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MOLECULAR MARKERS FOR PARENTAGE ANALYSIS IN THE GROUPER F1 HYBRID *EPINEPHELUS COIOIDES* × *EPINEPHELUS LANCEOLATUS* (ACTINOPTERYGII: PERCIFORMES: SERRANIDAE)

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Background. The aquaculture industry is driven by the need to develop novel interspecific hybrids with improved culture characteristics, however currently no molecular markers are available for the validation of F1 hybrids in the case of groupers. The presently reported study was directed toward the development of molecular markers for F1 hybrids obtained by artificial spawning of eggs derived from orange-spotted grouper, *Epinephelus coioides* (Hamilton, 1822), with sperm from giant grouper, *E. lanceolatus* (Bloch, 1790).
Materials and methods. DNA was extracted and purified from the fin clip of a single F1 hybrid of *E. coioides* × *E. lanceolatus*. Small insert shotgun genomics libraries were constructed, 40 positive transformants were sequenced, and the sequences deposited at the GenBank. Thirty locus-specific primer pairs were designed and tested across 64 F1 hybrid specimens as well as DNA samples extracted from coral grouper, *Epinephelus corallicola* (Valenciennes, 1828), and brown-marbled grouper, *E. fuscoguttatus* (Forsskål, 1775).
Results. A panel of thirty molecular markers was developed to verify parentage, of these eleven and six of the markers were inherited from the sire and dam respectively. Two markers were specific to the F1 hybrid and none of the markers could be assigned to the closely related groupers *E. corallicola* and *E. fuscoguttatus*.
Conclusion. The markers developed can be applied to assign parentage and determine the degree of introgression in interspecific grouper hybrids developed from *E. coioides* and *E. lanceolatus*. The methods developed in this study can be extended to characterize Quantitative Trait Loci (QTLs) and to identify interspecific F1 hybrids in the wild.

Keywords: Mendelian inheritance, heterosis, Dobzhansky–Muller incompatibility, single locus DNA markers, genomic library

INTRODUCTION

The aquaculture industry is increasingly turning towards the cultivation of interspecific grouper hybrids, which have been reported to exhibit higher growth rates and resistance to disease, characteristics which are likely to be the result of heterosis. Grouper hybrids have been documented in the case of goldblotch grouper, *Epinephelus costae* (Steindachner, 1878) × dusky grouper, *E. marginatus* (Lowe, 1834) (see Glamuzina et al. 2001); brown-marbled grouper, *E. fuscoguttatus* (Forsskål, 1775) × camouflage grouper, *E. polyphekadion* (Bleeker, 1849) (see James et al. 1999); as well as an F1 hybrid which has been developed from the giant grouper, *E. lanceolatus* (Bloch, 1790) and orange-spotted grouper, *E. coioides* (Hamilton, 1822) (see Kiriyakit et al. 2011). Natural hybrids have been reported in leopard coral-grouper, *Plectropomus leopardus* (Lacepède, 1802) and spotted coralgrouper, *P. maculatus* (Bloch, 1790) (see van Herwerden et al. 2002) and in reef fishes of the family Serranidae (see Yaakub et al. 2006). Molecular markers are ideal for mapping inheritance patterns in fish (Liu and Cordes 2004) and have been developed and characterized for many species of groupers which include the humpback grouper, *Cromileptes altivelis* (Valenciennes, 1828) (see Na-Nakorn et al. 2010); the giant grouper, *E. lanceolatus* (see Yang et al. 2011); the Hawaiian grouper, *Hyporthodus quernus* (Seale, 1901) (see Rivera et al. 2003); and the brown-marbled grouper, *E. fuscoguttatus* (see Renshaw et al. 2012), however these molecular markers have been used in population genetic studies and not for inferring

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parentage and inheritance as was done in the case of tilapia hybrids developed from *Oreochromis niloticus* (Linnaeus, 1758) and *Sarotherodon melanotheron* Rüppell, 1852 (see Bezault et al. 2012). The current study was based on the F1 hybrid developed at the Borneo Marine Research Institute between an *E. coioides* (Female) and *E. lanceolatus* (Male) (Koh et al. 2010). The objective of this study, which was the determination of parentage, necessitated the development of single locus genomic molecular markers, which could then be applied to verify inheritance patterns. This was done using a bottom-up approach which involved the construction of a small insert genomic library for the F1 hybrid followed by mapping of the genomic loci against the parental genotypes.

MATERIALS AND METHODS

Sample collection and DNA extraction. Specimens of *Epinephelus lanceolatus*, *E. fuscoguttatus*, *E. coioides*, *E. corallicola* (Valenciennes, 1828) and F1 hybrids of *E. coioides* (Female) and *E. lanceolatus* (Male) are currently being maintained at the hatchery of the Borneo Marine Research Institute, Universiti Malaysia Sabah. Anal fin clips of approximately 1 cm^2 were collected from each of the parental genotypes. An additional 64 samples were collected from one year-old F1 hybrid specimens. Ten samples were derived from *E. corallicola* and six from *E. fuscoguttatus*. DNA was extracted using the salting out protocol (Aljanabi and Martinez 1997) and the concentration of DNA was adjusted with sterile nuclease free water to 200 ng · μL^{-1} and verified using a single drop spectrophotometer (GE Life Sciences).

Development of small insert genomic library. A small insert genomic library was constructed by digesting 200 ng of genomic DNA from the F1 hybrid and 200 ng of the circularized cloning vector pUC19 with the following combinations of restriction enzymes *Bam* HI/*Hind* III (5 Units each), *Eco* RI/*Bam* HI (5 Units each), *Eco* RI/*Hind* III (5 Units each), *Xba* I /*Bam* HI (5 Units each), and *Xba* I/*Hind* III (5 Units each) in a reaction volume of 20 µL comprising 2× Fermentas buffer Tango (4.0 µL), restriction enzymes (5 Units), DNA (200 ng) and nuclease free water. Reaction mixtures were incubated at 37ºC for 3 h followed by heating to 80ºC for 20 min to inactivate the restriction enzymes. The DNA fragments were resolved on a 1× Tris-Boric acid-EDTA (TBE) agarose gel (Promega) followed by gel purification using a gel extraction kit according to the manufacturers instruction (Qiagen) and subsequently ligated onto the cloning vector pUC19 in a reaction mixture comprising 2× T4 DNA ligation buffer (Fermentas) (2.0 µL), T4 DNA ligase (5 Units), genomic DNA fragments (100 ng), linearized pUC19 cloning vector (200 ng), and nuclease free water to a final volume of 20 µL. Ligation was carried out at 4ºC for 16 h. The ligation mixture was transformed into chemically competent *Escherichia coli* (TOP 10) by immersion in a water bath set at 37ºC for 40 s followed by plating of 100 µL of the transformed cells onto lysogeny agar containing 100 µg · mL^{-1} of ampicillin and 5 µg · mL^{-1} of X-Gal followed by incubation for 16 h at 42ºC. Positive transformants were selected by blue-white colony screening and single colonies were cultured in 10 mL of lysogeny broth containing ampicillin (100 µg · mL^{-1}). Plasmids were extracted using the GenJET plasmid minipreparation kit (Fermentas) followed by sequencing using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Sequences were trimmed using the Vec Screen software (NCBI) and 40 of the edited sequences were deposited at the NCBI GenBank. Sequence similarity was determined using the blast tool (Zhang et al. 2000) and sequences with a coverage in excess of 50% were deemed to be significant matches to the fish genome database.

Design of locus specific primers and testing in parental genotypes, F1 hybrids and related groupers. Locus specific primers were designed for each of the 40 loci using the online primer design tool Primer 3.0 (Rozen and Skaletsky 1999) and 30 primer pairs were synthesized (First Base, Singapore). Primers were reconstituted in nuclease free water to a final concentration of 100 µM. PCR amplification was performed in final volume of 20 µL which consisted of 1.2 µL $MgCl_2$ (1.5 mM), 0.4 µL dNTPs (0.2 mM), 4 µL 1× Go*Taq* buffer (Promega, USA), 1 U *Taq* DNA polymerase (Fermentas, USA), 1 µL of each primer (5 µM), 3 µL template and nuclease free water. Amplification was performed in the thermal cycler (MJ research, PTC-200) with the parameters of the cycler pre-denaturation at 95ºC for 3 min, this was followed by 30 cycles of denaturation (30 s at 95ºC), annealing (40 s at 56ºC), extension (2 min at 72ºC) and final extension (10 min at 72ºC). PCR products were resolved by gel electrophoresis on a 1.5% TBE Agarose gel (Promega) followed by staining for 10 min in a solution of ethidium bromide (5 µg · mL^{-1}). Gels were then rinsed by immersion in water for 5 min and visualized and scored using a gel documentation system ALPHAIMAGER 2000 (Alpha Innotech Corp. USA).

Ethical issues. The samples were isolated from specimens which are currently being maintained at the Borneo Marine Research Institute, Universiti Malaysia Sabah, in compliance to the institutional Animal Care and Use Committee (ACUC) guidelines.

RESULTS

Sample collection and DNA extraction. The sample collection and DNA extraction protocols yielded DNA, which was of the required quality (A_{260} : A_{280} = 1.8–2.0) and concentration (100–300 µg · mL^{-1}) for genomic library construction and polymerase chain reaction.

Development of small insert genomic library. A total of 40 genomic loci were deposited at the NCBI GenBank (Accession numbers JQ732815–JQ732778). Sequence similarity searches revealed that the following eight accessions could be mapped back to fish genomes with sequence coverage in excess of 50%: JQ732778 [*Danio rerio* (Hamilton, 1822)], JQ732785 (*E. lanceolatus*), JQ732786 (*E. fuscoguttatus*), JQ732785 (*E. lanceolatus* × *E. fuscoguttatus*), JQ732794 [*Perca flavescens* (Mitchill,

1814)], JQ732795 (*E. fuscoguttatus*), JQ732798 [*E. fuscoguttatus*; *Dicentrarchus labrax* (Linnaeus, 1758); *Gasterosteus aculeatus* Linnaeus, 1758], JQ732814 (*Oreochromis niloticus*), JQ732809 (*E. coioides* × *E. fuscoguttatus*), and JQ732815 (*Cromileptes altivelis*). The remaining 32 accessions could be mapped back to the fish genome database, however the level of coverage was less than 50% and these matches were considered not significant.

Design and application of locus specific genomic molecular markers. A panel of 30 locus specific molecular markers were designed, of which 29 generated PCR amplicons of the expected size, one of the primer pairs (ECELEB008) failed to amplify in the F1 hybrid or the parents, 13 loci were found to be common to both the parents, 11 and 6 were inherited from *E. lanceolatus* and *E. coioides*, respectively (Fig. 1). Two of the loci, ECELEH007 and ECELEB001 were specific to the F1 hybrid and not associated with the parents. Three of the primers ECELBH003, ECELB013 and ECELXB003 produced two distinct bands in the F1 hybrid and one in each of the parental genotypes which were designated as A, B, and AB (Table 1). None of the molecular markers could cross-amplify in the closely related groupers *E. fuscoguttatus* and *E. corallicola*. Primer amplification was uniform across the entire set of samples which were tested.

DISCUSSION

Genomic library development. Small insert genomic libraries constructed using a shotgun approach provide a widely dispersed set of genomic DNA fragments which can be sequenced in order to develop single locus molecular markers. The current approach utilized five combinations of restriction enzymes in order to reduce the level of redundancy in genomic coverage. The sequences, which had a significant level of similarity to fish genomes represented non-coding regions of DNA and can be considered as Type II molecular markers.

Design and application of locus specific genomic molecular markers. The commercial aquaculture industry has directed a significant amount of effort towards the development of grouper hybrids as it is generally assumed that there will be an increase in the levels of allelic diversity and the associated development of characteristics which are desired in aquaculture systems. One of the major driver of this effort has been the increase in the incidence of disease and the need to develop resistant varieties (Harikrishnan et al. 2010). In conventional breeding approaches involving interspecific grouper hybrids, the selection of the parental genotypes is based purely on phenotypic characterization, for instance an F1 hybrid was developed from *E. fuscoguttatus* × *E. polyphekadion* (see James et al. 1999) solely on the basis of the higher growth rate of the hybrid when compared to the dam and sire. Contemporary approaches using molecular markers extend the scope of selection beyond the phenotype and provide a more accurate indicator of genetic compatibility and hybrid viability. Three key criteria have to be considered when selecting parental genotypes for hybridization; the first relates to the genetic distance between species, the second concerns interspecific genetic viability which is governed by the Dobzhansky–Muller Incompatibility (DMI) model and the third pertains to variations in the number of chromosomes, which can result in complications in chromosomal rearrangements after the formation of the zygote. The first criterion, which relates to genetic distance, can be resolved using molecular phylogenetics. A reconstruction of grouper phylogenetic trees using molecular data (Craig and Hastings 2007) clearly delineates the relation between groupers and *E. coioides* and *E. lanceolatus* clustered together in one clade with a high level of confidence. This is supported by the fact that 13 of the 29 markers tested were shared between both *E. coioides* and *E. lanceolatus* indicating a high level of genomic similarity at randomly selected loci.

The second criterion which concerns DMI needs to be further examined in view of the evidence that indicates a lower percentage of survival among hybrids (Kiriyakit et al. 2011), this phenomenon has also been reported in hybrids developed in Sunfish (López-Fernández and Bolnick 2007) and has been attributed to incomplete penetrance. Similar evidence for attenuation of hybrid vigor has been reported in a study involving Flounder hybrids (Xu et al. 2011) and these findings must be carefully considered prior to hybrid breeding programs involving groupers. The markers developed for this study amplified consistently in all of the F1 samples tested, these represent the individuals which have survived for a period of one year after hatching and may be indicative of the alleles which confer fitness in the case of this particular hybrid. One of the major ecological concerns associated with F1 hybrids is the possibility of their escape from containment followed by inbreeding with native populations (Allendorf et al. 2001). Two of the molecular markers developed were specific to the F1 hybrid and these can be applied for the analysis of individuals from wild grouper populations in order to detect any possible breach of containment at the aquaculture facility.

The third criterion is the uniformity in the number of chromosomes. Karyotype analysis (Wang et al. 2009) of groupers indicates a consistency in the number of chromosomes ($2n = 48$) within the genus *Epinephelus* further supporting the premise that interspecific grouper hybrids will be viable (Stelkens et al. 2009). The application of Haldane's rule in the case of protogynous hermaphrodites is further complicated as there was no evidence of sexual dimorphism in the F1 hybrids and sex reversal can take several years (Yeh et al. 2003).

None of the markers developed in this study could be assigned to the groupers *E. corallicola* and *E. fuscoguttatus*, this may provide a strong molecular basis for not selecting these two species for hybridization with *E. coioiodes* or *E. lanceolatus* owing to the genetic distance as evinced using multiple genomic loci. The study clearly indicated that molecular markers developed using the bottom up approach could serve as a useful tool in

determining parentage in F1 hybrids developed from groupers.

CONCLUSIONS

This study was carried out to verify parentage in F1 hybrids developed from *E. coioides* and *E. lanceolatus*. A small insert genomic library was constructed based on the F1 hybrid and single locus molecular markers could be assigned to each of the parental genotypes and not to two other closely related species of grouper. The approach adopted in this study can be applied to develop breeding programs for interspecific hybrids based on molecular markers.

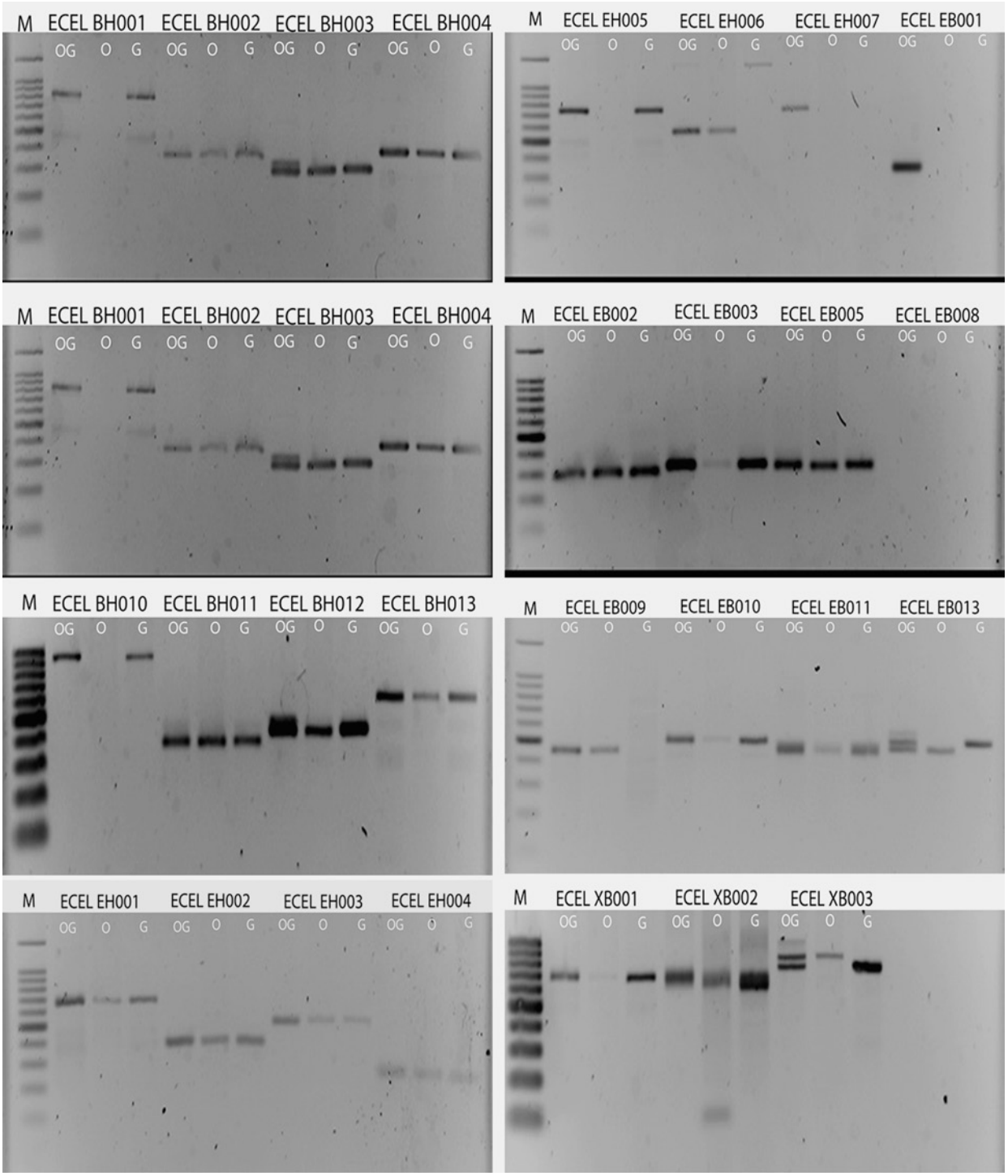

Fig. 1. Gel image of cross amplification comparing the FI grouper hybrid of *Epinephelus coioides* and *Epinephelus lanceolatus* against parental species that are *Epinephelus coioides* and *Epinephelus lanceolatus*; Lane M represents the 100 bp DNA ladder (Promega); lanes OG represent the amplification products of FI grouper hybrid of *Epinephelus coioides* and *Epinephelus lanceolatus*; Lanes O represents the amplification products of *Epinephelus coioides* and lanes G represents the amplification products of *Epinephelus lanceolatus* for the labelled genomic locus respectively

Table 1

Polymerase Chain Reaction (PCR) amplification profiles of locus specific molecular markers developed for the F1 hybrid of *Epinephelus coioides* and *E. lanceolatus*

No.	Primer name	Sequence from 5′ through 3′	Tm	Product size [bp]	F1	EC	EL	GenBank
1	ECELBH001F	CAGATGCCTGACAACCTCAA	59.83	814	+	–	+	JQ732777
	ECELBH001R	ACTCTCCTTGCCTCTGACCA	59.99					
2	ECELBH002F	TAGCCTCATGCCCTCCTAGA	59.93	367	+	+	+	JQ732778
	ECELBH002R	GTGCCATACGGCTTTGAAAT	59.97					
3	ECELBH003F	CCTGTCAGTGGCAACAACAA	60.76	311	AB	B	A	JQ732779
	ECELBH003R	GGATGGCCTGAAAATACACG	60.33					
4	ECELBH004F	GATGAACCAAACACCCCAAC	60.07	399	+	+	+	JQ732780
	ECELBH004R	TCGCCACAATCTCTGAACTG	59.98					
5	ECELBH005F	CACCACAACAGAATGGCAAC	60.01	712	+	–	+	JQ732781
	ECELBH005R	AAACGATCTCTCGCCAAGAA	59.96					
6	ECELBH006F	GAAGACGGGGTGACATGTTT	59.83	374	+	+	–	JQ732782
	ECELBH006R	GGTGGGCATTCTTCTTTGTG	60.50					
7	ECELBH008F	CCTGTTCCCACTGTGACTGA	59.70	761	+	–	+	JQ732784
	ECELBH008R	TGACCTCTTCCATTCCCTTG	60.04					
8	ECELBH010F	ACGTGGCTTCGAGGAGTAAA	59.88	958	+	–	+	JQ732786
	ECELBH010R	AGAACGACCAGCCTCTTTGA	59.99					
9	ECELBH011F	TCGCGTAACCTCTCTGTCCT	60.01	383	+	+	+	JQ732787
	ECELBH011R	CCTGTGTGGAAACCAAAGGT	59.86					
10	ECELBH012F	GCCTTTTGTTGAGGGAACTG	59.71	438	+	+	+	JQ732788
	ECELBH012R	CCCACCCTCATTCTCTTTCA	60.04					
11	ECELBH013F	GGGAAAGAGAAGGTGGAAGG	60.04	611	+	+	+	JQ732789
	ECELBH013R	CGTTAGTGCATCCCCTTGAT	59.96					
12	ECELEB001F	TCAGGAAAGCGTTGGAGAGT	59.99	367	+	–	–	JQ732790
	ECELEB001R	CAGGGGTAAAGGCACTTCAG	59.73					
13	ECELEB002F	GGCAGAGAGAGAGCGAGATG	60.39	290	+	+	+	JQ732791
	ECELEB002R	GCTATGCAGCTACAGGCAGA	59.35					
14	ECELEB003F	CATGGCGAGTAGGACACAGA	59.86	323	+	–	+	JQ732792
	ECELEB003R	CACCTCGGCTCTAAACTTCG	60.01					
15	ECELEB005F	TCCGTTACTTCCCACCAGAC	59.97	312	+	+	+	JQ732793
	ECELEB005R	CGACAGGAACAGCTGATGAA	59.98					
16	ECELEB008F	GTGCCTGACAACGCTAGAAG	59.68	674	–	–	–	JQ732795
	ECELEB008R	CCAGGGAAACCAGCTTATGA	60.07					
17	ECELEB009F	AAATGTGTGTGGGTGGGTTT	59.99	453	+	+	–	JQ732796
	ECELEB009R	ATGACGTGAATCCATCAGCA	60.08					
18	ECELEB010F	CTTTCCCTTTCCCTCACCTC	60.04	505	+	–	+	JQ732797
	ECELEB010R	CTGTCGTCAGGCATCTCTCA	60.14					
19	ECELEB011F	GATTACACGCCACTCAGCAA	59.87	451	+	+	+	JQ732798
	ECELEB011R	TGAAAAGAAACCTGGGGTTG	59.94					
20	ECELEB013F	CGATCCCAACCAGAGTCATT	59.93	506	AB	B	A	JQ732800
	ECELEB013R	GGTTTGTCCATTCTGGGCTA	59.93					
21	ECELEH001F	GATGTCGTGTTGGACTGTGG	60.00	658	+	+	+	JQ732801
	ECELEH001R	CTTCCAGCCTACCTTTGCAC	59.88					
22	ECELEH002F	AATCTGACACCCTGGAGCTG	60.26	385	+	+	+	JQ732802
	ECELEH002R	ACGGGACTCAACCAAACATC	59.83					
23	ECELEH003F	GCGACACAGGTCAAGTCTCA	60.03	506	+	+	+	JQ732803
	ECELEH003R	GCGATAGATCCCATCCTGAA	60.00					
24	ECELEHOO4F	ACTCAACTAGCAGCCCAGGA	60.01	234	+	+	+	JQ732804
	ECELEH004R	TGAGGACATTTGGGGCTTAG	60.07					
25	ECELEH005F	AGTCACTGCCGCTGAAAGAT	60.02	752	+	–	+	JQ732805
	ECELEH005R	CCACAGGTAGAAGGCATGGT	59.99					
26	ECELEH006F	CTAATCCTGGCTGGCATGTT	60.10	562	+	+	–	JQ732806
	ECELEH006R	CCACGATGACCCAGAAGAAT	59.93					
27	ECELEH007F	AACACACCAGAAGGGTCTGC	60.16	733	+	–	–	JQ732807
	ECELEH007R	TTTTTGGGGTGGCAGTAGTC	59.97					
28	ECELXB001F	CCCTCTGGGGTAGATTGTGA	59.92	715	+	–	+	JQ732808
	ECELXB001R	TATGTGGCGTGTCAGAGGAG	59.86					
29	ECELXB002F	GACTAATGATGGCAGCAGCA	59.98	621	+	+	+	JQ732809
	ECELXB002R	CTGGCTCCCTGTAAAATCCA	60.07					
30	ECELXB003F	ATGTGCGTCTTCGAGAGGTT	59.87	763	AB	A	B	JQ732810
	ECELXB003R	CACTGCAGCGCTAATAACCA	60.04					

No. = number, Tm = annealing temperature, F1 = F1 hybrid, EC = *Epinephelus coioides*, EL = *Epinephelus lanceolatus*, GenBank = GenBank accession numbers for loci; positive amplification (+), no amplification (–), alleles are indicated by A, B, and AB.

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