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High-throughput simple sequence repeat (SSR) markers development for the kelp grouper (*Epinephelus bruneus*) and cross-species amplifications for Epinephelinae species

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ABSTRACT

The kelp grouper (*Epinephelus bruneus*), belonging to one of the largest genera among the subfamily Epinephelinae, is a commercially important fish in Japan. There are limited data about the genomics of this species. To provide tools for addressing both population genetics studies and gene mapping, di- to penta-nucleotide simple sequence repeat (SSR) markers were developed using 454 pyrosequencing. Among the 1466 SSR markers developed, 1244 primer sets produced strong PCR products, of which 905 (72.7%) were polymorphic in kelp grouper. Cross-species utility of the 905 polymorphic SSR markers was tested in four additional Epinephelinae species of *Hyporthodus septemfasciatus*, *Plectropomus leopardus*, *Epinephelus lanceolatus* and *Epinephelus coioides*. Results revealed that, respectively, 401 (44.3%), 136 (15.0%), 434 (49.0%) and 538 (59.4%) SSRs showed specific polymorphic products. Of these, 40 SSR markers (33 di-, 1 tri- and 6 tetra-nucleotides) showed polymorphism in all species tested. Additionally, three AGAT SSR motifs which accounted for 42.9% of the non-di-nucleotide markers were found in the 40 SSR markers. This indicates that the AGAT SSR motif has a high potential as a highly versatile SSR marker in grouper Epinephelinae. The SSR markers developed in this study can be employed to obtain reliable genetic variability estimates for groupers (Epinephelinae).

KEYWORDS

Kelp Grouper; *Epinephelus bruneus*; Repeat Motif; Simple Sequence Repeat (SSR); 454 Pyrosequencing; Cross-Species Amplification

1. INTRODUCTION

Groupers (family Serranidae, subfamily Epinephelinae) comprising about 159 marine fish species in fifteen genera are the most intensively exploited group in marine fishing [1,2]. Groupers are also in demand as new aquaculture species in East and South-East Asia because of their high market price, rapid growth and adaptability to high density, rearing conditions [3,4]. The high market price has led to overexploitation of these fish and increased aquaculture. Among the subfamily Epinephelinae, the genus *Epinephelus* is one of the largest genera among bony fish with 98 species including many economically important species for both capture fisheries and aquaculture [1,5]. The kelp grouper (*Epinephelus bruneus*) is one of the most economically important species in Japan. However, it has been listed as Vulnerable in the IUCN Red List Category & Criteria (www. iucnredlist.org) due to the sharp decline in catch. Although aquacultural production of kelp grouper has been attempted in response to the decreases in natural resources and increases in price, analysis of the genomics of this species has rarely been explored.

Simple sequence repeat (SSR) has been particularly valuable in parentage analyses, population genetics,

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conservation or management of biological resources and gene mapping because of their ease of use, co-dominance and high levels of polymorphism [6,7]. Relatively large numbers of SSR markers have been recently developed and characterized for grouper species e.g., *Plectropomus maculatus* [8], *E. lanceolatus* [9,10], *E. fuscoguttatus* [11], *Mycteroperca tigris* [12], *Cromileptes altivelis* [13]. However, there has been only one published research study on the SSR primers for *E. bruneus* in which di-nucleotide markers are developed [14]. Although di-nucleotide SSR markers are powerful tools for parentage analyses and gene mapping, analyses are frequently complicated by the presence of stutter bands caused by polymerase slippage during PCR amplification. This results in secondary products containing one or more repeat units less than the primary allelic band. The stutter bands are sometimes equivalent to the intensity of the primary band which decreases the accuracy of the genotypic characterization, particularly in population genetics studies [6]. To overcome this problem, tri-, tetra- or more nucleotide SSR which possess fewer problems with stutter bands should be used, however, these SSR markers are much less abundant in the genome compared to di-nucleotide SSR markers. Recently developed next-generation sequencing (NGS) technologies have allowed reassessment of the technical aspects of SSR search techniques [15]. A notable merit of NGS-based SSR search technique is that various repetitive elements can be explored in shotgun libraries, which enables simultaneous search for nondi-nucleotide SSR markers without implementing special procedures for SSR-enrichment [16]. In human forensic DNA, a consensus was reached that tetra-nucleotide repeat markers should be used as the gold standard for individual identification [17]. Currently, only tetra- and penta-nucleotide SSR markers are acceptable for routine human forensic casework [18].

SSR markers can be transferable from different genotypes within or between species or between genera [19]. Such interspecific or intergeneric transferability makes SSR markers a useful tool for genetic studies, such as fingerprinting, genetic mapping, and molecular marker identification. In the present study, we report the development of 1290 SSR markers from whole genome sequences of *E. bruneus* using 454 pyrosequencing to obtain SSR markers for addressing both population genetics studies and gene mapping. In addition, the developed SSR markers were used to conduct amplification tests with five grouper species *i.e*., *E. bruneus*, *Hyporthodus septemfasciatus*, *P. leopardus*, *E. lanceolatus* and *E. coioides* in order to characterize cross-species polymorphism as well as find SSR motifs that have potential as a highly versatile SSR marker in groupers (Epinephelinae).

2. MATERIALS AND METHODS

2.1. Whole Genome Shotgun Sequences Assembly

Large whole genome shotgun (WGS) sequences were generated from wild kelp grouper. A quarter plate of DNA sequencing was performed with 454 pyrosequencing on a Genome Sequencer FLX-454 System (GS FLX sequencer). Sample preparation and DNA sequencing was performed according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The raw reads from the GS FLX454 were assembled using Newbler software version 2.3 (Roche Diagnostics) and the WGS contigs and singletons were generated. Briefly, the adapters and poor sequence data were removed by using the built-in adapter removal tools in Newbler. After sequence trimming and clean-up, *de novo* assembly was performed using the default parameters. The sequences of contigs and singletons were used for SSR identification.

2.2. SSR Identification

A pipeline program, Auto-primer [20], which automatically runs two software programs *i.e*., Tandem Repeats Finder ver. 4.0.4 [21] and Primer3 ver. 2.2.2 beta [22], was used to identify sequences containing repeat motifs (di-, tri-, tetra- and penta-nucleotide) and to predict their appropriate PCR primers from WGS contigs and singletons. In Tandem Repeats Finder, alignment weights for match, mismatch and indels were set as two, seven and seven, respectively. Matching and indel probabilities were set as 0.8 and 0.1, respectively. Since nonunique repeat motifs such as reverse-complement repeat motifs (e.g., AC and GT) and translated or shifted motifs (e.g., AAT, ATA, ATT, TAA, TAT and TTA) were grouped together [23], a total of three unique di-nucleotide repeats, ten unique tri-nucleotide repeats, 25 unique tetra-nucleotide repeats and 47 unique penta-nucleotide repeats were obtained from the kelp grouper genome. SSR markers containing genomic DNA sequences with flanking sequences greater than 30 bp on either side of SSR markers were collected for primer design.

2.3. SSR Marker Development

In order to adjust the PCR product size, primers were redesigned using web software WebSat, which is accessible through the Internet [24]. The thresholds for di-, tri-, tetra-, penta-nucleotide repeat minimum were set as six in all motif length. The parameters were employed to design primers except for the product size was set as 100 - 250 bp. Each forward primer was 5' labeled with fluorescent dye: Tetrachloro-fluorescein (TET).

2.4. Sample Collection and Polymorphism Test

To examine SSR markers, newly-designed PCR primer pairs were tested for amplification using the samples from eleven kelp groupers. Those fish are already known the relationship between the parental fish and progenies. Three individuals each of *H. septemfasciatus*, *P. leopardus*, *E. lanceolatus* and *E. coioides* were used to characterize cross-species amplification from wild resources. These species were chosen as they are of economic and current research importance.

2.5. SSR Marker Validation

PCR amplification of the SSR markers was performed on MJ PTC-100 thermal cycler (Bio-Rad, USA) in 11 µl reaction volume containing 0.5 pmol/µl of unlabeled primer, 0.05 pmol/µl of fluorescence-end-labeled (TET) primer, 1× Ex Taq buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTP, 1% BSA, 0.25 U of Taq DNA polymerase (TaKaRa: Ex-Taq) and 50 ng template DNA. Suitable annealing temperatures for each SSR marker were used. PCR conditions were 95˚C for 5 min for initial denaturation, followed by 36 cycles at 95˚C for 30 s, 56˚C for 1 min, 72˚C for 1 min, with a final extension at 72˚C for 10 min. Amplification products were mixed with an equal volume of loading buffer (98% formamide, 10 mM EDTA (pH 8.0), 0.05% bromophenol blue), heated for 10 min at 95˚C and then immediately cooled on ice. The mixture was loaded onto 6% PAGE-PLUS gel (Amresco, OH, USA) containing 7 M urea and 0.5× TBE buffer. The PCR products were visualized using FMBIO III Multi-View fluorescence image analyzer (Hitachi-soft, Tokyo, Japan). SSR markers that showed more than one alleles per locus were recognized as polymorphic markers.

3. RESULTS AND DISCUSSION

3.1. 454 Sequencing Result

The raw sequence data from a quarter-plate run of the 454 sequencing yielded 84.1 Mbp containing 213,073 reads or sequences with an average length of 395 bp (maximum: 677 bp, minimum: 40 bp) (**Table 1**). A total of 10,766 reads (approximately 5.1%) were assembled into 4551 contigs with an average length of 385 bp (maximum: 6510 bp, minimum: 100 bp), leaving 166,867 singletons. The mean length of these 171,418 sequences (4551 contigs plus 166,867 singletons) was 392 bp, which was similar to that of the raw sequences. This number also agreed with the results from previous studies on 454 sequencing run in nonmodel species including the bream, *Megalobrama pellegrini*, with average read length of 404 bp [25] and the abalone, *Haliotis diversicolor supertexta*, with average length of 385 bp [26]. To our knowledge, this is the first large scale study of genomic data from *E. bruneus*.

Table 1. Sequencing statistics using 454 sequencing platforms.

Primary sequence data	Number
Total number of reads	213073
Total read length (bp)	84135800
Mean length of all reads (bp)	395
Number of contigs	4551
Number of singletons	166867
Total number of reads after assembly	171418
Total read length after assembly (bp)	67207137
Mean length of read after assembly (bp)	392
Number of SSR with primers designed	2348

3.2. SSR Loci Isolation

Of the 171,418 unique sequences, 2348 (1.37%) sequences comprising 1118 (47.6%), 488 (20.8%), 473 (20.1%) and 269 (11.5%) of di-, tri-, tetra- and penta-nucleotide repeat motifs, respectively, were suitable for primer design (**Table 2**). In general, the number of repeats decreased with motif length. Of 2348 sequences, the average number of repeats with di-, tri-, tetra- and penta-nucleotide repeat motifs were eighteen, twelve, ten and eight, respectively. Currently, CAG and AGAT repeat types predominate in vertebrate SSR markers while di-nucleotide (CA) repeats are the common SSR markers developed for genetic studies in fish [27, 28]. The most common repeat motifs of di-, tri-, tetra- and penta- nucleotide in *E. bruneus* included AC/GT (93.3%), AAT/ATT (37.5%), ACAG/CTGT (13.7%) and AAAAT (12.3%), respectively. Although the relative frequency of SSR motif types was different among the species, AC/GT, AAT/ATT and AGAT/ATCT were found as the common repeat motifs with a high percentage of loci suitable for primer design in the North American fish (*Etheostoma okaloosae*) [29]. These SSR motifs are also abundant in the *E. bruneus* genome.

3.3. SSR Marker Development and Validation

A total of 1466 primer sets were redesigned from 2348 sequences using the web software WebSat (Table S1) of which four types of SSR markers *i.e.*, di-, tri-, tetra- and penta-nucleotide repeat motifs were included with the primer numbers of 826 (56.3%), 317 (21.6%), 254 (17.3%) and 69 (4.7%), respectively. Since the threshold for all repeat minima was set as six, 71 penta-nucleotide repeat motifs (26.4% of identified penta-nucleotide repeat motifs from the kelp grouper genome) were ignored as SSR marker in this study. Consequently, only 25.7%

Table 2. Summary of SSR motifs with suitable for primer design of kelp grouper.

Di-nucleotide	Number	Di-nucleotide	Number
AC/GT	1043	AT	35
AG/CT	40	Total	1118
Tri-nucleotide	Number	Tri-nucleotide	Number
AAT/ATT	183	ACT/AGT	22
ATC/GAT	70	ACC/GGT	12
AGG/CCT	69	GGC	1
AGC/GCT/CAG	52	TCG	1
AAC/GTT	45		
AAG/CTT	33	Total	488
Tetra-nucleotide	Number	Tetra-nucleotide	Number
ACAG/CTGT	65	ACAC	8
AGAT/ATCT	62	ACAT/ATGT	6
ATCC/GGAT	61	AACT	5
AATC/GATT	49	AAGG/CCTT	5
AAAT/ATTT	47	CTAC/ACCT	4
AAAC/GTTT	41	AAGT	3
AATG/CATT	34	AGGC	3
AGTG/ACTC/GAGT	16	CCAG	2
CACG/ACGC/GCGT	16	TAAT	2
ACTG/CAGT	12	GCTA	1
AGGG/CCCT	10	GGTG	1
CTTT/AAAG	10	GTCC	1
AACC/GGTT	9	Total	473
Penta-nucleotide	Number	Penta-nucleotide	Number
AAAAT	33	ACGTA	3
AATTC	21	ATGAC	3
CTAAG	21	ACATT	2
AAGAT	19	ACCAC	2
AGAGG	18	ATAAC	2
AATAG	17	CATAC	2
AAAAC	14	CTCTG	2
AACTA	10	GATCA	2
ATATA	8	TGACT	2
AAATT	7	AAATG	1
AAGAA	7	AATAC	1
AGATG	7	AGTTG	1

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ATCGT	6	CACAT	1
CTCTT	6	CCCCA	1
AAGGA	5	CTGAG	1
ACTGA	5	GACGA	1
AGGTA	5	GAGGG	1
CCAAA	5	GAGTG	1
ATAGT	4	GTGAA	1
ATCAT	4	TCCAA	1
ATTTG	4	TGCTA	1
AATCT	3	TGGAA	1
ACAAG	3	TGTTG	1
ACCTC	3	Total	269

of identified penta-nucleotide repeat motifs from kelp grouper genome could be used for redesigning the primer. Newly-designed primers were tested in the eleven kelp groupers. Among the primer sets tested, 1244 primer sets produced strongly amplified expected fragment size, of which 905 (72.7%) primer sets showed clear amplification with polymorphic patterns (**Table 3**).

The amplified and polymorphic marker results for each nucleotide repeat motif are summarized in **Tables 4(a)** and **(b)**. In each of di- and tri-nucleotide repeat motifs, there are only slight differences between proportion of amplification and polymorphism among species. It seems that each di- and tri-nucleotide repeat motif has a similar potential for amplification to polymorphic marker conversion ratio. ACAG, AGAT, ATCC, AATC and AAAC repeat markers accounted for 61.5% among the successfully amplified 20 tetra-nucleotide SSR motif markers, of which ACAG, AGAT and ATCC repeat markers totally accounted for more than 50% of the polymorphism in *E. bruneus*. In addition, AGAT and ATCC repeat markers exhibit high amplification to polymorphic marker conversion ratio (more than 90%). A/T-rich motif, containing A or T nucleotides > 50%, accounted for 69.6% of polymorphic tri-nucleotide SSR markers, while G/C-rich motifs, containing G or C nucleotides > 50%, were 30.4% of polymorphic tri-nucleotide SSR markers. Similarly, in polymorphic tetra- and penta-nucleotide SSR markers, the A/T-rich SSR markers were the vast majority (more than 82%) compared with G/C-rich SSR markers in *E. bruneus*.

Prior to our study, twelve SSR markers had been developed in *E. bruneus* using the traditional method [14], and they reported on development of di- nucleotide markers. Di-nucleotide SSR markers, while providing powerful discrimination, do not provide high-precision genotyping needed for comparative multi-locus profiling. In this study, 905 polymorphic SSR markers were developed using 454 pyrosequencing of which nondinucleotide repeat motifs including 184 tri-, 176 tetra- and 52 penta-nucleotide markers were successfully developed. Although unclear amplification or monomorphic pattern was obtained for 561 loci, a comparably high primer to polymorphic marker conversion ratio (62%) was achieved which was similar to that observed in the mottled skate (*Raja pulchra*) [30]. Of the 1244 primer sets, a total of 742 (59.6%) were successfully validated as polymorphic markers in the future mapping family (Table S2). The polymorphic SSR markers were genotyped in the mapping panel for a future linkage map project.

Table 3. Statistics of SSR markers developed in *E. bruneus*.

Species	Amplification	Polymorphism	%
E. bruneus	1244	905	72.7%
H. septemfasciatus	1066	508	47.7%
P. leopardus	523	185	35.4%
E. lanceolatus	1132	551	48.7%
E. coioides	1124	704	62.6%

%, percentage of polymorphic markers in each species.

3.4. Characterization of Cross-Species Amplification

The cross-species amplification of the 1466 SSR loci were conducted in an additional four species, *H. septemfasciatus*, *P. leopardus*, *E. lanceolatus* and *E. coioides* using three individuals for each species. These grouper species are economically important for both capture fisheries and aquaculture. The number of SSR loci which

Table 4. (a) Summary of cross-species amplification test based on SSR motifs in 1466 SSR markers for *E. bruneus*, *H. septemfasciatus* and *P. leopardus*; (b) Summary of cross-species amplification test based on SSR motifs in 1466 SSR markers for *E. lanceolatus* and *E. coioides*.

(a)

	Epinephelus bruneus			*Hyporthodus septemfasciatus*			*Plectropomus leopardus*		
Motif type	Amp (F)	Poly (F)	A/P	Amp (F)	Poly (F)	A/P	Amp (F)	Poly (F)	A/P
Di	638	493	77.3%	555	294	53.0%	317	148	46.7%
AC (1043)	599 (93.9)	466 (94.5)	77.8%	521 (93.9)	287 (97.6)	55.1%	303 (95.6)	145 (98.0)	47.9%
AG (40)	21 (3.3)	13 (2.6)	61.9%	20 (3.6)	3 (1.0)	15.0%	10 (3.2)	3 (2.0)	30.0%
AT (35)	18 (2.8)	14 (2.8)	77.8%	14 (2.5)	4 (1.4)	28.6%	4 (1.3)	0 (0)	0%
Tri	300	184	61.3%	267	103	38.6%	99	20	20.2%
AAT (183)	91 (30.3)	55 (29.9)	60.4%	74 (27.7)	22 (21.4)	29.7%	19 (19.2)	2 (10.0)	10.5%
ATC (70)	54 (18.0)	33 (17.9)	61.1%	48 (18.0)	23 (22.3)	47.9%	13 (13.1)	4 (20.0)	30.8%
AGG (69)	48 (16.0)	28 (15.2)	58.3%	44 (16.5)	15 (14.6)	34.1%	23 (23.2)	5 (25.0)	21.7%
AGC (52)	40 (13.3)	25 (13.6)	62.5%	39 (14.6)	19 (18.4)	48.7%	23 (23.2)	4 (20.0)	17.4%
AAC (45)	26 (8.7)	20 (10.9)	76.9%	27 (10.1)	13 (12.6)	48.1%	10 (10.1)	2 (10.0)	20.0%
AAG (33)	21 (7.0)	11 (6.0)	52.4%	18 (6.7)	6 (5.8)	33.3%	4 (4.0)	0 (0)	0%
ACT (22)	12 (4.0)	9 (4.9)	75.0%	8 (3.0)	3 (2.9)	37.5%	2 (2.0)	1 (5.0)	50.0%
ACC (12)	8 (2.7)	3 (1.6)	37.5%	9 (3.4)	2 (1.9)	22.2%	5 (5.1)	2 (10.0)	40.0%
Tetra	239	176	73.6%	199	96	48.2%	93	16	17.2%
ACAG (65)	36 (15.1)	25 (14.2)	69.4%	32 (16.1)	15 (15.6)	46.9%	13 (14.0)	4 (25.0)	30.8%
AGAT (62)	34 (14.2)	33 (18.8)	97.1%	24 (12.1)	21 (21.9)	87.5%	14 (15.1)	4 (25.0)	28.6%
ATCC (61)	36 (15.1)	34 (19.3)	94.4%	32 (16.1)	20 (20.8)	62.5%	15 (16.1)	1 (6.3)	6.7%
AATC (49)	27 (11.3)	17 (9.7)	63.0%	14 (7.0)	7 (7.3)	50.0%	9 (9.7)	1 (6.3)	11.1%
AAAT (47)	14 (5.9)	7 (4.0)	50.0%	14 (7.0)	5 (5.2)	35.7%	6 (6.5)	2 (12.5)	33.3%
AAAC (41)	25 (10.5)	11 (6.3)	44.0%	23 (11.6)	5 (5.2)	21.7%	7 (7.5)	1 (6.3)	14.3%
AATG (34)	17 (7.1)	13 (7.4)	76.5%	13 (6.5)	3 (3.1)	23.1%	4 (4.3)	0 (0)	0%
AGTG (16)	9 (3.8)	6 (3.4)	66.7%	9 (4.5)	5 (5.2)	55.6%	5 (5.4)	1 (6.3)	20.0%
CACG (16)	6 (2.5)	6 (3.4)	100%	6 (3.0)	2 (2.1)	33.3%	4 (4.3)	1 (6.3)	25.0%
ACTG (12)	6 (2.5)	6 (3.4)	100%	5 (2.5)	1 (1.0)	20.0%	2 (2.2)	0 (0)	0%
AGGG (10)	6 (2.5)	3 (1.7)	50.0%	7 (3.5)	3 (3.1)	42.9%	5 (5.4)	0 (0)	0%
CTTT (10)	4 (1.7)	3 (1.7)	75.0%	4 (2.0)	3 (3.1)	75.0%	2 (2.2)	1 (6.3)	50.0%
AACC (9)	5 (2.1)	4 (2.3)	80.0%	5 (2.5)	1 (1.0)	20.0%	1 (1.1)	0 (0)	0%
ACAC (8)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
ACAT (6)	4 (1.7)	2 (1.1)	50.0%	3 (1.5)	0 (0)	0%	1 (1.1)	0 (0)	0%
AACT (5)	2 (0.8)	2 (1.1)	100%	2 (1.0)	2 (2.1)	100%	1 (1.1)	0 (0)	0%
AAGG (5)	2 (0.8)	0 (0)	0%	1 (0.5)	0 (0)	0%	0 (0)	0 (0)	0%
CTAC (4)	1 (0.4)	1 (0.6)	100%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
AAGT (3)	1 (0.4)	0 (0)	0%	1 (0.5)	0 (0)	0%	1 (1.1)	0 (0)	0%
AGGC (3)	2 (0.8)	2 (1.1)	100%	2 (1.0)	2 (2.1)	100%	2 (2.2)	0 (0)	0%

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CCAG (2)	2 (0.8)	1 (0.6)	50.0%	2 (1.0)	1 (1.0)	50.0%	1 (1.1)	0 (0)	0%
Penta	67	52	77.6%	45	15	33.3%	14	1	7.1%
AAAAT (33)	3 (4.5)	3 (5.8)	100%	2 (4.4)	1 (6.7)	50.0%	0 (0)	0 (0)	0%
AATTC (21)	3 (4.5)	3 (5.8)	100%	1 (2.2)	0 (0)	0%	0 (0)	0 (0)	0%
CTAAG (21)	4 (6.0)	3 (5.8)	75.0%	3 (6.7)	2 (13.3)	66.7%	3 (21.4)	0 (0)	0%
AAGAT (19)	5 (7.5)	5 (9.6)	100%	3 (6.7)	1 (6.7)	33.3%	0 (0)	0 (0)	0%
AGAGG (18)	9 (13.4)	6 (11.5)	66.7%	6 (13.3)	4 (26.7)	66.7%	2 (14.3)	0 (0)	0%
AATAG (17)	6 (9.0)	4 (7.7)	66.7%	5 (11.1)	1 (6.7)	20.0%	1 (7.1)	0 (0)	0%
AAAAC (14)	1 (1.5)	1 (1.9)	100%	1 (2.2)	0 (0)	0%	0 (0)	0 (0)	0%
AACTA (10)	4 (6.0)	4 (7.7)	100%	3 (6.7)	0 (0)	0%	0 (0)	0 (0)	0%
ATATA (8)	3 (4.5)	2 (3.8)	66.7%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
AAATT (7)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
AAGAA (7)	3 (4.5)	2 (3.8)	66.7%	2 (4.4)	0 (0)	0%	1 (7.1)	0 (0)	0%
AGATG (7)	1 (1.5)	1 (1.9)	100%	1 (2.2)	0 (0)	0%	0 (0)	0 (0)	0%
ATCGT (6)	1 (1.5)	1 (1.9)	100%	1 (2.2)	0 (0)	0%	0 (0)	0 (0)	0%
CTCTT (6)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
AAGGA (5)	1 (1.5)	1 (1.9)	100%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
ACTGA (5)	2 (3.0)	0 (0)	0%	2 (4.4)	0 (0)	0%	0 (0)	0 (0)	0%
AGGTA (5)	1 (1.5)	1 (1.9)	100%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
CCAAA (5)	1 (1.5)	0 (0)	0%	1 (2.2)	1 (6.7)	100%	1 (7.1)	0 (0)	0%
ATAGT (4)	2 (3.0)	2 (3.8)	100%	1 (2.2)	0 (0)	0%	0 (0)	0 (0)	0%
ATCAT (4)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
ATTTG (4)	2 (3.0)	0 (0)	0%	2 (4.4)	0 (0)	0%	1 (7.1)	0 (0)	0%
AATCT (3)	1 (1.5)	1 (1.9)	100%	1 (2.2)	0 (0)	0%	0 (0)	0 (0)	0%
ACAAG (3)	2 (3.0)	2 (3.8)	100%	1 (2.2)	1 (6.7)	100%	1 (7.1)	0 (0)	0%
ACCTC (3)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
ACGTA (3)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
ATGAC (3)	2 (3.0)	2 (3.8)	100%	2 (4.4)	2 (13.3)	100%	0 (0)	0 (0)	0%
ACATT (2)	1 (1.5)	1 (1.9)	100%	1 (2.2)	0 (0)	0%	1 (7.1)	0 (0)	0%
ACCAC (2)	1 (1.5)	1 (1.9)	100%	1 (2.2)	0 (0)	0%	1 (7.1)	0 (0)	0%
ATAAC (2)	1 (1.5)	1 (1.9)	100%	1 (2.2)	0 (0)	0%	0 (0)	0 (0)	0%
CATAC (2)	1 (1.5)	1 (1.9)	100%	1 (2.2)	1 (6.7)	100%	0 (0)	0 (0)	0%
CTCTG (2)	1 (1.5)	0 (0)	0%	0 (0)	0 (0)	0%	1 (7.1)	1 (100)	100%
GATCA (2)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
TGACT (2)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
AAATG (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
AATAC (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%

Continued

AGTTG (1)	1 (1.5)	0 (0)	0%	1 (2.2)	0 (0)	0%	0 (0)	0 (0)	0%
CACAT (1)	1 (1.5)	1 (1.9)	100%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
CCCCA (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
CTGAG (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
GACGA (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
GAGGG (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
GAGTG (1)	1 (1.5)	1 (1.9)	100%	1 (2.2)	1 (6.7)	100%	1 (7.1)	0 (0)	0%
GTGAA (1)	1 (1.5)	1 (1.9)	100%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
TCCAA (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
TGCTA (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
TGGAA (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
TGTTG (1)	1 (1.5)	1 (1.9)	100%	1 (2.2)	0 (0)	0%	0 (0)	0 (0)	0%

Motif type, each nucleotide repeat and number of the identified SSR markers in the whole genome sequencing of *E. bruneus*; Amp (F), number of the amplified SSR markers and the frequency (%) in each nucleotide repeat; Poly (F), number of the polymorphic SSR markers and the frequency (%) in each nucleotide repeat; A/P, percentage of number of the SSR amplified / number of polymorphic SSR markers (amplification to polymorphic marker conversion ratio).

(b)

	Epinephelus lanceolatus			*Epinephelus coioides*		
Motif type	Amp (F)	Poly (F)	A/P	Amp (F)	Poly (F)	A/P
Di	584	342	58.6%	593	424	71.5%
AC (1043)	555 (95.0)	334 (97.7)	60.2%	564 (95.1)	406 (95.8)	72.0%
AG (40)	18 (3.1)	3 (0.9)	16.7%	18 (3.0)	8 (1.9)	44.4%
AT (35)	11 (1.9)	5 (1.5)	45.5%	11 (2.0)	10 (2.4)	90.9%
Tri	279	118	42.3%	271	151	55.7%
AAT (183)	83 (29.7)	43 (36.4)	51.8%	77 (28.4)	48 (31.8)	62.3%
ATC (70)	48 (17.2)	24 (20.3)	50.0%	46 (17.0)	26 (17.2)	56.5%
AGG (69)	49 (17.6)	19 (16.1)	38.8%	48 (17.7)	26 (17.2)	54.2%
AGC (52)	40 (14.3)	9 (7.6)	22.5%	40 (14.8)	20 (13.2)	50.0%
AAC (45)	25 (9.0)	9 (7.6)	36.0%	25 (9.2)	15 (9.9)	60.0%
AAG (33)	17 (6.1)	10 (8.5)	58.8%	18 (6.6)	9 (6.0)	50.0%
ACT (22)	9 (3.2)	2 (1.7)	22.2%	10 (3.7)	3 (2.0)	30.0%
ACC (12)	8 (2.9)	2 (1.7)	25.0%	7 (2.6)	4 (2.6)	57.1%
Tetra	214	69	32.2%	208	101	48.6%
ACAG (65)	32 (15.0)	14 (20.3)	43.8%	33 (15.9)	16 (15.8)	48.5%
AGAT (62)	28 (13.1)	17 (24.6)	60.7%	23 (11.1)	18 (17.8)	78.3%
ATCC (61)	32 (15.0)	9 (13.0)	28.1%	31 (14.9)	18 (17.8)	58.1%
AATC (49)	21 (9.8)	4 (5.8)	19.0%	20 (9.6)	6 (5.9)	30.0%
AAAT (47)	13 (6.1)	3 (4.3)	23.1%	14 (6.7)	4 (4.0)	28.6%
AAAC (41)	25 (11.7)	4 (5.8)	16.0%	22 (10.6)	7 (6.9)	31.8%

Continued

AATG (34)	15 (7.0)	4 (5.8)	26.7%	14 (6.7)	6 (5.9)	42.9%
AGTG (16)	9 (4.2)	2 (2.9)	22.2%	9 (4.3)	7 (6.9)	77.8%
CACG (16)	5 (2.3)	3 (4.3)	60.0%	5 (2.4)	3 (3.0)	60.0%
ACTG (12)	4 (1.9)	1 (1.4)	25.0%	6 (2.9)	4 (4.0)	66.7%
AGGG (10)	8 (3.7)	2 (2.9)	25.0%	8 (3.8)	4 (4.0)	50.0%
CTTT (10)	4 (1.9)	3 (4.3)	75.0%	4 (1.9)	1 (1.0)	25.0%
AACC (9)	5 (2.3)	1 (1.4)	20.0%	5 (2.4)	1 (1.0)	20.0%
ACAC (8)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
ACAT (6)	4 (1.9)	1 (1.4)	25.0%	3 (1.4)	0 (0)	0%
AACT (5)	2 (0.9)	0 (0)	0%	3 (1.4)	2 (2.0)	66.7%
AAGG (5)	2 (0.9)	0 (0)	0%	2 (1.0)	1 (1.0)	50.0%
CTAC (4)	0 (0)	0 (0)	0%	1 (0.5)	1 (1.0)	100%
AAGT (3)	1 (0.5)	0 (0)	0%	1 (0.5)	0 (0)	0%
AGGC (3)	2 (0.9)	0 (0)	0%	2 (1.0)	0 (0)	0%
CCAG (2)	2 (0.9)	1 (1.4)	50.0%	2 (1.0)	2 (2.0)	100%
Penta	55	22	40.0%	52	28	53.8%
AAAAT (33)	3 (5.5)	2 (9.1)	66.7%	3 (5.8)	2 (7.1)	66.7%
AATTC (21)	2 (3.6)	0 (0)	0%	2 (3.8)	0 (0)	0%
CTAAG (21)	4 (7.3)	1 (4.5)	25.0%	4 (7.7)	3 (10.7)	75.0%
AAGAT (19)	3 (5.5)	1 (4.5)	33.3%	3 (5.8)	1 (3.6)	33.3%
AGAGG (18)	7 (12.7)	2 (9.1)	28.6%	7 (13.5)	5 (17.9)	71.4%
AATAG (17)	6 (10.9)	4 (18.2)	66.7%	3 (5.8)	0 (0)	0%
AAAAC (14)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
AACTA (10)	4 (7.3)	1 (4.5)	25.0%	4 (7.7)	2 (7.1)	50.0%
ATATA (8)	2 (3.6)	1 (4.5)	50.0%	1 (1.9)	1 (3.6)	100%
AAATT (7)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
AAGAA (7)	2 (3.6)	1 (4.5)	50.0%	2 (3.8)	2 (7.1)	100%
AGATG (7)	1 (1.8)	0 (0)	0%	1 (1.9)	0 (0)	0%
ATCGT (6)	1 (1.8)	0 (0)	0%	1 (1.9)	0 (0)	0%
CTCTT (6)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
AAGGA (5)	0 (0)	0 (0)	0%	1 (1.9)	0 (0)	0%
ACTGA (5)	2 (3.6)	1 (4.5)	50.0%	1 (1.9)	1 (3.6)	100%
AGGTA (5)	1 (1.8)	1 (4.5)	100%	1 (1.9)	0 (0)	0%
CCAAA (5)	1 (1.8)	0 (0)	0%	1 (1.9)	0 (0)	0%
ATAGT (4)	2 (3.6)	2 (9.1)	100%	2 (3.8)	2 (7.1)	100%
ATCAT (4)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
ATTTG (4)	2 (3.6)	0 (0)	0%	2 (3.8)	1 (3.6)	50.0%

Continued

AATCT (3)	1 (1.8)	1 (4.5)	100%	1 (1.9)	0 (0)	0%
ACAAG (3)	2 (3.6)	1 (4.5)	50.0%	2 (3.8)	1 (3.6)	50.0%
ACCTC (3)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
ACGTA (3)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
ATGAC (3)	2 (3.6)	0 (0)	0%	1 (1.9)	1 (3.6)	100%
ACATT (2)	1 (1.8)	0 (0)	0%	1 (1.9)	1 (3.6)	100%
ACCAC (2)	1 (1.8)	0 (0)	0%	1 (1.9)	0 (0)	0%
ATAAC (2)	1 (1.8)	1 (4.5)	100%	1 (1.9)	1 (3.6)	100%
CATAC (2)	1 (1.8)	1 (4.5)	100%	1 (1.9)	1 (3.6)	100%
CTCTG (2)	1 (1.8)	1 (4.5)	100%	1 (1.9)	1 (3.6)	100%
GATCA (2)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
TGACT (2)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
AAATG (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
AATAC (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
AGTTG (1)	1 (1.8)	0 (0)	0%	1 (1.9)	1 (3.6)	100%
CACAT (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
CCCCA (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
CTGAG (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
GACGA (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
GAGGG (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
GAGTG (1)	0 (0)	0 (0)	0%	1 (1.9)	1 (3.6)	100%
GTGAA (1)	1 (1.8)	0 (0)	0%	1 (1.9)	0 (0)	0%
TCCAA (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
TGCTA (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
TGGAA (1)	0 (0)	0 (0)	0%	0 (0)	0 (0)	0%
TGTTG (1)	0 (0)	0 (0)	0%	1 (1.9)	0 (0)	0%

Motif type, each nucleotide repeat and number of the identified SSR markers in the whole genome sequencing of *E. bruneus*; Amp (F), number of the amplified SSR markers and the frequency (%) in each nucleotide repeat; Poly (F), number of the polymorphic SSR markers and the frequency (%) in each nucleotide repeat; A/P, percentage of number of the SSR amplified / number of polymorphic SSR markers (amplification to polymorphic marker conversion ratio).

were successfully amplified from *H. septemfasciatus*, *P. leopardus*, *E. lanceolatus* and *E. coioides* were 1066, 523, 1132 and 1124 SSR markers, respectively. Of these, 508 (47.7%), 185 (35.4%), 551 (48.7%) and 704 (62.6%) SSR markers, respectively, showed specific polymorphic products (**Table 3**).

There are some of the loci possessing unclear amplification or monomorphic pattern in *E. bruneus*. Although unclear amplification is lacking available loci for the species tested, monomorphism markers are likely to be due to the critically small sample size. In addition, it has been reported that sequences within protein-coding regions generally show lower levels of polymorphism due to functional selection pressure [7]. Hence, a total of 1290 SSR loci (671 di-, 307 tri-, 245 tetra- and 67 penta-nucleotide) that produced amplified products in more than one species were lodged with the DDBJ database (Accession numbers: AB755818 - AB757107). The SSR locus ID, sequence, sequence length, repeat type, number of repeats, repeat position, primer sequences, primer position and expected PCR fragment size are summarized in the supplement file (Table S1). BLAST search was conducted to compare each of the 1290 SSR loci against the database for 'nucleotide collection (nr/nt)' optimized for highly similar sequences. As the result of the BLAST searches (E value < e^{-48}), although fourteen SSR sequences identified from other grouper species including *E. akaara* (one SSR locus), *E. corallicola* (one SSR lo-

cus), *E. fuscoguttatus* (six SSR loci), *E. lanceolatus* (two SSR loci) and the hybrid between *E. coioides* and *E. lanceolatus* (four SSR loci) were not novel loci, the sequence composing SSR markers identified in our study did not match with the published sequences for *E. bruneus* in the database. In contrast, the sequence homologies are indicative of close evolutionary relationships between *E. bruneus* and other species of the genus *Epinephelus*. Results indicate that we successfully developed new SSR markers for *E. bruneus*. However, it should be noted that the number of *E. bruneus* sequences currently available in the NCBI database is still limited (twelve SSR loci are available) [14].

As shown in **Tables 4(a)** and **(b)**, the frequencies of amplification or polymorphism among the additional four species are similar to *E. bruneus* except for that of *P. leopardus*. This result is probably a reflection of the different genera between *Epinephelus* and *Plectropomus* [31]. ACAG, AGAT and ATCC repeats accounted for more than 40% of the successfully amplified tetra-nucleotide SSR motifs among the four species tested, which is not markedly different from that of *E. bruneus* (44.4%). These three motifs totally have more than 50% of the polymorphism. AGAT repeats exhibit relatively high amplification to polymorphic marker conversion ratio expect for *P. leopardus*. Also, SSR polymorphism is based on size differences due to varying number of repeat units contained by alleles at given locus [6]. These results suggest that sequence composed AGAT repeat motif is conserved, high SSR mutation rate and could be used as a target for SSR marker development for the genus *Epinephelus*. Although we could not find a unique motif in the genus *Plectropomus*, there are different A/T-rich and G/C-rich frequencies between *Epinephelus* and *Plectropomus*. G/C-rich motifs accounted for 51.5% of the successfully amplified tri-nucleotide repeat motifs in *P. leopardus*. This is relatively high percentage compared with *E. bruneus* (32.0%), *H. septemfasciatus* (34.5%), *E. lanceolatus* (34.8%) and *E. coioides* (35.1%). Similarly, in successfully amplified tetra- and penta-nucleotide SSR markers, the G/C-rich SSR markers were the higher percentage in comparison to A/T-rich SSR markers (about 1.43 - 2.18 times).

Although the cross-species transferability of SSR markers is unevenly distributed among taxa, over 40% of polymorphic marker transfers have been observed in different genera of fish within the same family, and 25% of families within the same order [19]. In our case, among the 905 SSR markers polymorphic in *E. bruneus*, only 15.0% were polymorphic in *P. leopardus* even though this species also belongs to the same family (**Table 5**). The sample size for recognition of marker polymorphism was limited, which probably reflects the relatively low transferability of polymorphic SSR markers. Cross-species amplification success is only achieved when the primer sequences are conserved between species and there is a decrease in the number of amplified loci with the increase in divergence between species [32]. It has been reported that a significant negative correlation was found between genetic divergence (based on mitochondrial DNA 16S rRNA) and SSR transferability from multiple cross-species amplification studies in Sparidae [33]. In order to compare the sequences with differences of mitochondrial DNA, the alignment of 16S rRNA gene sequences between *E. bruneus* (AY947562) and *H. septemfasciatus* (AY947559), *P. leopardus* (AF297298), *E. lanceolatus* (AY947588) or *E. coioides* (AY947608) were conducted using BLASTN. Results indicated that comparably high transferability is observed when the genetic divergence of 16S between *E. bruneus* and each tested species is less than 5% (**Table 5**) which is in accordance with the results of Carreras-Carbonell *et al.* [34]. These results support the phylogenetic tree of groupers based on DNA sequence data from two mitochondrial and two nuclear genes [31]. Considering this phylogenetic tree of the groupers, the genera *Epinephelus*, *Mycteroperca*, *Hyporthodus* may have a sequence composing AGAT motif repeat as a conserved region and polymorphism and the sequence comprise G/C rich motif might be an important parameter regarding grouper evolution.

Table 5. Statistics of polymorphic SSR markers in *E. bruneus* in related-species.

Species	Polymorphism	%	Identities
H. septemfasciatus	401	44.3%	94%
P. leopardus	136	15.0%	87%
E. lanceolatus	434	48.0%	96%
E. coioides	538	59.4%	97%

%, percentage of polymorphic markers in each species. Identities, percentage of mitochondrial DNA 16S rRNA consistency compared with *E. bruneus*.

Additionally, 40 SSR markers showed polymorphism in all the other species we tested (Table S3). The results suggested that these markers have a possibility to be used as universal markers for groupers. Interestingly, there are three SSR markers composing the AGAT SSR motif which accounts for 42.9% of the nondi-nucleotide markers among the 40 SSR markers (33 di-, 1 tri- and 6 tetra-nucleotides). Although we could not find a unique motif in the genus *Plectropomus*, the AGAT SSR motif has a high potential as a highly versatile SSR marker in grouper Epinephelinae. These results suggest that the AGAT SSR motif might be a target for further screening of larger sets of SSR markers. However the number of

genera and number of samples for each genus are limited, the 1290 SSR markers (671 di-, 307 tri-, 245 tetra- and 67 penta-nucleotides) developed in this study will be useful for future genetic studies such as parentage analyses, population genetics, conservation or management of biological resources and gene mapping in groupers (Epinephelinae).

4. CONCLUSIONS

The pyrosequencing method was applied to develop di- to penta-nucleotide markers for addressing population genetics studies and gene mapping for kelp grouper (*E. bruneus*). A total of 213,073 raw reads were obtained and 171,418 unique sequences were generated with an average length of 392bp, of which 2348 (1.37%) sequences contained SSR motifs which were suitable for primer design. AC, AAT, ACAG, AGAT and ATCC were found as the common repeat motifs in kelp grouper. A total of 1466 primer sets were designed from 2348 sequences. Among 1466 SSR markers, 1244 primer sets produced strong PCR products, of which 905 (72.7%) were polymorphic in kelp grouper. A relatively high ratio of primer to polymorphic marker conversion (62%) was achieved by this method. In the cross-species amplification, over 40% of the markers amplified specific polymorphic products in the fish belonging to the same subfamily including *H. septemfasciatus*, *E. lanceolatus* and *E. coioides*; however, only 15% were polymorphic in *P. leopardus*. G/C-rich motifs accounted for 51.5% of successfully amplified tri-nucleotide repeat motifs in *P. leopardus*, which was relatively high percentage compared with *E. bruneus* (32.0%), *H. septemfasciatus* (34.5%), *E. lanceolatus* (34.8%) and *E. coioides* (35.1%). ACAG, AGAT and ATCC repeats accounted for more than 40% of the successfully amplified tetra-nucleotide SSR motif, and these nucleotide repeat motifs exhibited more than 50% of polymorphism in all species tested. In addition, there are three SSR markers containing the AGAT SSR motif (42.9% of nondi-nucleotide markers) among the 40 SSR markers (33 di-, 1 tri- and 6 tetra-nucleotide) that showed polymorphism in all the species we tested. Results indicate that the AGAT SSR motif has a high potential as a highly versatile SSR markers in groupers since the genus *Epinephelus* is the largest group among grouper Epinephelinae. The SSR markers developed in this study can be employed to obtain reliable genetic variability estimates for groupers (Epinephelinae).

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