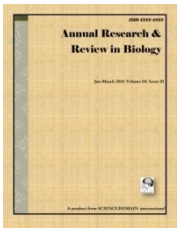

Annual Research & Review in Biology
4(1): 93-104, 2014

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Genomic Molecular Markers for the DNA Fingerprinting of Sperm Derived from *Epinephelus lanceolatus* and *E. fuscoguttatus*

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Authors' contributions

Author KFR designed and carried out the experiments, Authors SS and KFR were involved in manuscript preparation, author S Senoo is responsible for maintenance of brood stock and extraction of DNA from fin clip samples. All authors read and approved the final manuscript.

Research Article

Received 18th July 2013
Accepted 14th September 2013
Published 4th October 2013

ABSTRACT

Groupers belong to the subfamily Epinephelinae of the family Serranidae. They are an economically important marine fisheries resource and are commercially cultivated throughout the tropical and temperate regions of the world. The aquaculture industry relies on artificial breeding of groupers in order to obtain fingerlings which are free of pathogens and demonstrate a uniform growth rate. Rapid validation of sperm and eggs is a major challenge to breeders. Single locus DNA markers are ideal for the authentication of germplasm as they generate single PCR amplicons which do not require further sequencing. This study focused on the development of single locus DNA markers for genotyping of sperm samples derived from two species of grouper, the Giant grouper (*Epinephelus lanceolatus*) and the Tiger grouper (*E. fuscoguttatus*). Single locus molecular markers were developed using DNA sequences obtained from shotgun genomic libraries and tested against sperm samples derived from each of the species and the closely related groupers *E. coioides* and *E. corallicola*. A total of 54 molecular markers were developed of

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which six were found to be specific to *E. fuscoguttatus* and seven to *E. lanceolatus*. The remaining markers generated PCR products in all of the four species and were rejected as suitable candidates for genotyping. The markers developed as a result of this study are relevant to fish breeders and fish farmers as they species specific, inexpensive and augment traditional methods of identification based on phenotypic characterization.

Keywords: Epinephelus lanceolatus; E. coioides; E. fuscoguttatus; E. corallicola; molecular markers.

1. INTRODUCTION

Captive breeding of groupers is the practice of choice for a majority of commercial aquaculture farms in South East Asia. This ensures sustainability of wild stocks which are an important source of wild germplasm. Currently adopted practices involve the procurement of cryopreserved sperm [1] from commercial suppliers followed by in vitro fertilization of eggs collected from females at the hatchery. Validation of sperm received from diverse sources is not possible using conventional methods for identification including microscopy or biochemical tests. Molecular markers are ideal for applications in aquaculture [2] and have been developed for a wide range of groupers including the Giant grouper, *E. lanceolatus* [3], the Hump-backed grouper, *Cromileptes altivelis* [4] and the orange spotted grouper *E. coioides* [5,6]. Validation of grouper germplasm using DNA barcoding protocols [7] is a technically challenging process which involves DNA sequencing of PCR amplicons, followed by similarity searches using bioinformatic tools and these resources may not be available at breeding stations. A majority of the molecular markers available for groupers [8,9] have been developed based on microsatellite DNA loci for application in population genetic studies [10]. Microsatellites have the ability to cross-amplify DNA within a genus [11] and this characteristic hinders their application as a marker to distinguish germplasm derived from closely related species. Genotyping of sperm necessitates the development of markers which demonstrate the ability to amplify specific DNA loci within a single species [12]. This study focused on the development of single copy number DNA markers for application in genotyping of germplasm derived from two species of grouper, the Giant grouper *E. lanceolatus* and the Tiger grouper (*E. fuscoguttatus*). Genomic DNA loci were isolated from a small insert shotgun genomic library for each of the two species followed by the design of single locus DNA markers for validation. The markers were tested for species specificity in the closely related Orange spotted grouper (*E. coioides*) and the Coral grouper (*E. corallicola*).

2. METHODOLOGY

2.1 Sample Collection and DNA Extraction

DNA was extracted from 200 µl of sperm samples obtained from *E. lanceolatus* (4), *E. fuscoguttatus* (9), *E. coioides* (6) and *E. corallicola* (8) brooders using a DNeasy Blood and Tissue Kit® (Qiagen) according to the manufacturer's instructions. DNA concentration was adjusted to 50 ng/µl using a single drop spectrophotometer (GE Healthcare Life Sciences). In addition to the above samples, fin-clips were obtained from *E. lanceolatus* (23), *E. fuscoguttatus* (28), *E. coioides* (9) and *E. corallicola* (12) specimens which were randomly collected from fishermen in Sabah, Malaysia.

2.2 Genomic Library Construction

Genomic libraries were constructed following the protocol of [13] with minor modifications. Genomic DNA from *E. lanceolatus*, *E. fuscoguttatus* and the cloning vector pUC19 were digested separately using different combinations of restriction endonucleases (Fermentas) for five hours at 37°C followed by thermal inactivation at 80°C (Table 1). DNA fragments were resolved by electrophoresis on 1.5% TBE agarose gels. Digested pUC19 was excised and purified using QIAquick® gel extraction kit (Qiagen). The ligation reaction was set up with 300 ng of genomic DNA and 50 ng plasmid vector in a volume of 20µl using 0.8 units of T4 DNA ligase (Fermentas) according to the manufacturer's instructions. Ligation was done at 4°C for 16 hours. The products were transformed into chemically competent *E. coli* (TOP10) and screened on Lysogeny Agar containing Ampicillin (50 µg/ ml) and X-Gal (20 mg/ml). White colonies were chosen and screened using colony Polymerase Chain Reaction (PCR) and those with an insert size in excess of 500 bp were subjected to plasmid extraction and purification with the GeneJET™ Plasmid Purification Kit (Fermentas) and sequenced using an ABI Big Dye Terminator cycle sequencing kit on an ABI Prism 377 auto-sequencer (First Base, Singapore) using M13 (-20) as the forward primer. Sequences were edited using DNAstar, annotated, deposited at the NCBI GenBank and assigned accession numbers.

Table 1. Combinations of restriction endonucleases used to digest DNA for construction of the genomic library

No.	Restriction Enzyme Combinations
1	BamHI and HindIII
2	BamHI&PstI
3	EcoRI&BamHI
4	EcoRI&XbaI
5	EcoRI&HindIII
6	EcoRI&SalI
7	XbaI&PstI
8	XbaI&HindIII
9	XbaI&BamHI

2.3 Primer Design and Polymerase Chain Reaction

A total of 27 genomic sequences from each species were selected randomly for the purpose of designing primer pairs. Locus specific primers were designed using the online primer design software PRIMER 3.0 [14]. The first two letters of the primer code designate the species for which it has been designed *E. lanceolatus* (EL) and *E. fuscoguttatus* (EF). A Standard protocol of polymerase chain reaction (PCR) was used to test and identify the amplification pattern for the primers against *E. lanceolatus* and *E. fuscoguttatus* as well as cross-amplification against *E. coioides* and *E. corallicola*. PCR was performed on a MJ Research Thermal Cycler in a 20µl containing approximately 50 ng template DNA, 1X PCR buffer, 0.2 mM each dNTP, 0.25 units TaqDNA polymerase (Fermentas), 10pmol of forward and reverse primer, with the following cycle profile: initial denaturing at 96°C for 3 minutes followed by 30 cycles at 96°C for 10 s, annealing at 58°C for 20 s and 72°C for 30 s extension, with a final extension step at 72°C for 10 min. Amplification products were separated by electrophoresis on a 2% TBE agarose gel with a 100 bp DNA ladder (Promega) as a size standard. The gel was stained with Ethidium Bromide (5 µg/ml) and

bands were scored using the gel documentation system, Alphaimager 2000 (Alpha Innotech, USA).

3. RESULTS AND DISCUSSION

3.1 DNA Extraction and Genomic Library Construction

Concentration of DNA extracted from fresh sperm ranged from 100 – 300 µg/ml with an A_{260}:A_{280} between 1.8 and 2.0. DNA extraction from fish sperm samples is relatively easy as compared with extraction of tissue samples. The current study used 200 µl of sample and the concentration of DNA isolated (100 µg/ml) was sufficient for genomic library construction and routine PCR. Considering the high cost of grouper sperm, it is recommended that for the purposes of routine PCR, volumes of less than 50µl will suffice. The protocols applied in this study enabled the rapid isolation of genomic fragments for the subsequent development of molecular markers. A total of 119 sequences were isolated from each of the two parental genotypes *E. lanceolatus* and *E. fuscoguttatus*. The complete data set that comprised 59 sequences from *E. lanceolatus* (Accession Numbers: JN007470 - JN007411) and 60 from *E. fuscoguttatus* (Accession Numbers: JN944352 - JN944337 and JN159898 - JN048827) is currently available in the GenBank. A conventional shotgun cloning approach was adopted due to its low cost as compared to whole genome [15] sequencing approaches.

3.2 Primer Design and Polymerase Chain Reaction

The current study necessitated the development of genomic library which consisted of a large set of dispersed genomic fragments for the purpose of marker development. The high rate of attrition resulted in the rejection of a majority of the markers due to their ability to cross-amplify in closely related species. The primers were designed (Table 2) in order to generate PCR amplicons which ranged in size from approximately 150 to 800 base pairs (Table 3). The primer pairs ELJPK0010, ELJKE003, ELJKE005, ELJKE008, ELJS03, ELJE01 and EL103 were specific to *E. lanceolatus* DNA (Fig. 1) and did not produce PCR amplicons in any of the other species tested. Similarly, the primer pairs EFP007, EFJAAC1, EFJCTT1, EFJ007, EFJ009 and EFJ021 were specific to *E. fuscoguttatus* (Fig. 2). The remainder of the 41 loci amplified DNA from all four species tested and were not considered species specific DNA markers for the purpose of genotyping. Phylogenetic studies based on mitochondrial and genomic loci in the family Serranidae indicate a high degree of genetic similarity among the groupers with a distinct phylogenetic resolution of the four species in which *E. lanceolatus* and *E. fuscoguttatus* are more closely related as compared to *E. coioides* and *E. corallicola* [16]. The results of this study support this evidence as a higher number of genomic loci were shared between *E. lanceolatus* and *E. fuscoguttatus* as compared to *E. corallicola* and *E. coioides*.

3.3 Application of Molecular Markers for the Recruitment of Broodstock

Groupers are relatively difficult to breed as compared with most other fish species. The average time required for males to attain sexual maturity can range between one to five years [17]. Under these circumstances it is a common practice for breeders to maintain mature females at the hatchery and to obtain cryopreserved sperm from commercial suppliers. Authentication of sperm samples using PCR based approaches offers a reliable and cost effective solution to commercial hatcheries. The markers developed have to be species-specific and should not exhibit PCR products in closely related species. A majority

of the molecular markers developed generated PCR products of the expected size in all the four species which were tested indicating that these genomic loci are common. Previous studies [18] have indicated that the four species are closely related and cluster together as one group. The current assay was developed to facilitate DNA fingerprinting in laboratories with limited access to high end equipment such as a DNA sequencer. Validation of sperm prior to artificial fertilization is a critical step in fish breeding and the markers developed can be applied to validate sperm samples prior to artificial spawning.

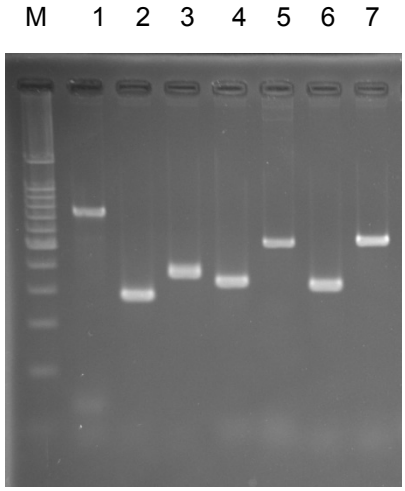

Fig. 1. Single locus genomic molecular markers specific to *Epinephelus lanceolatus*. Lane 1: ELJPK0010; 2: ELJKE003; 3: ELJKE005; 4: ELJKE008; 5: ELJS03; 6: ELJE01; 7: EL103; Lane M: 100 bp DNA marker (Promega).

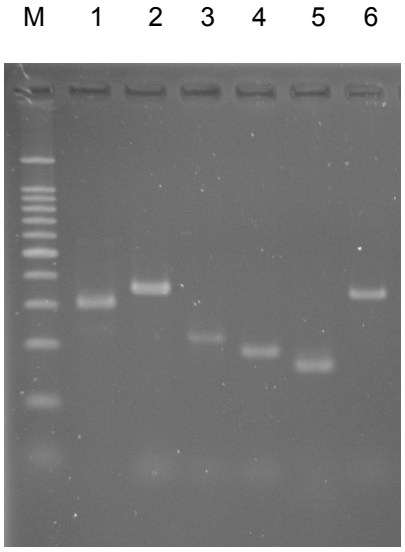

Fig 2. Single locus genomic molecular markers specific to Epinephelus fuscoguttatus. Lane 1: EFP007; 2: EFJAAC1; 3: EFJCTT1; 4: EFJ007; 5: EFJ009; 6: EFJ021; Lane M: 100 bp DNA marker (Promega).

Table 2. Primers developed for testing in four species of groupers indicating locus, primer sequence, expected size of PCR product and GenBank accession number

No.	Locus	Primer Sequence (5'- 3')	Expected size of PCR product (bp)	GenBank Accession No.
1	ELJPK001	F: GTG TAA TCC CTC CAG CGT GT R: CTC ATG TCT GTG CGC TCA AT	737	JN007453
2	ELJPK002	F: GGT GCC TAA ATG TGG GAA AA R: CAG CGT GCA GAT TGA TGT TC	276	JN007454
3	ELJPK003	F: AGC AGG GCT GAA ATG TGT CT R: AAC CGA ACG AAA TGA AGG TG	356	JN007455
4	ELJPK004	F: TTC TCT GGT GGT TGG GTT TC R: GCT CCG AAC TCC TCT GTG TC	329	JN007456
5	ELJPK006	F: GCT TCC TGC CTG CTT GTA AC R: GGG TGG AAT TGC ATC AGT CT	513	JN007458
6	ELJPK007	F: CTC GTT AGC CAT TCA GCA CA R: GGA GCC ATC AGA CTC AAA GC	314	JN007459
7	ELJPK008	F: GCT GCC AAA GAG AAC GAA AC R: GTA ACC ATG CAA AGC TGC AA	528	JN007460
8	ELJPK009	F: TCT GTC CAT GAG CTG AAA CG R: TGT CTG TCA GTG GGG GTA CA	777	JN007461
9	ELJPK0010	F: ACT GCT ACC CGA CTC GTG AC R: GCA AGG AAA GTG GAG AGA GC	434	JN007462
10	ELJKE001	F: GCT CTG TTA ACG TGC GAT GA R: ATT CCC GAC AAA ACA CAG AG	309	JN007463
11	ELJKE002	F: CAA TGG GGA GAC AAA GCA GT R: GAA AGG CAA GGC AGA GAA TG	288	JN007464
12	ELJKE003	F: AGT TTG AGG GGG AAA AGC AT R: CTG GCA TTG ACG AGA GCA TA	354	JN007465
13	ELJKE004	F: CAA CAC ATG GCA GTG GAC TAA R: GGT TGC AAG TGA GCT TTT CC	285	JN007466
14	ELJKE005	F: CCC ATG TTA AAA TGC CCA AC R: GGA TCG GTT GCG TAA GTT GT	387	JN007467
15	ELJKE006	F: GGG ACA CAA CAA ACA CGA GA R; CCT GTT CCT GAG GGA GAG AA	278	JN007468

16	ELJKE007	F: GTC TTT GGA CTG TGG GAG GA R: CGT TCC TCG GTG GTG ATA CT	490	JN007469
17	ELJKE008	F: GGG AGG CAT TTG GTC AGA TA R: ACA CAC AGG CTG CTG ACA AG	355	JN007470
18	EL104	F: CCG GAC CAA ATA CAA AGC AG R: CTC GGG GTA ATC CTC GTG TA	376	JN007414
19	ELJK01	F: CAC GGC TGT TTT GTC TCT GA R: GAT TGA GCA GCA TCC ACA GA	369	JN007431
20	ELJK02	F: GAG GAA TGG GCT TTT GAT GA R: AAG TTT CGC GAC TAC CCA GA	411	JN007432
21	ELJK03	F: CTG CCA TGT TTT GGG TTT TT R: GTG TAG GGG GAG GTC TGT GA	253	JN007433
22	ELJS01	F: CGT TCC CAC AAA TGT CAC TG R: GTG TCT ACG CCC ACT TGG AT	790	JN007435
23	ELJS02	F: CCC CAA TTT TAA GGA AAA CG R: CAG TCA ATA GCA AGC AGG TCA	258	JN007436
24	ELJS03	F: ACG TCA GGG AGA AAT TGT GC R: CCA GAC AGT GTG CTC CAT TG	252	JN007437
25	ELJE01	F: TCC TGT GTG AAG CTG AAT GC R: TCA CAC GGG ACA TGA ACA CT	474	JN007438
26	ELJE02	F: TTC AAT TTT CCC TGC CTT GT R: TGG TCC TGT TGT CAT GTG GT	270	JN007439
27	EL103	F: GAG GGC AGG AAC ACT GAG AA R: ACT CTG CAG GTC CCA GCT T	434	JN007413
28	EFJ002	F: TTG GGA TGG GTT CTA AGA GA R: ACC CCA GGT TTC TTT TCA GC	294	JN048832
29	EFJ005	F: TTT CGT TGT AGC GCT TGA TG R: TGC ACA CTC TTG GCA TTC TC	345	JN048835
30	EFJ006	F: CGT CTC TCC ACG GGA TAT TT R: CCG TGA CAA CTT TGA GCA TC	253	JN048836
31	EFJ016	F: GGG CAG CAT TAT GTC TCC AT R: TGT CTG TCC CTC CCT ACA CC	185	JN048846
32	EFJ018	F: CCT GTC TCT GGA AGC CTC AC R: CCT GCA ACG TAG TGT GGG TA	157	JN048848
33	EFJ022	F: ATG TGC CAT GCA ATC TGT GT	332	JN048852

		R: ACT GCT GTC CAT CCA TCT CC		
34	EFP007	F: GAA GTA TGG GGG CAA TGA TG	624	JN048861
		R: TTT TTG TGG GGC TTT GCT AC		
35	EFP009	F: GCT GAG TGA TCT GGC ATC AA	178	JN048863
		R: ATG CTC CAG AAG ACG AGG AA		
36	EFP010	F: CAT GGC AGC AGA ATA AAC CA	192	JN048864
		R: CAG GAA GAG GGG AAG AAG TG		
37	EFP013	F: ACG GAC CTC TGG GAG AAA CT	379	JN048867
		R: GAT GTC CCA GAA AGG CAA AA		
38	EFPHI003	F: ACA AGG CCA AAG CAA AGA GA	550	JN159896
		R: GGG TGG AGG AAG AAC ACA AA		
39	EFPHI004	F: TTT GTC TCC CTC CCT CAA TG	265	JN159897
		R: GCT AGC ATG ATC CCG ATG TT		
40	EFPHI006	F: CTA GCT GTG GCA GAC AGA CG	214	JN159899
		R: AGG GAC ACT GGT TGT GGA AC		
41	EFJAAC2	F: TGT GAA AAT GGG TGA AGT CG	161	JN048828
		R: GTA TGG CCC TGC AAA GGT AA		
42	EFJCTT1	F: TCC TGC ACA ACT CCA CAG AG	288	JN048829
		R: CAA GCA TGT CTG CCT TTT GA		
43	EFJ003	F: TTT GCA GTG TAG GCC AGA TG	340	JN048833
		R: GTA AGC AGG GCA AGG AAA AC		
44	EFJ007	F: AAG ATC GCT GGA GAC CAG AA	288	JN048837
		R: AAT CGT CAG TCG CTT CAC CT		
45	EFJ008	F: GGG GAA GCT CTG TCT GAA AA	173	JN048838
		R: TTC ATT CTG TCC CCA GAA CC		
46	EFJ009	F: GCA AAC TCT GCA CTC CC AG	203	JN048839
		R: TCT GGG ATG CCT ACG TGA AT		
47	EFJ013	F: GCA CCT TGA GGG AGC TAG TG	244	JN048843
		R: GTC AGC AGA AGC CAC TTT CC		
48	EFJ020	F: TCA GTG ACC CCT GTG TGT GT	372	JN048850
		R: GTG CTT GTT TTT GCC ACT GA		
49	EFJ021	F: GTC ACA ACA CTG GGA ACG TG	476	JN048851
		R: GGC AGC CAT GGT TTA TGT CT		
50	EFP003	F: TCA TCT AAT GTG CGC TGC TC	185	JN048857
		R: TGC TGT TAA TGC GTG AGG AC		

51	EFP005	F: AGA GCG GAG CTT GTT CTC AC R:GAG TGT GCC TGC ATG AGT GT	358	JN048859
52	EFP006	F: AGC ACG TTT GAG CAG GAG AT R: CAG GGA GGG TCA AGA TTT CA	466	JN048860
53	EFPHI002	F: AGA CTG GAC ATC TAA TCC AC R: AGA GGT CTG TTT TGC GCA TT	217	JN159895
54	EFJAAC1	F: CAT CGT GGT ATG CAC CTC TG R: TCA AAC AGG TCG TCC ACA AA	285	JN048827

Table 3. PCR amplification profiles for primers tested against four species of groupers E. lanceolatus (EL), E. fuscoguttatus (EF), E. coioides (EC) and E. corallicola (CC), the presence of a PCR band at the expected size is indicated by (1) and no amplification is indicated by (0)

No.	Primer	EL	EF	EC	CC
1	**EL JPK001**	**1**	**1**	**0**	**0**
2	**EL JPK002**	**1**	**1**	**0**	**1**
3	**EL JPK003**	**1**	**0**	**0**	**1**
4	**EL JPK004**	**1**	**1**	**0**	**1**
5	**EL JPK006**	**1**	**1**	**0**	**1**
6	**EL JPK007**	**1**	**1**	**0**	**1**
7	**EL JPK008**	**1**	**1**	**1**	**1**
8	**EL JPK009**	**1**	**1**	**0**	**1**
9	**EL JPK0010**	**1**	**0**	**0**	**0**
10	**EL JKE001**	**1**	**0**	**0**	**1**
11	**EL JKE002**	**1**	**1**	**0**	**1**
12	**EL JKE003**	**1**	**0**	**0**	**0**
13	**EL JKE004**	**1**	**1**	**0**	**1**
14	**EL JKE005**	**1**	**0**	**0**	**0**
15	**EL JKE006**	**1**	**1**	**1**	**1**
16	**EL JKE007**	**1**	**1**	**0**	**0**
17	**ELJKE008**	**1**	**0**	**0**	**0**
18	**EL 104**	**1**	**1**	**0**	**1**
19	**EL JK01**	**1**	**1**	**0**	**1**
20	**EL JK02**	**1**	**0**	**0**	**1**
21	**EL JK03**	**1**	**0**	**0**	**0**

22	**EL JS01**	**1**	**0**	**0**	**0**
23	**EL JS02**	**1**	**0**	**0**	**0**
24	**EL JS03**	**1**	**0**	**0**	**0**
25	**EL JE01**	**1**	**0**	**0**	**0**
26	**EL JE02**	**1**	**0**	**0**	**0**
27	**EL 103**	**1**	**0**	**0**	**0**
28	**EF J002**	**1**	**1**	**0**	**0**
29	**EF J005**	**1**	**1**	**0**	**0**
30	**EF J006**	**1**	**1**	**0**	**0**
31	**EF J016**	**1**	**1**	**0**	**0**
32	**EF J018**	**0**	**1**	**1**	**0**
33	**EF J022**	**1**	**1**	**0**	**0**
34	**EF P007**	**0**	**1**	**0**	**0**
35	**EF P009**	**1**	**1**	**0**	**0**
36	**EF P010**	**1**	**1**	**0**	**0**
37	**EF P013**	**1**	**1**	**0**	**0**
38	**EF PHI003**	**1**	**1**	**0**	**0**
39	**EF PHI004**	**1**	**1**	**0**	**0**
40	**EF PHI006**	**1**	**1**	**0**	**0**
41	**EF JAAC2**	**1**	**1**	**0**	**0**
42	**EF JCTT1**	**0**	**1**	**0**	**0**
43	**EF J003**	**1**	**1**	**0**	**0**
44	**EF J007**	**0**	**1**	**0**	**0**
45	**EF J008**	**1**	**1**	**0**	**0**
46	**EF J009**	**0**	**1**	**0**	**0**
47	**EF J013**	**1**	**1**	**0**	**0**
48	**EF J020**	**0**	**1**	**0**	**1**
49	**EF J021**	**0**	**1**	**0**	**0**
50	**EF P003**	**1**	**1**	**0**	**0**
51	**EF P005**	**1**	**1**	**0**	**0**
52	**EF P006**	**1**	**1**	**0**	**0**
53	**EF PHI002**	**1**	**1**	**0**	**0**
54	**EF JAAC1**	**0**	**1**	**0**	**0**

4. CONCLUSION

This study successfully developed single locus genomic DNA markers for the rapid and reproducible genotyping of germplasm derived from *E. lanceolatus* and *E. fuscoguttatus*. These markers will find application in the aquaculture and fish breeding industry and facilitate the identification of fish stocks prior to recruitment to a breeding program.

ACKNOWLEDGEMENTS

The project was funded by the Ministry of Science Technology and Innovation (MOSTI), Government of Malaysia research grant number SCF0074.

COMPETING INTERESTS

The authors have declared that no competing interests exist.

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