syngnathiformes  
Family: Syngnathidae  
Genus: Hippocampus  
Species: Hippocampus comes (Cantor, 1849)  
Common name: Tiger tail Seahorse

Seahorses are generally occupying temperate to tropical waters. This species can be found in coral habitats, macroalgae, mangrove roots, seagrass, or water with an open sandy base to muddy. Certain species can be found in the estuary or lagoon. The abundance of seahorses in each habitat tends to be low (Lourie et al. 2004).

Seahorse food is a small animal (according to the mouth openings) that moves, especially crustacean and small fish. This species is an active predator. Male seahorses will tend to eat plankton when experiencing a phase of embryo development in his pocket. This is due to the slow-motion speed so it is impossible to capture bigger and faster prey (Vincent 1996).

3.2. Total DNA isolation

The total DNA isolates that visualized in agarose are shown in figure 2a. Total DNA of 11 seahorse samples characterized by the presence of smears on each of the electrophoresis agarose wells. The
principle of electrophoresis is the separation of fragments based on molecular size, smaller fragments will migrate away from agarose wells, while large fragments will be close to agarose wells. This causes a smear in the agarose gel. Smear indicates that DNA isolates are heterogeneous. Fragments between DNA targets and non-target DNA are mutually joined to form smears (Nicholl 2008). This indicates that the total DNA isolates obtained have a low purity level.

3.3. Amplification and visualisation DNA fragment 16S rRNA gene
The annealing temperature (46°C) produced a good DNA fragment amplification product of the 16S rRNA gene, indicated by the presence of one ribbon in each agarose well. The result of target gene measured is 600 bp. The base length for intact genome 16S rRNA is about 1500 pb (Myer et al 2016). This indicates that the amplification result corresponds to the length of the gene base 16S rRNA.

3.4. Restriction enzyme cutting pattern
The EcoRI restriction enzyme was not able to produce different allele cutting types, which can be called a monomorphic type (figure 3a). AluI enzymes can produce different enzyme cutting patterns or called dimorphic type. The enzyme AluI cuts the DNA fragment at a base length of 250 and 350 BP (figure 3b). The enzyme-based cutting type is illustrated in figure 4.

![EcoRI enzyme cutting pattern](a), and AluI enzyme cutting pattern (b), M = marker, A, B, C, E, F, G, H = female, and D, I, J, K = male.
Different enzymes are used to demonstrate difference DNA cuts of male and female seahorses. It was done to saw the difference in the seahorse based on molecular about the production system. Results showed that the EcoRI and AluI enzymes produced a uniform cutting pattern between males and females. This is possible because 16S rRNA genes are not the gene sequences derived from the genital Koromosom (Suryo 1984).

Chromosomes in an organism are divided into autosomes (body chromosomes) and genital chromosomes (Suryo 1984). An overview of the autosomal can provide information about the chromosome of an organism as an individual. Differences in genital chromosomes can be used as useful information for conservation (Allendorf and Luikart 2009).

A specific nucleotide (restriction site) is not contained in amplification products for the EcoRI enzyme causing none allele separation formed in the amplification product that cut by EcoRI enzyme (Pingoud and Jeltsch 2001). AluI enzyme produces an allele separation pattern indicating that there was a specific nucleotide in its amplification product. This can be caused by an AluI enzyme capable of producing a good DNA cutting pattern (Vereijken et al 1975).

![Figure 3 Cutting pattern of restriction enzyme, M=marker, A= EcoRI, B= AluI.](image)

### 3.5. Number of haplotype

Haplotype differences are different types of DNA site cuts. Different haplotypes of both enzymes can be produced from this study. Table 1 provides differences in haplotypes result from cutting patterns with EcoRI and AluI restriction enzymes.

| Restriction Enzyme | Cutting Pattern | Number of Haplotype | Σ sample |
|---------------------|-----------------|---------------------|----------|
| EcoRI               | A               | 1                   | 11       |
| AluI                | B               | 2                   | 11       |

### 3.6. Population's genetics

The diversity of haplotype *H. comes* of the Bintan waters based on the EcoRI enzyme presented in table 2. The calculation of heterozygosity based on EcoRI is less than one. It is shown that the genetic diversity of the seahorse type *H. comes* is low. The diversity of the *H. comes* haplotype in the Bintan waters based on AluI enzymes presented in table 3. The intensity of heterozygosity with the enzyme AluI is less than one. It is shown that the genetic diversity of the seahorse type *H. comes* is low.
Table 2. Haplotype diversity of *H. comes* in Bintan waters based on EcoRI enzyme.

| Parameters                | Value |
|---------------------------|-------|
| Σ Sample                  | 11    |
| Σ Haplotype               | 1     |
| Haplotype Frequency       | 1     |
| Heterozigosity            | 0.0   |
| Haplotype Diversity       | 0.0   |

Table 3. Haplotype diversity of *H. comes* in Bintan waters based on AluI enzyme.

| Parameters                | Value |
|---------------------------|-------|
| Σ Sample                  | 11    |
| Σ Haplotype               | 2     |
| Haplotype Frequency       | 0.5   |
| Heterozigosity            | 0.5   |
| Haplotype Diversity       | 0.2   |

Cutting of the resulting enzyme comes from autosomes. An overview of autosomes is used to obtain information that can be used as a determinant of a population's genetic diversity (Allendorf and Luikart 2009). The genetic diversity and population genetic structure is the result of complex interactions between species in certain geographical ranges and dynamics (Parra *et al* 2018). Genetic diversity can show estimates of genes that reside in a population of certain organisms, which can indicate an estimate of phenotype variation in a population. The value of heterozygosity levels can describe genetic diversity in a population (Irawan 2010). The heterozygosity of *H. comes* in the Bintan waters based on the EcoRI restriction enzyme was 0.00, and 0.50 based on AluI restriction enzyme. It shows that the genetic diversity of *H. comes* in the Bintan waters is relatively low. It is also reinforced with the results of a low-value haplotype diversity parameter, with a value of haplotype diversity based on EcoRI of 0.00, and AluI of 0.2.

The low genetic diversity of *H. comes* in the Bintan waters can be caused by the seahorse adaptation to a highly fluctuating environment, which involves the selection of a flexible genotyping against variations in environmental change that allows the survival of organisms (Rodrigues *et al* 2015). It is also supported by research (Pratomo and Irawan 2015), that the seahorse in the Bintan waters is found on the sidelines of macroalgae with varying environmental conditions.

Large, abundant, and long-lived species have a high likelihood of genetic variation. Meanwhile, the species has a low distribution and abundance, and a relatively long age has low genetic variability (Parra *et al* 2018). According to Pratomo and Irawan (2015), Seahorse distribution is not extensive. The seahorse has the characteristics of abundance, mobility, and low habitat Distribution (Vincent 1996). The spread and density of the seahorses also relatively low (Lourie *et al* 2004). It can also be the cause of the diversity of the *H. comes* in the Bintan waters low.

The expansion of the distance between populations becomes one of the causes of decreasing genetic diversity and increasing genetic differences between populations due to increased distances. In addition, fluctuating climate can also cause significant changes in population abundance as well as genetic diversity (Parra *et al* 2018). Genetic diversity in a population plays an important role in the population's response to natural selection or other disorders. Populations that have high genetic diversity have a greater chance of sustaining their population life from extinction (Yusron 2005). Therefore, there needs to be a conservation effort to improve the genetic diversity of the population *H. comes* in the Bintan waters.
Genetic conservation was done to prevent the extinction of a population. The causes of extinction can be habitat damage, pollution, high catch, species translocation, and also climate change. Other causes may be random changes in genetic structures (genetic drift) and increased inbreeding processes (Allendorf and Luikart 2009). The decline in seahorse populations is generally caused by habitat damage, excessive arrest, illegal smuggling and trading, high population decline, lack of oversight and enforcement, and the lack of research on seahorses (Sadili et al 2015).

Conservation efforts can be made in order to have a population of *H. comes* in Bintan waters. First, the management and restocking species have a high resistance to environmental change. Second, the addition of *H. comes* to research on biologic conditions (life cycle, parent size, parental care, and sea horse spreading) in order to be formulated with the right conservation measures. Third, improved surveillance of the arrest and trading of *H. comes*.

4. Conclusion

The result of the value of heterozygosity levels was less than one, indicating that a population of *H. comes* in the Bintan waters has low genetic diversity. Conservation efforts need to be undertaken. This was done to prevent the seahorse population, especially *H. comes* in Bintan waters did not suffer extinction.

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