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MOLECULAR IDENTIFICATION OF INDONESIAN-ORIGIN *CHANNA STRIATA* USING MULTIPLEX-PCR

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ABSTRACT

Striatin is a bioactive protein fraction extracted from *Channa striata* fillet. The identity of raw material of *C. striata* is a critical issue that must be confirmed for the consistency of its quality. PCR-based identification technique is prospective to be used to get accurate identity of *C. striata*. Till date, there are no specific primers been developed to identify fillet of *C. striata*. In this study, we have developed a specific pair of primer for identification of *C. striata*, namely CSTR primers. The CSTR primers were designed based on cytochrome c oxidase subunit 1 (COI) gene sequence. They were combined with beta actin (BAM) primers as internal control. The PCR assay produced two amplicons i.e. CSTR product of 269 base pairs (bp) and BAM product (138 bp) at the optimum annealing temperature (Ta) of 50°C. Seven samples of *C. striata* from several areas in Indonesia were positively amplified by those primers, while others popular freshwater fishes in Indonesia (nile tilapia, common carp, catfish, mozambique tilapia, pangasius and giant gouramy) and a brackish water fish (milkfish) as negative controls only produced 138 bp DNA fragment. It indicates that the CSTR primer pair detects specifically *C. striata*. Therefore, PCR technique using these specific primers is potentially applied for a routine analysis of *C. striata* for Striatin in the production process.

KEYWORDS: ***Channa striata, Beta actin, Striatin, Polymerase Chain Reaction, cytochrome c oxidase subunit 1***

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INTRODUCTION

Channa striata or snakehead murrel from family *Channidae* is an indigenous predatory fish in Southeast Asia, South Asia and China.[1] In Indonesia, it is widespread from Sumatera to Papua.[2] *C. striata* is commonly found in the ditches, swamps, lakes, paddy fields, irrigation canals, small streams, mining pools and old ponds. It has some characteristics like air breathing ability, hardiness and high tolerance to adverse environmental conditions.[3-4] The fish is usually consumed as daily food and has high value for medicinal purposes such as wound healing, postpartum meal, antipain, antifungal and antibacterial, anti-oxidant, anti-inflammatory and antipyretic, cardiological or hematological treatments, as well as neurological and neurophysiological therapies.[5] Commercial medicinal products of *C. striata* produced using biotechnology are Posafit and Inbumin both developed by Dexa Laboratories of Biomolecular Sciences (DLBS), PT Dexa Medica, a pharmaceutical company in Indonesia which explore various natural compounds for medicinal applications.[6-9] Posafit and Inbumin are potentially applied to increase serum albumin level for hypoalbuminemia condition and serve as nutritional support for post-operative and postpartum patients. These products contain Striatin, a bioactive protein fraction isolated from *C. striata* fillet. The product consistency of Posafit and Inbumin is determined by the quality of Striatin, thus appropriate raw materials of *C. striata* are strictly required. Therefore, it is necessary to confirm the identity of *C. striata*. A common way to identify fish species is based on external morphological features, including body shape, feature of the head, the number and relative position of fins, the number and types of rays composing of fins, lateral line, scales and other dermal structures, pigmentation and color patterns.[10] Identification using those methods needs specific skill and experience and is highly subjective. On the other hand, in the market, *C. striata* is not only available as intact fish but also as a fillet. The physical appearance of some fish fillets is similar to that of *C. striata*. Hence, it requires a more objective and easy way to identify the fish and fillet such as by a molecular technique particularly when used as a routine assay. In addition, this technique has more advantages in terms of reliability, speed, accuracy and sensitivity.[11-12] Usually, the target of DNA fragments for animal identification is mainly originated from mitochondrial genome, i.e. COI, cytochrome b, 16S rRNA, 12S rRNA genes and mitochondrial D-loop region.[13] The COI gene sequence is reliably used to identify at species level where interspecific variation (differences between species) does not overlap intraspecific variation (the differences within species).[14] Identification of fish species is conducted by amplification of DNA fragment in COI gene with a primer pair that recognizes conserved region. The amplification product is sequenced and the sequence is aligned with those deposited in GenBank. However, it is not a quick and simple process, for laboratories that are not equipped with DNA sequencer should obtain the data from the external service laboratory. Therefore, specific primers that can specifically identify *C. striata* without DNA sequencing offers a better solution to overcome the problem. Unfortunately, to date no specific primer has been developed to identify *C. striata*. Therefore, in this study, we developed a multiplex PCR by using primers derived from COI gene and BAM primers as an internal control for specific identification of *C. striata*.

MATERIALS AND METHODS

Materials

The fishes of *C. striata* were collected from seven areas in Indonesia comprising IND (Indramayu, West Java), BAN (Bandung, West Java), PAN (Pandeglang, Banten), MUS (Musi Banyuasin), South sumatera), TAN (Tanah laut, South Kalimantan), OGA (Ogan Ilir, South Sumatera) and LAM (Lamongan, East Java). Other types of fish including nile tilapia (*Oreochromis niloticus*), common carp (*Cyprinus carpio*), catfish (*Clarias batrachus*), mozambique tilapia (*Oreochromis mossambicus*), pangasius (*Pangasius pangasius*), giant gouramy (*Osphronemus goramy*) and milkfish (*Chanos chanos*) that were obtained from local markets in Bogor and Bekasi (West Java), were used as negative controls.

DNA isolation

The fish fillet was frozen in -80°C for an hour and crushed into powder. The DNA was extracted using UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, USA). Approximately 200-500 mg of sample powder was homogenized with 300 µL MicroBead Solution. The mix solution was transferred into MicroBead tube. Fifty-milliliter of solution MD1 was added into the tube. The MicroBead tube was incubated at temperature of 65°C for 10 minutes with occasional bump vortexing for a few seconds every 2-3 minutes. The aqueous supernatant was collected by centrifugation at 10,000 x g for 30 seconds and carefully transferred to a new 2.0 mL collection tube. A hundred mL of solution MD2 was added then inverted several times, incubated at temperature of 4°C for 5 minutes and centrifuged at 10,000 x g for a minute. Three hundred milliliters of the supernatant were then added to 900 mL of solution MD3. The mix solution was pipetted into the spin column and centrifuged at 10,000 x g for 30 seconds. Three hundred-milliliters of solution MD4 were then added to the spin column, and then the mixture was centrifuged as described above. Thereafter, each flow-through was discarded. Fifty-milliliters of solution MD5 were added to the spin column, centrifuged at 10,000 x g for a minute and the flow-through was retained for PCR.

Primers design

The CSTR-F (5'-GTCATTCCTGCTTTTACTAGC) and CSTR-R (GTAGAAGTAGTAC GGCTGTAA) primers were designed based on conserved region present in 43 COI gene sequences from *C. striata* deposited in GenBank (Accession number JF781193 - JF781225 and JN695688 - JN695697), and absent in twelve COI sequences from other *Channa* families (*C. lucius* - KJ937433, *C. pleurophthalma* - KJ937345, *C. barca* -. KJ847151, *C. burmanica* - KJ937381, *C. marulius* - JX983244, *C. bankanensis* - KJ937371, *C. asiatica* - KC819602, *C. orientalis* - KJ936643, *C. argus* - KJ937427, *C. maculata* - KJ937459, and *C. micropeltes* - KJ937458) and six popular freshwater fishes in Indonesia (*O. niloticus*- KU565860, *C. carpio* -

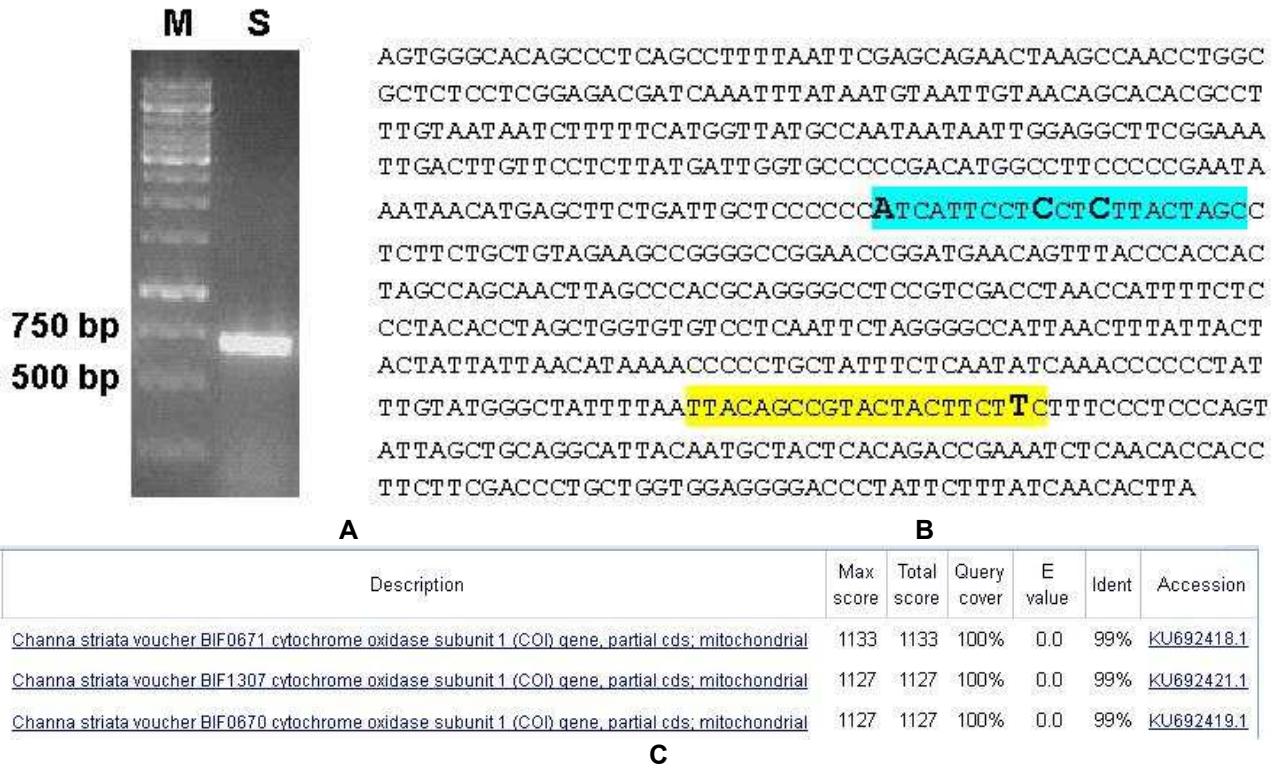
JX983284, *C. batrachus* - KF604653, *P. pangasius* - KM232626, *O. goramy* - KU692701 and *C. macropomum* - KU692455). All sequences were aligned by multiple sequence alignment using Muscle program (http://www.ebi.ac.uk/Tools/msa/muscle/), then the PCR primers were generated manually. The primer specificity was determined with Primer-blast program (http://www.ncbi.nlm.nih.gov/tools/primer-blast). All primer sequences were tested for the potential secondary structure and dimer formation using Oligo Analyzer 3.1 program (https://sg.idtdna.com/calc/analyzer).

Polymerase chain reaction

The universal primers of COI gene of fish i.e Fish F1 (5'-TCAACCAACCACAAAGACATTGGCAC-3') and Fish R1 (5'-TAGACTTCTGGGTGGCCAAAG AAT CA-3') were used to ensure the identity of snakehead fish in this study, i.e *C. striata*.[15] The PCR product was sequenced and analyzed with Basic Local Alignment Search Tool (BLAST) method. Meanwhile, the CSTR primer pair was combined with BAM primer pair as an internal control of PCR comprising BAM-F (5'-AGAGGGAAATCGTGCGTGAC-3') and BAM-R (5'-CAATAGTGATGACCTGGCCGT-3') to obtain an accurate result. PCR reactions were carried out using a Biometra® T3000 thermocycler. A typical 20 µL PCR reaction contained 1x GoTaq® Green Master Mix (Promega, USA), 5 pmol of each primer, 2 µL DNA samples (5 – 40 ng/µL) and Nuclease free water up to 20 µL. The thermal cycles consisted of 10 minutes initial denaturation at 95°C, followed by 35 cycles of denaturation at 94˚C for 30 seconds, annealing for 30 seconds, and extension at 72°C for 30 seconds. Final extension was carried out at 72°C for 10 minutes. Five microliters aliquot of each amplified PCR product was subjected to electrophoresis on a 2.0% of agarose gel stained with NEW EcoDye™ DNA Staining Solution (Solgent, Korea), and visualized on a Gel Doc (Biorad, USA).

RESULT AND DISCUSSION

Fish DNA barcoding based on the sequence of COI gene has received significant interest in taxonomy study and an accurate tool for species identification.[16-17] Fish F1 and R1 primers developed from COI gene have been used for fish identification. It amplifies a DNA fragment approximately of 700 bp.[15,18] The fish primers were used to ensure the identity of snakehead fish in this study are *C. striata*. The primers positively amplified COI gene of *C. striata* from Pandeglang, Banten (PAN). Based on the analysis of PCR product nucleotide sequence using BLAST method, it showed 99% identical to *C. striata* with query coverage of 100% (Figure 1-A, 1-B and 1-C). Therefore, PAN could be used as a positive control.



A) Agarose electrophoresis gel of PCR product of PAN using Fish primers. M) 1 kb DNA ladder. S) PAN; B) COI gene sequence of PAN; C) BLAST analysis result of COI gene sequence of PAN. The highlighted letters are sequences recognized by the CSTR primers.

Figure 1
Confirmation of PAN based on COI gene sequence.

Identification of *C. striata* using fish primers is not a quick and simple process because it requires sequencing of the PCR product. In order to simplify the identification of *C. striata*, a specific pair of primers was designed, namely CSTR. The primers were designed based on conserved region in 43 COI gene sequences from *C. striata* deposited in GenBank. The nucleotide sequence of the CSTR primers were significantly different to those of COI genes from eleven other species of *Channa* genus and six other popular

freshwater fishes in Indonesia. The designed forward primer wasn't exactly matched with recognition site of all *C. striata*. The forward primer recognition site exactly matched with COI genes of 23 *C. striata* (COI *C. striata* 1), while the primer had mismatch with the remaining (COI *C. striata* 2 and COI *C. striata* 3). There was no mismatch found between the nucleotide sequences of reverse primer with those of COI genes of all *C. striata* (Figure 2). As previously reported, primer mismatch up to 4 bases were tolerable and did not contribute to irreproducibility of PCR replicates.[19-20] On the other hand, mismatch can produce biased product but can be eliminated by applying low Ta.[21] Determination of optimum Ta is an essential step to obtain reliable results and efficient amplification of a specific target.[22] PAN was used for CSTR primers testing. It has some mismatches (showed by bold alphabets) for both forward and reverse primers (Figure 1-B).

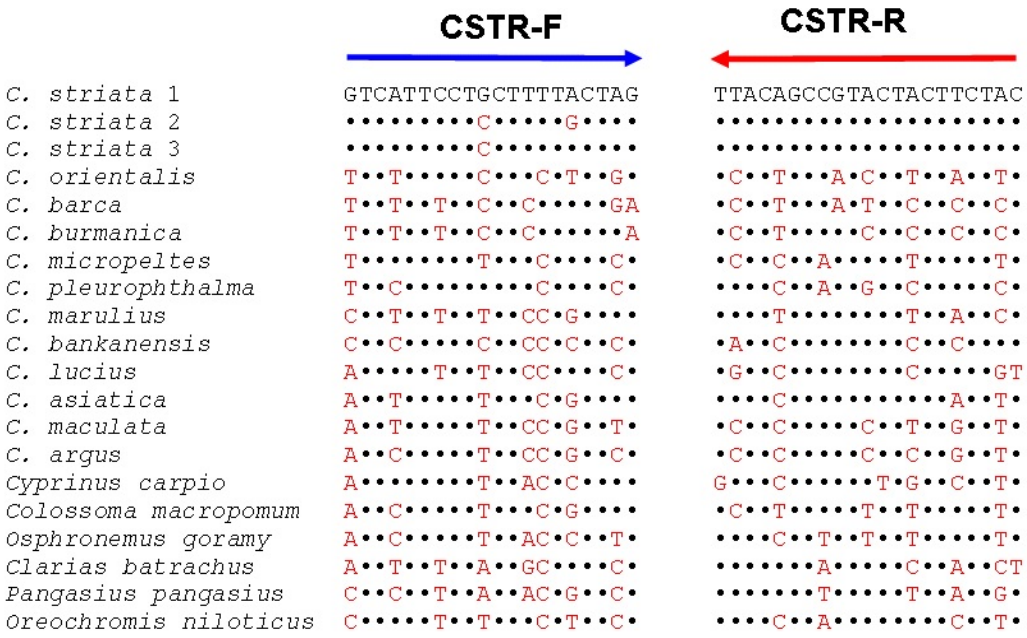
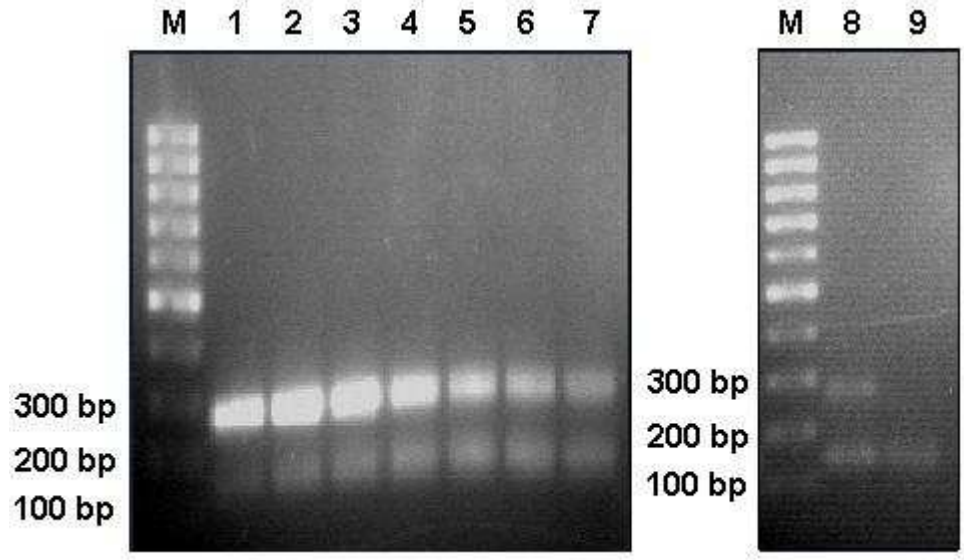


Figure 2
The alignment of COI gene sequence for design of CSTR primer

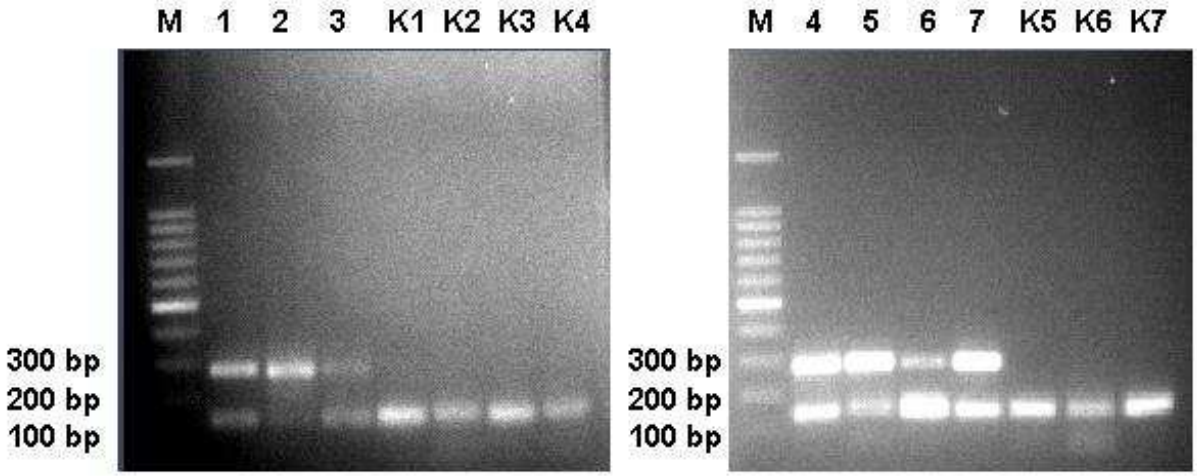
Based on COI gene sequence, as expected the CSTR primers only amplified *C. striata* DNA of 269 bp in size (Figure 1-B). In this study, we combined the CSTR and BAM primers in a multiplex PCR. BAM primers are usually utilized for normalization of gene expression in a Real-time PCR.[23] These primers can be also used to amplify genomic DNA from many organisms, including *C. striata* (data not shown). BAM primers serve as an internal control to detect the presence of PCR inhibitor. A 138 bp PCR product of BAM primers confirms good quality of DNA template and the absence of PCR inhibitor.



The 269 bp band is CSTR primers product, while 138 bp band is BAM primers product. M) 100 bp DNA ladder; 1) 44°C; 2) 46°C; 3) 48°C; 4) 50°C; 5) 52°C; 6) 54°C; 7) 56°C; 8) 58°C; 9) 60°C.

Figure 3
Effect of different annealing temperatures on selective amplification of PAN's DNA in a multiplex PCR using CSTR-BAM primers.

Primer analysis by OligoAnalyzer 3.1 program showed that melting temperature (Tm) of CSTR primer pair was around 51°C. Applicable Ta is typically 5°C below the true Tm, but optimal Ta is often higher (5-10°C) than the Tm of the primers.[24] In this study, optimization of Ta was conducted in the range of 44-60°C. The Ta with range of 44-50°C produced a thick specific DNA band of 269 bp, while above 50°C it produced thin DNA band and no band at 60°C. BAM DNA band of 138 bp in size was present in all Ta used in this study (Figure 3). Therefore, 50°C was chosen as optimum Ta.



M) 100 bp DNA ladder; 1-7) C. striata from IND, BAN, MUS, OGA, TAN, LAM, and PAN, respectively; K1) Common carp; K2) Nile tilapia; K3) Catfish; K4) Giant gouramy; K5) Mozambique tilapia; K6) Pangasius; K7) Milkfish.

Figure 4
Agarose electrophoresis gel of PCR product from fish DNA by multiplex PCR using CSTR-BAM primers.

Specificity of CSTR primers was determined by amplification of DNAs from seven different sources of *C. striata* collected from several areas in Indonesia (IND, BAN, MUS, OGA, TAN, LAM, and PAN), nile tilapia, common carp, catfish, giant gouramy, mozambique tilapia, pangasius and milkfish. Result of PCR and electrophoresis of *C. striata* from seven areas and seven other fishes are shown in Figure 4. The expected DNA band of 269 bp was specifically produced in all *C. striata* samples but this DNA band was absent in seven other fishes. It indicates the specificity of CSTR primer pair for *C. striata*.

CONCLUSION

Taken together all these data, a multiplex PCR using specific PCR primers for a rapid and specific identification of *C. striata* and BAM primers to detect the presence of PCR inhibitor is developed in this study. This PCR technique is potentially applied as a tool for identification of *C. striata*.

AUTHORS CONTRIBUTION STATEMENT

YPN designed the primers, performed the experiments, analyzed data and wrote the paper. PR participated in supervising the whole experiment, and reviewed the entire data and the manuscript. RRT conceived the original idea, supervised the project and contributed to the final version of the manuscript.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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