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Microsatellite Markers for the Identification of Commercially Important Groupers *Epinephelus lanceolatus*, *Cromileptes altivelis* and *Epinephelus fuscoguttatus*

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ABSTRACT

The Giant Grouper (*Epinephelus lanceolatus*), Mouse Grouper (*Cromileptes altivelis*) and the Brown Marbled Grouper (*Epinephelus fuscoguttatus*) are all found in the coastal waters of Sabah, Malaysia; they are listed as vulnerable, threatened and near-threatened, respectively, according to the IUCN Red Data list. Based on microsatellite loci, molecular markers can be applied as versatile tools for forensic detection, population genetic studies and the development of genomic databases which can subsequently be incorporated into viable conservation programmes. This paper describes the development of molecular markers for the three species of grouper. DNA was extracted from adult specimens of the three species, which are currently maintained at the Borneo Marine Research Institute Aquaculture facility, subject to DNA amplification using a multiplex PCR enrichment procedure, and the PCR products were ligated onto a pJET 1.2 blunt cloning vector. This was followed by sequencing and the development of 24 specific molecular markers for each of the three species. These markers have a potential application for the identification and forensic detection of these species in transit and can be adopted within the context of a strategy for the conservation and management of Malaysian fisheries resources.

Keywords: *Epinephelus fuscoguttatus*, *Epinephelus lancelolatus*, *Cromileptes altivelis,* molecular markers

INTRODUCTION

Groupers belong to the subfamily *Epinephelinae* of the family *Serranidae* and inhabit reefs from the tropics to the sub-tropical waters. Many of the species have been classified as vulnerable as in the case of the Giant groupers (*E. lanceolatus*), according to the IUCN Red Data list. The high demand for reef fishes in the developing nations of Southeast Asia has further exacerbated the decline of wild Grouper populations and led to the necessity for interventions such as the protection of spawning sites (Cesar *et al.*, 2003). Meanwhile, the development of molecular markers for the forensic detection of these endangered and economically important species forms the basis of this investigation, which is directed towards developing specific DNA based molecular markers for the forensic identification of *E. lanceolatus*, *E. fuscoguttatus*, and *C. altivelis*.

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Several previous studies done on *E. lanceolatus* (Zeng *et al*., 2008) have focused on eight polymorphic microsatellite loci; however, for the purpose of forensic identification, emphasis has been placed on the development of molecular markers, based on the relatively stable non-hyper-variable genomic regions.

MATERIALS AND METHODS

The adult specimens of *C. altivelis*, *E. fuscoguttatus* and *E. lanceolatus* were selected for the sample collection from the fish hatchery at Borneo Marine Research Institute, Universiti Malaysia Sabah. Fin clips of approximately 1 cm^2 were excised. Meanwhile, total DNA was extracted using the method described by Doyle and Doyle (1987), with a minor modification of the extraction buffer, to which 500 µg/ml final concentration of Proteinase K (Sigma) was added. Amplicons containing genomic repeat motifs generated using three Inosine containing degenerate primers, with a range of repeat motifs, BRICTT10 [5'-GG$(CTT)_{10}$III-3'], BRICAA8 [5'-GG$(CAA)_8$III-3'] and BRIAGG8 [5'-GG$(AGG)_8$ III– 3'] (where I = Inosine), were used in PCR amplification to isolate the range of repeat motifs. PCR amplification was carried out in a total volume of 20 µL containing 50 ng of template DNA, 1.5 mM $MgCl_2$,1X PCR buffer containing 10 mM Tris-HCl (pH = 8.0), 50 mM KCl, 1 U Taq DNA polymerase (Qiagen), 20 pmol of primer (BRICTT10, BRICAA8 and BRIAGG8), 0.2 mM of dATP, dGTP, dCTP and dTTP. Amplification was carried out in a thermocycler (MJ Research thermal cycler) with an initial denaturation at 94°C for 3 minutes, and this was followed by 30 cycles of 30 s at 94°C, 40 s at 55°C or 58°C, 1 minute at 72°C and a final extension step of 5 min at 72°C. The amplification products were separated by electrophoresis on a 2% TBE Agarose gel with a 1 Kb DNA ladder (Promega) as a size standard. PCR products containing distinct amplicons, with a size in the excess of 200 bp, were purified using a PCR purification kit (Qiagen), while 2 µL of the purified products were cloned onto a pJET1.2 blunt cloning vector and transformed into chemically competent TOP10F E. coli cells, according to the instructions from the manufacturer (Fermentas). A total of 20 recombinant clones from each species were selected randomly from Luria-Bertani plates containing Ampicillin (100 mg/ litre) and X-Gal (50 mg/ litre). Plasmids were extracted and purified using GeneJET plasmid purification kit (Fermentas). Plasmids with an insert size in excess of 300 bp were sequenced using BigDye Terminator 2.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an ABI Prism 377 DNA sequencer. The characterization of specific genomic loci was done using specific primer pairs designed to flank the regions containing microsatellite repeat motifs using the online software PRIMER 3 (Rozen & Skaletsky, 2000). A total of 24 primer pairs were designed, synthesized, and tested across five representative specimens from each of the three species. The PCR amplification was carried out in a total volume of 20 µL containing 50 ng of template DNA, 1.5 mM $MgCl_2$,1X PCR buffer containing 10 mM Tris-HCl (pH = 8.0), 50 mM KCl, 0.25 U Proofreading Taq DNA polymerase (Qiagen), 10 pmol of each forward and reverse primer, 0.2 mM of dATP, dGTP, dCTP, and dTTP. The amplification was carried out in a thermocycler (MJ Research thermal cycler) with an initial denaturation at 94°C for 3 minutes, followed by 30 cycles of 30 s at 94°C, 40 s at the specific annealing temperature, 40 s at 72°C and a final extension step of 10 min at 72°C. The amplification products were separated by electrophoresis on a 2% TBE agarose gel (Promega), with a 100 bp DNA ladder (Promega) as a size standard. The gel was stained with Ethidium Bromide (5 µg/ ml) and the bands were scored using the gel documentation system ALPHAIMAGER 2000.

RESULTS AND DISCUSSION

The amplification of the DNA with the primers developed resulted in distinct amplification profiles for each of the three species of groupers (*Fig. 1*). The specificity of the amplification was improved by increasing the number of the

repeat motifs as in the case of primer BRICTT10 (*Fig. 2*); this is an indicative of the rarity of the repeat motifs within the genome in direct relation to the repeat frequency. Meanwhile, the application of degenerate primers containing inosine increased the probability of PCR amplification of microsatellites, and this can be attributed to the fact that microsatellite repeat units are likely to contain interruptions in the form of nucleotide additions and substitutions

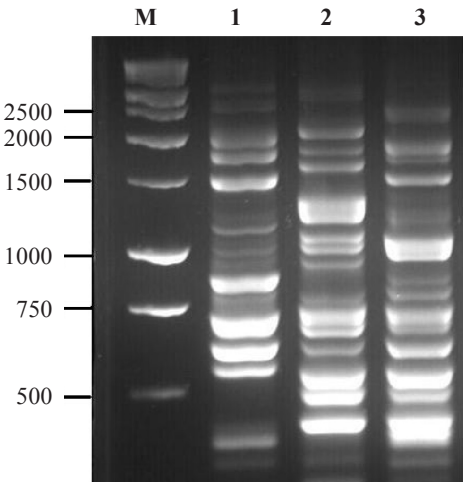

Fig. 1: 2% TBE agarose gel electrophoresis analysis of PCR amplicons generated as a result of the amplification of grouper DNA with multiplex primer BRICAA8, according to the conditions described in the text. Lane M: 1 Kb DNA ladder, Lane 1: C. altivelis*, Lane 2:* E. fuscoguttatus*, Lane 3:* E. lanceolatus *(all the marker sizes are indicated in base pairs)*

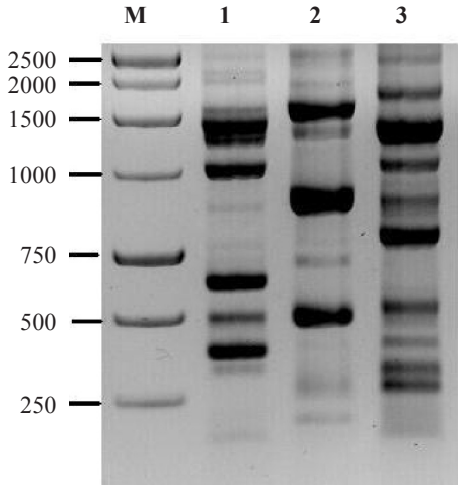

Fig. 2: 2% TBE agarose gel electrophoresis analysis of PCR amplicons generated as a result of the amplification of grouper DNA with multiplex primer BRICTT10, according to the conditions described in the text. Lane M: 1 Kb DNA ladder, Lane 1: C. altivelis*, Lane 2:* E. fuscoguttatus*, Lane 3:* E. lanceolatus

TABLE 1
Microsatellite markers developed for the forensic identification of *Epinephelus lanceolatus* (EL), *Epinephelus fuscoguttatus* (EF) and *Cromileptes altivelis* (CA) indicating specific locus, primer sequence (5' – 3'), observed size in base pairs (bp) and annealing temperature (°C)

No.	Locus	Primer sequence 5' – 3'	Observed size (bp)	Annealing temperature (°C)	GenBank accession number
1.	CAA01B	F: GAGGAGTTCAAAGCCTCCAA R: CCCTTTTCCCAAAATGACAA	311	58	GQ912309
2.	CAA02B	F: TAACTTGCCAAGCATCAGCA R: GCACTTTCCTCCGAAAAACA	312	60	GQ912310
3.	CAA03B	F: TGCTTGACCAAAACACAATGA R: GCTAGGCGGAAGTGACAAAG	371	60	GQ912311
4.	CAA04B	F: GCGGTTAAACAGAGGGGATT R: ACTCGTGTTCCCTCCTGCT	164	58	GQ912312
5.	CAA05B	F: TTTAACCCGGTCAACTCCAG R: GACGCAGTTTCAATGCAAAA	535	58	GQ912313
6.	CAA06B	F: ATCAGTGTCCTGCCACCTTC R: TACTCCACATGCTGGCTGTC	340	59	GQ912314
7.	CAB01A	F: GACTGAATTTGGGGACCAAG R: CAATGTCGACGTCGCTAAAC	222	58	GQ912315
8.	CAB06B	F: TTCCTTCAGCAACAAACACG R: CCGCAAACAGTGCTAAACAA	315	58	GQ912318
9.	EL09A	F: TGCTGGTTTTTACGGAGACC R: TGTCTGCACCACCTGTCATT	157	58	GQ912329
10.	EL10A	F: GCTCAGCTGTTGAAAACACG R: GCTCCTCCGAAATGTCTCTG	308	58	GQ912330
11.	EL16A	F: TTCTTCCTCTGCTGTCTTTTCC R: TTACGTTTCCAGAGCACCAA	301	58	GQ912331
12.	EL19A	F: GAATCTCCTGCACCTCTTGC R: TGCTGGAGCTGTATCCTCCT	397	58	GQ912332
13.	EL20A	F: CGACAAAACCGGGATTAAAA R: GGAAAGGGAAGTTGGGAGAG	304	60	GQ912333
14.	EL21A	F: ACTTCCCTCCAATGCTTCAA R: CCTTCGTCCACCATCAGTCT	302	58	GQ912334
15.	EL22A	F: GCACAAGCCTAGCCCTACTG R: TTGGGTCCAATGGAACATTT	399	58	GQ912335
16.	EL13A	F: AATGAGCACCTGGAGACCAC R: TTTTCAGCCTTCCTCTCCTG	352	59	GQ912336
17.	EFX2B	F: GCGCTGCTGTACAACAAGAA R: TCAGCAGGTGAACTGAGGTG	420	58	GQ912319
18.	EFX7A	F: AGCACGGTCTGTGTGTCTTG R: TGCCACAAAACTAAGAAAGGAA	185	58	GQ912320
19.	EFX8A	F: CGTCACTGACTGCCAAGAAA R: GAGCCAGGACCAGTTGTAGC	187	58	GQ912321

Table 1 (Continued)

20.	EFX10B	F: GTTGTTGTTGTTGCCAATCG R: GAGTCAGTGAGCGAGGAAGC	346	59	GQ912322
21.	EFX11B	F: TTGGCAGGTGTCTCTCTCCT R: ACAGCGGTGGAAGGTTACTG	211	58	GQ912323
22.	EFY1B	F: GAAGAGAACCAGTGGGGACA R: AGAGGTCGCCATCTGACATC	484	58	GQ912325
23.	EFY7A	F: CACAGGCTGTCAAACAGGAA R: TCGGGAGAATGTGAAGCTCT	228	58	GQ912326
24.	EFY8A	F: ACTGCTGCGTCAAGAAGACA R: ATCAGTGTCCTGCCACCTTC	216	58	GQ912327

resulting from strand slippage during replication (Schlötterer & Tautz, 1992) and the exclusion of degenerate bases which reduces the likelihood of amplification of loci containing imperfect microsatellite repeats. A similar approach was undertaken by Yaish & Pérez de la Vega (2003) for the isolation of microsatellite loci from *Phaseolus vulgaris*.

Meanwhile, the sequencing of each of the amplicons yielded distinct sequences for each of the species. A search for similarity using the NCBI blastn algorithm indicated that each of the sequences was novel and had not been previously described. Specific primer pairs were designed for each of the sequences (Table 1) and were determined to be species specific for each of the three species on which they were tested. These markers can be used for multiplex fingerprinting of the three grouper species, particularly for monitoring fisheries stocks and in the aquaculture industry. The ongoing focus of this investigation is the development of molecular markers used to determine Mendelian inheritance patterns and genetic recombination in grouper hybrids.

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