ISBN : 978-602-72574-5-0
Aplikasi Teknologi Sebagai Solusi Di Bidang Perikanan Secara Berkelanjutan
Seminar Nasional Perikanan Indonesia 19-20 November 2015, STP JAKARTA

PHYLOGENETIC EVIDENCE OF CRYPTIC SPECIES IN GIANT FETAHERBACK (*Chitala lopis* Bleeker 1851)[1]

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ABSTRACT

The successes of biology conservation effort rely on the ability to identify species level and determine of the population unit. Molecular marker can assist identification population unit precisely. Molecular technique revealed hidden diversity on species with similar morphology performed. The typical diverse specific habitat of giant featherback (reservoir, floodplain, tributary and river) and geography separation of the species have been contributed to the existed of the cryptic species. Research objective is to reveal the evidence cryptic species of giant featherback, in order to make contribution either for *in-situ* and *ex-situ* conservation and adding more information on giant featherback's biodiversity for breeding purpose. Sequence data were obtained from *Cytochrome Oxidase Subunit I (COI)* and *Cytochrome b* mtDNA. Samples total are 28 individuals collected from five different river. We compare 5 samples of *Chitala lopis* and *Notophterus notopterus* sequences from *Genebank* as standard samples. Results reveal molecular filogeny analysis from COI and Cyt b nucleotide of mtDNA indicated that Indonesia *Chitala lopis* can be distinguised minimal four clusters as cryptic species, which were *Chitala lopis,* Borneo, giant featherback (totol) dan giant featherback in acid environment. The evidence cryptic species added the importance of molecular analysis techniques beside morphological whilst cataloge biodiversity and determine conservation prioroties.

Keywords: *Chitala lopis,* mtDNA and cryptic species

INTRODUCTION

The success of conservation biology relies heavily on the ability to identify species and to descript the units of the population. This subject is closely related to systematic biology, the field that provides scientific names for organisms, describes them and provides classifications for the organisms, keys for their identification, data on their distributions and considers their environmental adaptations (Wheeler and Meier, 2000). The need for a strategic approach to the management of species has become urgent to maximise the effectiveness of conservation efforts, molecular markers can be used in the identification of management units, including migration patterns and life history strategies, such as the giant tortoise, Komodo dragon (Ciofi *et al.,* 1999).

There have been a number of major advances in molecular biology in the past few years, providing conservation biologists with additional instruments as species-diagnostic characters for accurate identification of organism(s) other than just to rely on morphological characters. Recent developments in the application of modern genetic techniques have revealed hidden species diversity of morphologically similar species (Remeire *et al.,* 2006). Molecular phylogeny analysis uncovers possible hidden cryptic diversity (disguised) in numbers of fish species (DeSalle *et al.,* 2005; Colborn *et al.,* 2001; Miya and Nishida, 1997) and unusual levels of intra-specific differentiation, more than 3%, among cryptic species (Lee, 2000). These cryptic species look very similar morphologically that they are hardly be distinguished solely by morphological characters. Cryptic species appears to represent distinct evolutionarily significant units (ESUs) for conservation, rather than accumulation of morphologically similar species, which may not be closely related to each other (Crandall *et al.,* 2000, Bickford *et al.*, 2007, Page *et al.,* 2007).

[1] Makalah dipresentasikan pada Seminar Nasional Perikanan Indonesia Sekolah Tinggi Perikanan, Jakarta, 19-20 November 2015

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Giant featherback is a member of the class Actinopterygii (*ray-finned fishes*), ordo Osteoglossiformes (*bony tongues*), family Notopteridae (*knife fishes*), genus Chitala dan species *Chitala lopis* (Kottelat *et al.,* 1997). There is a paucity of information with regard to current knowledge on taxonomy information for *Chitala lopis* from Indonesia, however Kottelat and Widjanarti (2006), tentatively mentioned Indonesian chitala as *C.hypselonotus, C. borneensis, C. lopis dan Chitala sp*., whereas *Chitala hypselonotus* dan *Chitala borneensis* have been found in Sumatra.

Giant featherback is distributed in the Sunda Shelf, the fish is native to reservoirs, lakes swamps, creeks and rivers. The site-specific environmental parameters for giant featherback, such as total dissolved solids (TDS), conducivity (DHL), air temperature, chlorophyll, current, biological oxygen demad (BOD), Oxygen, pH, alkalinity and carbon dioxide (CO2) (Wibowo *et al.,* 2009). Geographical separation and severe climatic condition became well known as stimulants on the process of cryptic species formation (Lefebure *et al.,* 2006; Bickford *et al.,* 2007).

The geographical separation and site-specific environmental parameters are believed to create a cryptic diversity in giant featherback. The objective of our study is to reveal the presence of cryptic species in giant featherback contributing to the in situ and ex situ conservation. This information is important for breeding program to improve and to strengthen aquaculture production.

MATERIALS AND METHODS

1. Specific sampling frame and sampling sites

The fresh tissue, muscles and blood sample preserved in 96% ethanol, these samples were incubated in room temperature during transportation and instantly frozen after returning to the laboratory before proceeding with DNA extraction and purification procedure.

Cytochrome b were analyzed in 14 samples of DNA from three different rivers (Kampar/Riau, Indragiri Hilir/Riau and Mahakam/East Kalimantan in Indonesia during 2006-2009 and 14 COI sequence data covering different river obtained from Wibowo (2014). Isolation, extraction, purification, amplification and DNA visualization were conducted in 2011 at Molecular Biology laboratory, Animal Science, Bogor Agricultural University and DNA sequences are performed by *Macrogen Corporation* in South Korea.

2. Isolation, extraction and purification DNA

DNA extraction using modified *Genomic DNA mini kit for blood (Geneaid)*. The Modified part is in destruction of tissue with additional of SDS and K Proteinase (Muladno, 2006). Blood cell sperm and blood of Giant Featherbacks are stored at alcohol absolute then washed with distilled water (molecular grade) twice and then they are suspended in STE buffer (NaCl 1M, Tris-HCL 10mM, EDTA 0.1mM, pH 8) until 350 µl. Blood cell is broken with 1% SDS and K Proteinase 0.125 mg/ml in 55^{o}C temperature for overnight while shaking slowly in the rotary. DNA extraction method then followed the instruction of *Genomic DNA mini kit for fresh blood (Geneaid)* (company instruction). DNA samples obtained are stored at 4^{o}C.

3. Amplification and visualization of mtDNA fragments

Amplification of partial fragment *Cytochrome Oxidase Subunit I (COI)* mtDNA used universal primer Ivanova *et al.* (2009) COI F (5' – TCT ACC AAC CAC AAA GAC ATC GG 3') and COI R (5' – TAC TTC TGG GTG TCC RAA GAA TCA 3'). While primer was used to amplified the complete gen fragment *Cytochrome b* (1140) were: L15930 (forward): 5´-CTT CGA TCT TCG rTT TAC AAG-3´. H14724 (reverse): 5´-TGA TAT

GAA AAA CCA TCG TTG-3 from Lavoue and Sullivan (2004). The Composition of PCR reaction was conducted with 50 µl final volume consisted of 5 µl DNA samples, 16 µl sterile DW, each primer 2 µl and 25 µl *Taq ready mix*. PCR reaction is performed using *PCR-Applied Biosystems* machine with conditions subsequent: stage pradenaturation 95°C for 10 minutes,the second stage consisting of 30 cycles, each of which include a denaturation stage 94°C for 1 minute, annealing at temperature 48 °C for 1 minute, extension at 72 ° C for 1.5 minutes, and the last stage is the elongation of the end (final extension) at 72 ° C for 7 minutes. PCR products are tested using a 6% PAGE in TBE 1x buffer (10 Mm Tris-HCl, 1 M boric acid, and EDTA 0.1 Mm) which run at 200 Mv conditions for 50 minutes. Further DNA was stained by sensitive silver colour (Tegelstrom, 1986). Sequences of DNA samples were conducted on two ways (forward and reverse) and sequence of DNA was performed by Macrogen Corporation in South Korea

4. Data analysis

Homologous side sequences of nucleotide base sequences of genes Cytochrome Oxidase Subunit I (COI) and Cytochrome b Giant Featherbacks mitochondrial DNA obtained, and then were juxtaposed (multiple alignment) are compared with sequences of genes Cytochrome Oxidase subunit I (COI) and Cytochrome b giant featherbacks (*Chitala lopis*) and Bronze Featherbacks (*Notopterus notopterus*) from Genbank completely. Sequence chromatograms displayed and edited manually used Bioedit (Applied Biosystems, Foster City, CA, USA). After the editing process, conducted multiple allignment using Clustal X 1.81 (Thompson et al., 1997). Analysis of kinship based on the nucleotide sequences, conducted using the program MEGA version 4.0 (Tamura et al., 2007) with the Bootstrapped Neighbour Joining methods with 1,000 repetitions.

Results and discussion

Cytochrome b gene amplification produces Cyt b gene fragments range from 606 - 1149 bp at position of 15008-16601 bp by Genbank reference, DNA amplification product profiles are presented in Figure 1. Phylogeny reconstruction of Cyt b and COI nucleotide bases sequences of giant featherbacks samples and their relatives are presented in Figure 2 and 3.

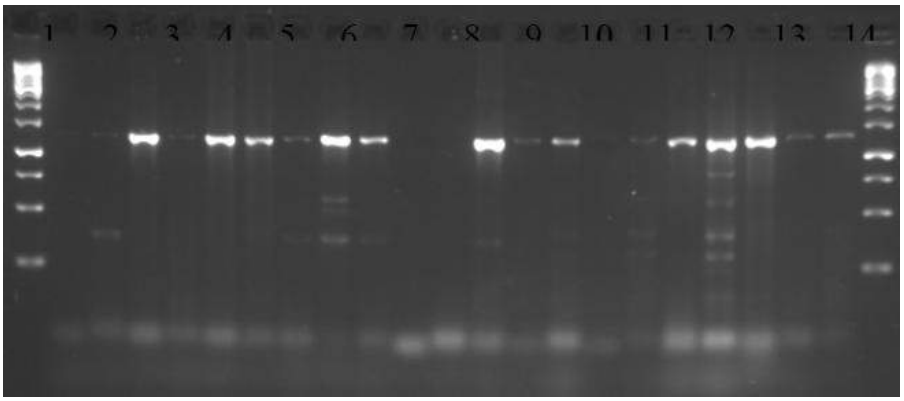

Figure 1. Profile of Giant Featherbacks DNA sample amplification product Cytochrome b using primers L15930 and H14724.

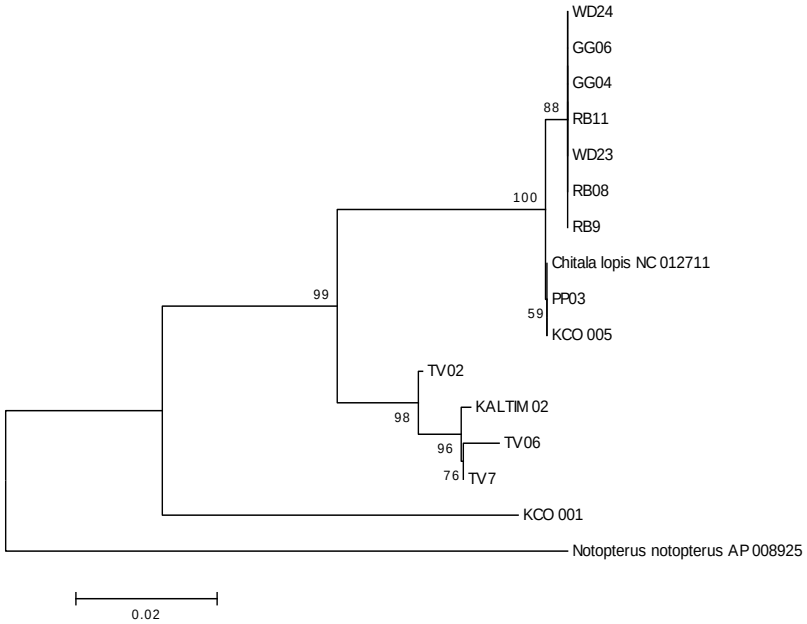

Figure 2. The Cyt b phylogeny reconstruction of giant featherbacks and their relatives comparison from *Genbank.*

The filogram shows that there are two groups of species, as described by Kottelat et al (1997), namely: *Chitala lopis* and *Notopterus notopterus*. The Cyt b fragments genes and COI gene fragment identifies the existence of four cryptic species in the species group *Chitala lopis* namely: *Chitala lopis* clusters, Borneo clusters, large size Giant Featherbacks clusters (> 30 kg / per tail) with a red pattern on the tail (spot) and clusters that live in acid waters, Figure 4.

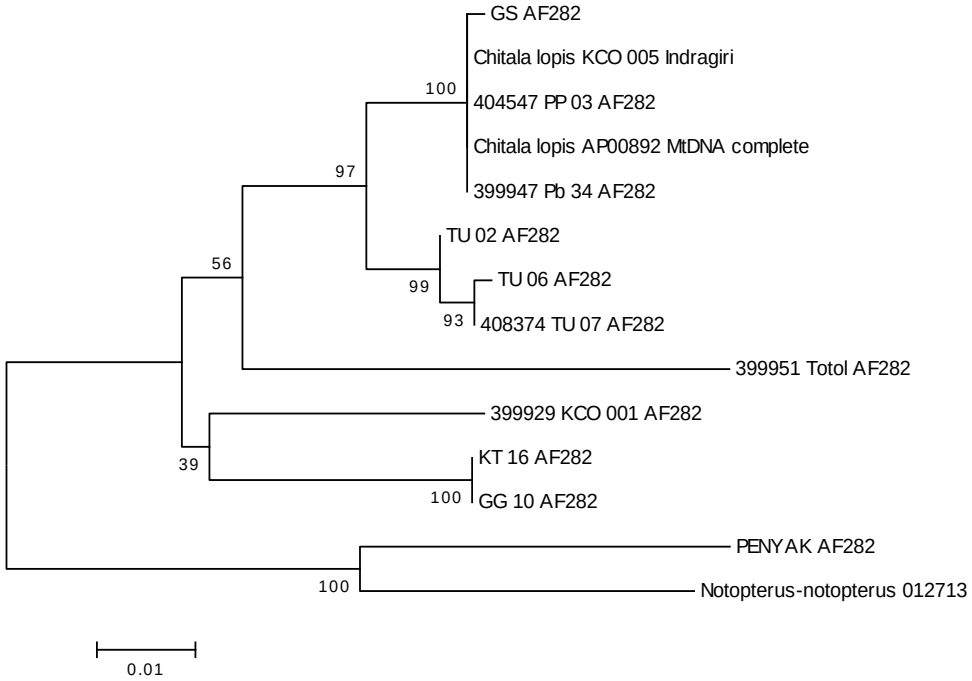

Figure 3. The COI phylogeny reconstruction of giant featherbacks and their relatives comparison from *Genbank*

Chitala lopis Cluster

Borneo Cluster

Spot Cluster

Acid Water Cluster

Figure 4. Phenotype of cryptic species of giant featherbacks

The discovery of cryptic species significantly increased with the development of molecular phylogeny analysis techniques (Harris and Froufe, 2005). In the complex species, as detailed morphological comparisons made, often found a small but clear differences. Furthermore, the new species (pseudo-cryptic species or pseudo-sibling) can be formally described (Saez and Lozano, 2005). Identification of cryptic species is an activity that is very important to do that effectively protects valid taxa evolution in program planning conservation of freshwater fish resources.

Phylogeny analysis of Giant Featherbacks on mtDNA Cyt b and CO1 nucleotide sequence indicates that *Chitala lopis* sample collection is divided into four groups. The group that emerged in addition to *Chitala lopis*, seems to have undergone a process of self-selection and length in time. As well as informed by Kottelat et al. (1997) giant featherbacks group, has several species morphologically indistinguishable.

The cryptic species classified taxonomically difficulty, this is because the diagnosis of morphology is a very important part "as standard" in the procedure of classification of animals. Realizing that many miniature fish, has diagnostic morphological characters that can not be distinguished, the number of species which are not named and cryptic will increase in line with the increasing number of DNA-based research. This means that more and more discovered cryptic species are officially yet to be named and accommodated in relation to conservation biology. It seems appropriate, if in addition to standard taxonomic procedures, identification using DNA should be done to taxa morphologically indistinguishable, although until now there is no agreement the use of this technique in the description of species (Moritz and Cicero, 2004).

Research reveals cryptic species of giant featherbacks identified in the ecosystem of the river (Kampar River). The diversity of species in the river has been reported in freshwater fish species and their importance for conservation (Dudgeon et al., 2006). These cryptic species usually endemic in specific areas, we found that clusters of acidic waters, specifically are found in the Kampar River system which has acidic waters. Berendzen et al. (2008), reported rosy face cryptic species, genus Notropis endemic in certain parts of the creek in the Mississippi River, USA.

Cluster cryptic species that adapt to the acidic environment, so perhaps genetically different but in the same morphology. Because in extreme environments (acidic environment), the organism will vary widely believed that restricted how they can adapt. They will adapt a psychic, leads to morphological static conditions (Bickford et al., 2007). Cluster spots cryptic species, large Giant featherbacks (> 30kg / per tail) despite living in the same habitat, but is thought to have evolved as a species sibling for example caused due to differences in mating signal (Bickford et al., 2007).

Cryptic species clusters of Giant featherbacks Borneo groups are clearly visible on both markers (CO1 and Cyt b) and also control region (Wibowo, 2010). Separation or isolation are the precursor in the formation of cryptic species (Bickford et al., 2007). Widespread physiological and behavioral adaptations in the evolution of these organisms may be very important in explaining the lack of morphological changes associated with adaptation to local habitat, diversifying population and cryptic speciation.

Cryptic species with a limited range of endemic, should really realize when implementing any system of conservation and management strategies. Our studies reveal the importance of adding a taxonomic description is only based on morphology with molecular analysis, when catalogs diversity and conservation priority determiner.

CONCLUSION

Molecular phylogeny analysis on the nucleotide sequence of mtDNA Cyt b and CO1 indicates that *Chitala lopis* Indonesia *Chitala lopis* has at least four groups that are cryptic species, namely the *Chitala lopis* cluster, Borneo cluster, large Giant featherbacks cluster (spots) and acid water cluster.

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