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Recruitment of grouper broodstock on the basis of single locus DNA markers

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Abstract
Scientific breeding programs are founded on the screening and recruitment of genetically diverse broodstock, with the ultimate aim of developing heterogeneous breeding populations that host a collection of desirable traits. Single locus DNA markers can be applied to facilitate the process of selection as they are species specific, reliable, reproducible and easy to use. This study set forth to develop a library of single locus DNA markers for two commercially cultured species of groupers, *Epinephelus fuscoguttatus* and *Epinephelus corallicola*. DNA was isolated from one representative specimen of each species and utilized to construct shotgun genomic libraries. DNA sequences derived from the library were selected for the development of 42 and 41 single locus DNA markers for *Epinephelus fuscoguttatus* and *Epinephelus corallicola* respectively. The markers were then tested against randomly selected specimens obtained from the wild. Genotyping results revealed that the species specific primers demonstrated the ability to distinguish between individuals from the same species into distinct operational taxonomic units (OTUs) on the basis of their differential DNA profiles, thus establishing a basis for selection based on genetic heterogeneity. The findings of this study present a strong case for the application of single locus DNA markers as molecular tools for the selection of broodstock on the basis of genotyping.

Keywords: Grouper, *Epinephelus fuscoguttatus*, *Epinephelus corallicola*, Single locus DNA markers

1. Introduction
The economic success of the aquaculture industry is founded on the selection and recruitment of high quality broodstock from genetically diverse wild populations. However, the process of selection of wild specimens is confounded by phenotypical similarity and paucity of genetic information. Genomic molecular markers [1] have the potential to revolutionize the aquaculture industry when applied in conjunction with conventional breeding techniques [2]. This has formed the basis for approaches that recruit broodstock and seed on the basis of data obtained from Quantitative Trait Loci (QTLs) [3] and Marker Assisted Selection (MAS) [4]. These two molecular approaches can have a significant impact on the economics of a fish breeding operation as they offer an avenue for the elimination of genetically inferior germplasm prior to recruitment. Scientists have initiated the process of marker development for finfish by focusing on several commercially exploited species such as *Orecohromis niloticus* [5], *Ictalurus punctatus* [6], *Salmo salar* [7] and *Cyprinus carpio* [8]. They have relied upon an assortment of molecular markers such as microsatellites [9], Single Nucleotide Polymorphisms (SNPs) [10], Amplified Fragment Length Polymorphisms [11] and Short Sequence Repeats [12] in order to detect genetic polymorphisms that can be exploited by breeders. The practical application of molecular markers to breeding have been discussed in detail [13] and it is interesting to note that highly informative markers such as microsatellites and SNPs require specialized laboratories and analytical techniques, thus putting them beyond the technological reach of commercial breeders. Single locus DNA markers [14] are less informative than microsatellites and SNPs, however they make up for this shortcoming by their simplicity, reproducibility and ability to distinguish individuals within a population. This study set forth to develop single locus DNA markers could be applied for the selection of broodstock purely on the basis of Boolean DNA profiling. The groupers *Epinephelus fuscoguttatus* and *Epinephelus corallicola* were selected as the target species as they are commercially exploited by the aquaculture industry.

2. Material and methods

2.1 Sample collection and DNA isolation

The samples for this study were all collected from broodstock being maintained at the Borneo Marine Research Institute, Universiti Malaysia Sabah from December 2010 to February 2011. DNA was extracted from Fin clips collected from *Epinephelus fuscoguttatus* (N=12) and *Epinephelus corallicola* (N=10) using the DNeasy extraction kit (Qiagen), the concentration of DNA was assessed using a micro-volume UV-Vis Spectrophotometer (GE Healthcare Life Sciences) and the final concentration was adjusted to 50 ng/µl using sterile nuclease free water. DNA samples were stored at -80 °C.

2.2 Construction of genomic libraries

Partial small insert genomic libraries [15] were constructed using one DNA sample derived from each of the two species of groupers, *Epinephelus fuscoguttatus* and *Epinephelus corallicola*. Restriction digests were carried out using a combination of six restriction enzymes, *Eco*RI/*Hind*III, *Eco*RI/*Bam*HI, *Bam*HI/*Hind* III, *Kpn*I/*Sal*I, *Sac*I/*Kpn*I and *Sac*I/*Xba*I. (New Englan Biolabs), following which the digested DNA was ligated onto a pUC19 Vector and transformed into chemically competent *Escherichia coli* (TOP10). Recombinant clones from each library were selected randomly from Luria–Bertani plates containing Ampicillin (100 mg/l) and X-Gal (50 mg/l). Plasmid DNA were extracted and purified using Gene JET plasmid purification kit (Fermentas) and sequenced using Big Dye Terminator 2.0 Cycle Sequencing Ready Reaction Kit (Life Technologies, USA) on an ABI Prism 3770 automated DNA sequencer (Applied Biosystems). Sequences were deposited at the NCBI Gen Bank and assigned accession numbers (Tables 1.0 and 3.0).

2.3 Primer design

One pair of primers was designed per individual DNA sequence using the online Primer3 software [16]. The annealing temperature was set (Ta) at 60 °C and regions with potential secondary structures, high G: C ratio and runs of a single nucleotide, were excluded. PCR grade primers were synthesized (IDT Technologies, Singapore).

2.4 Genotyping

PCR amplification was performed in final volume of 20 µl containing 1.2 µl $MgCl_2$ (1.5mM), 0.4 µl dNTPs (0.2mM each), 4 µl 1x Go*Taq* buffer (Promega), 1 U *Taq* DNA polymerase (Promega), 1µl of each primer (5µM), 2µl template DNA and nuclease free water. Amplification was performed using a thermal cycler (MJ research, PTC-200) under the following conditions: pre-denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation (30 sec at 95 °C), annealing (40 sec at 58 °C), extension (2 min at 72 °C) and final extension (10 min at 72 °C). PCR products were resolved by electrophoresis on a 1.5% Tris-Boric Acid EDTA agarose gel, stained with Ethidium bromide and the gel was analyzed using a gel documentation system (Alpha Innotech, San Leandro, CA).

2.5 Scoring of PCR amplicons and data analysis

PCR amplicons were resolved on a 1.5% Tris – Boric Acid – EDTA (TBE) agarose gel, stained in a solution of Ethidium Bromide (50 µg/ml) for 10 min following which the gels were viewed using a UV transilluminator (Alpha Innotech, USA). Bands were scored as "1" for present and "0" for absent resulting in a binary score matrix. To test the reproducibility of the amplification pattern the PCR was repeated at least twice. Only unambiguous and clear amplicons were scored and chosen for Jaccard's similarity coefficient analysis. The UPGMA phenogram was constructed using the online software Dendro UPGMA (http://genomes.urv.cat/UPGMA/) [17]. Distance and Similarity matrices were computed based on the Dice Coefficient with 100 replicates. Operational Taxonomic Units (OTUs) were determined on the basis of the Cophenetic Correlation Coefficient (CPCC) [18] and phenograms were rendered graphically using the online software Phylo Widget (http://www.phylowidget.org/) [19].

3. Results

3.1 Single Locus DNA Markers for *Epinephelus fuscoguttatus*

Six genomic libraries yielded 341 clones of which 68 were randomly selected for sequencing. Primers were designed to amplify 42 loci (Table 1.0). 37 primers yielded single amplicons whereas the remaining 5 primers (EFJ006, EFJ018, EFP010, EFJAAC2 and EFP003) amplified more than one locus. The total number of polymorphic loci was 21 (Table 2.0). The phenogram of *Epinephelus fuscoguttatus* graphically depicts the OTUs (Figure 1.0). The individuals clustered into three major clades. Clade 1 consisted of OTUs EF001, EF006, EF008, EF005, EF004 and EF010. Clade 2 consisted of OTUs EF002, EF009, EF03X, EF02X and EF003 and Clade 3 contained one OTU EF007. Within clade 1, OTUs EF001 and EF006 were grouped together with a similarity of 96%. Both of these OTUs were connected to 4 nodes containing 4 different OTUs namely; EF008, EF005, EF004 and EF010. OTU EF006 indicating highly a higher degree of similarity to two inter-joining nodes containing OTUs EF008 and EF005 with a similarity of 96% and 95% respectively. OTU EF005 was highly similar to OTU EF004 with 93% similarity. Within clade 2, OTUs EF009 and EF03X clustered with a similarity of 97%. Both OTUs were connected with three other nodes. OTU EF009 was 95% similar to OTUs EF02X, EF002 and EF003. Clade 3 was represented by only one individual OTU EGF007.

Table 1: Molecular markers developed for genotyping of *E. fuscoguttatus* indicating the genomic locus, GenBank accession number, primer designation, sequences of forward and reverse primers, expected product size and annealing temperatures.

No.	Genomic Locus	Gen Bank Accession No.	Primer designation	Primer Sequences 5' – 3'	Expected Product Size (bp)	Annealing Temperature Ta (°C)
1	EFJ002A	JN048832	EFJ002F	TTG GGA TGG GGT CTA AGA GA	294	58
			EFJ002R	ACC CCA GGT TTC TTT TCA GC		
2	EFJ005	JN048835	EFJ005F	TTT CGT TGT AGC GCT TGA TG	345	58
			EFJ005R	TGC ACA CTC TTG GCA TTC TC		
3	EFJ006	JN048836	EFJ006F	CGT CTC TCC ACG GGA TAT TT	253	55
			EFJ006R	CCG TGA CAA CTT TGA CCA TC		

4	EFJ016	JN048846	EFJ016F	GGG CAG CAT TAT GTC TCC AT	185	58
			EFJ016R	TGT CTG TCC CTC CCT ACA CC		
5	EFJ018A	JN048848	EFJ018F	CCT GTC TCT GGA AGC CTC AC	157	58
			EFJ018R	CCT GCA ACG TAG TGT GGG TA		
6	EFJ022	JN048852	EFJ022F	ATG TGC CAT GCA ATC TGT GT	332	58
			EFJ022R	ACT GCT GTC CAT CCA TCT CC		
7	EFP007	JN048861	EFP007F	GAA GTA TGG GGG CAA TGA TG	624	58
			EFP007R	TTT TTG TGG GGC TTT GCT AC		
8	EFP009	JN048863	EFP009F	GCT GAG TGA TCT GGC ATC AA	178	58
			EFP009R	ATG CTC CAG AAG ACG AGG AA		
9	EFP010	JN048864	EFP010F	CAT GGC AGC AGA ATA AAC CA	192	58
			EFP010R	CAG GAA GAG GGG AAG AAG TG		
10	EFP013	JN048867	EFP013F	ACG GAC CTC TGG GAG AAA CT	379	59
			EFP013R	GAT GTC CCA GAA AGG CAA AA		
11	EFPHI003	JN159896	EFPHI003F	ACA AGG CCA AAG CAA AGA GA	550	58
			EFPHI003R	GGG TGG AGG AAG AAC ACA AA		
12	EFPHI004	JN159897	EFPHI004F	TTT GTC TCC CTC CCT CAA TG	265	58
			EFPHI004R	GCT AGC ATG ATC CCG ATG TT		
13	EFPHI006	JN159899	EFPHI006F	CTA GCT GTG GCA GAC AGA CG	214	58
			EFPHI006R	AGG GAC ACT GGT TGT GGA AC		
14	EFJAAC2	JN048828	EFJAAC2F	TGT GAA AAT GGG TGA AGT CG	161	58
			EFJAAC2R	GTA TGG CCC TGC AAA GGT AA		
15	EFJCTT1	JN048829	EFJCTT1F	TCC TGC ACA ACT CCA CAG AG	288	58
			EFJCTT1R	CAA GCA TGT CTG CCT TTT GA		
16	EFJ003	JN048833	EFJ003F	TTT GCA GTG TAG GCC AGA TG	340	55
			EFJ003R	GTA AGC AGG GCA AGG AAA AC		
17	EFJ007	JN048837	EFJ007F	AAG ATC GCT GGA GAC CAG AA	288	55
			EFJ007R	AAT CGT CAG TCG CTT CAC CT		
18	EFJ008	JN048838	EFJ008F	GGG GAA GCT CTG TCT GAA AA	173	55
			EFJ008R	TTC ATT CTG TCC CCA GAA CC		
19	EFJ009	JN048839	EFJ009F	GCA AAC TCT GCA CTC ACC AG	203	55
			EFJ009R	TCT GGG ATG CCT ACG TGA AT		
20	EFJ013	JN048843	EFJ013F	GCA CCT TGA GGG AGC TAG TG	244	55
			EFJ013R	GTC AGC AGA AGC CAC TTT CC		
21	EFJ020	JN048850	EFJ020F	TCA GTG ACC CCT GTG TGT GT	372	55
			EFJ020R	GTG CTT GTT TTT GCC ACT GA		
22	EFJ021	JN048851	EFJ021F	GTC ACA ACA CTG GGA ACG TG	476	55
			EFJ021R	GGC AGC CAT GGT TTA TGT CT		
23	EFP003	JN048857	EFP003F	TCA TCT AAT GTG CGC TGC TC	185	55
			EFP003R	TGC TGT TAA TGC GTG AGG AC		
24	EFP005	JN048859	EFP005F	AGA GCG GAG CTT GTT CTC AC	358	55
			EFP005R	GAG TGT GCC TGC ATG AGT GT		
25	EFP006	JN048860	EFP006F	AGC ACG TTT GAG CAG GAG AT	466	55
			EFP006R	CAG GGA GGG TCA AGA TTT CA		
26	EFPHI002	JN159895	EFPHI002F	AGA GGC TGG CTG TGT CAA CT	217	55
			EFPHI002R	AAA CAT CCC ATC AGG CTG TT		
27	EFJAAC1	JN048827	EFJAAC1F	CAT CGT GGT ATG CAC CTC TG	285	55
			EFJAAC1R	TCA AAC AGG TCG TCC ACA AA		
28	EFPHI001	JN159894	EFPHI001BF	GTT CAG ACG TGC TCA GCG TA	189	58
			EFPHI001BR	TTA GTA AAC GCA CGC TGG TG		
29	EFJ014	JN048844	EFJ014AF	ACT GCG ACA TCG TAT CGT GA	119	60
			EFJ014AR	GGC CTT TAC AGC ACT GAG ATG		
30	EFP012	JN048866	EFP012AF	AAT GGC CAG ACT GGT TCA AG	112	62
			EFP012AR	CAA GGT TGG ACG TGT TGT TG		
31	EFEH001	JN944339	EFEH001B1F	AAA GAG CCC GTT CTG TCT CA	111	58
			EFEH001B1R	GGG GGT GTT GAT GCT GTA AA		
32	EFEX002	JN944340	EFEX002YBF	TTG AGT GTG CTT GCT GAT CC	147	62
			EFEX002YBR	ATG CGA GCT CCA GGT AGA AA		
33	EFEX003	JN944342	EFEX003YBF	CCC AAA GTG CTG TTG CAG TA	142	58
			EFEX003YBR	TTC GCC AAG ATC TGG TA GC		
34	EFHX003	JN944347	EFHX003YBF	GGG TTC GCT TGT CAC ACT TT	243	58
			EFHX003YBR	GTG ACC TTT CCT CCA CCA GA		
35	EFXB003	JN944350	EFXB003YBF	GCC TAA TAG CAT GCC AGG TG	244	58
			EFXB003YBR	GCC CAA CAC AGG TCA GAA GT		
36	EFXP001	JN944352	EFXP001YBF	CAA TGG ACT CAG GAC CTG CT	114	60
			EFXP001YBR	ACG GTG GAT AAA CCA AGA CG		
37	EFBP001	JN944336	EFBP001AF	GAC CAA CAC CAA TCC AGT CC	126	55
			EFBP001AR	GAG ACG AGA ACC AGG ACA GC		

38	EFEX001	JN944340	EFEX001AF	CAG CCG ACC AAC ACT CAC TA	344	55
			EFEX001AR	CCT AGT GAC GCG AGG CTA TC		
39	EFEX004	JN944343	EFEX004AF	GGC CCG TGT CTC ACT ATG TT	149	55
			EFEX004AR	TGG GGG AAG ACA ACA CTT TC		
40	EFEX005	JN944344	EFEX005AF	CTT AGG CAG ACG GTG TGT GA	288	55
			EFEX005AR	CTG GGG GAG GTT TTA CGT TT		
41	EFXB001	JN944348	EFXB001AF	CTG CAT GCA CCT GAC AGA CT	182	55
			EFXB001AR	CAG GCA TTT GAT CTG GTC CT		
42	EFXB003	JN944350	EFXB003AF	CCA GAT GGC CTC AAA TCC TA	400	55
			EFXB003AR	TGT GAC TCT GCA GGA ACA GG		

Table 2: Intra-specific genetic profiling of *E. fuscoguttatus* broodstock collected from the wild. '1' indicates the presence of a PCR amplicon of the expected product size, '0' indicate the absence of a PCR amplicon. Primers EFJ006, EFJ018, EFP010, EFJAAC2 and EFP003 yielded more than one PCR amplicon and these were scored separately.

No.	Genomic Locus	Observed Product Sizes (bp)	EF02X Control DNA	EF 001	EF 002	EF 003	EF 004	EF 005	EF 006	EF 007	EF 008	EF 009	EF 0010	EF 03X
1	EFJ002	294	1	1	0	0	1	1	1	1	1	1	1	1
2	EFJ005	345	1	1	1	1	1	1	1	1	1	1	1	1
3	EFJ006A	253	1	1	1	0	1	1	1	1	1	1	1	1
3a	EFJ006B	~200	0	1	1	1	1	1	1	1	1	1	1	0
4	EFJ016	185	1	1	1	1	1	1	1	1	1	1	1	1
5	EFJ018A	157	1	1	1	1	0	0	1	1	0	1	1	1
5b	EFJ018B	~180	0	1	0	0	1	1	1	0	1	0	0	0
6	EFJ022	332	1	1	1	1	1	1	1	1	1	1	1	1
7	EFP007	624	1	1	1	0	1	1	1	0	1	1	1	1
8	EFP009	178	1	1	1	1	1	1	1	1	1	1	1	0
9	EFP010A	192	1	1	1	1	1	1	1	1	1	1	1	1
9a	EFP010B	~210	0	1	1	1	1	1	1	1	1	1	1	1
9b	EFP010C	~270	0	1	0	0	0	0	0	0	1	0	1	0
10	EFP013	379	1	1	1	1	1	1	1	1	1	1	1	1
11	EFPHI003	550	1	1	1	1	1	1	1	1	1	1	1	1
12	EFPHI004	265	1	1	1	1	1	1	1	1	1	1	1	1
13	EFPHI006	214	1	1	1	1	1	1	1	1	1	1	1	1
14	EFJAAC2A	161	1	1	1	1	1	0	0	0	0	1	0	1
14a	EFJAAC2B	~180	0	1	0	0	0	1	1	1	1	0	0	0
15	EFJCTT1	288	1	1	1	1	1	1	1	0	1	1	0	1
16	EFJ003	340	1	1	1	1	1	1	1	1	1	1	1	1
17	EFJ007	288	1	0	1	0	0	0	1	1	1	1	0	1
18	EFJ008	173	1	1	1	1	1	1	1	1	1	1	1	1
19	EFJ009	203	1	1	1	1	0	1	1	1	1	1	1	1
20	EFJ013	244	1	1	1	1	1	1	1	1	1	1	1	1
21	EFJ020	372	1	1	1	1	1	1	1	0	1	1	1	1
22	EFJ021	476	1	1	1	1	1	1	1	0	1	1	1	1
23	EFP003A	185	1	1	0	1	0	1	1	1	0	1	0	1
23a	EFP003B	~210	0	1	1	0	0	0	1	0	1	0	1	0
24	EFP005	358	1	0	0	0	0	0	0	0	0	0	0	0
25	EFP006	466	1	0	1	0	0	1	0	0	0	0	0	0
26	EFPHI002	217	1	1	1	1	1	1	1	1	1	1	1	1
27	EFJAAC1	285	1	1	1	1	1	1	1	1	1	1	1	1
28	EFPHI001	189	1	1	1	1	1	1	1	1	1	1	1	1
29	EFJ014	119	1	1	1	1	1	1	1	1	1	1	1	1
30	EFP012	112	1	1	1	1	1	1	1	1	1	1	1	1
31	EFEH001	111	1	1	1	1	1	1	1	1	1	1	1	1
32	EFEX002	147	1	1	1	1	1	1	1	1	1	1	1	1
33	EFEX003	142	1	1	1	1	1	1	1	1	1	1	1	1
34	EFHX003	243	1	1	1	1	1	1	1	1	1	1	1	1
35	EFXB003	244	1	1	1	1	1	1	1	1	1	1	1	1
36	EFXP001	114	1	1	1	1	1	1	1	1	1	1	1	1
37	EFBP001	126	1	1	1	1	1	1	1	1	1	1	1	1
38	EFEX001	344	1	1	1	1	1	1	1	1	1	1	1	1
39	EFEX004	149	1	1	1	1	1	1	1	1	1	1	1	1
40	EFEX005	288	1	1	1	1	1	1	1	1	1	1	1	1
41	EFXB001	182	1	1	1	1	1	1	1	1	1	1	1	1
42	EFXB003	400	1	1	1	1	1	1	1	1	1	1	1	1
		TOTAL	15/21	18/21	15/21	11/21	12/21	15/21	17/21	11/21	16/21	15/21	13/21	13/21
	Similarity	%	71	86	71	52	57	71	81	52	76	71	62	62

3.2 Single Locus DNA Markers for *Epinephelus corallicola*

Six genomic libraries yielded 220 clones of which 52 were randomly selected for sequencing. Primers were designed to amplify 41 loci (Table 3.0) which yielded single amplicons (Table 4.0). The phenogram of *Epinephelus corallicola* graphically depicts the OTUs (Figure 2.0). The phenogram depicts three major clades. Clade 1 was composed of OTUs CC001, CC009, CC006 and CC008. Clade 2 comprised OTUs CC003, CC004, CC002, CC010, CC007 and CC005. In clade 1, OTUs CC001 and CC009 exhibited 81% similarity. Both OTUs were linked to another set of 2 nodes containing 2 different OTUs namely; CC006 and CC008.

Table 3: Molecular markers developed for genotyping of *E. corallicola* indicating the genomic locus, GenBank accession number, primer designation, sequences of forward and reverse primers, expected product size and annealing temperatures.

No	Genomic Locus	Gen Bank Accession No.	Primer designation	Primer Sequences 5' – 3'	Expected Product Size (bp)	Annealing Temperature Ta (°C)
1	CCBP001	JX684019	CCBP001AF	GGT GTG AGA TGG GCT ACC AG	144	58
			CCBP001AR	TTG CCT CAC AGA GTT TGC AC		
2	CCBP002	JX684020	CCBP002AF	TCA TTC TTC CCT GGA AGA GG	178	58
			CCBP002AR	CTG TTG AGC GTG TGT GTG TG		
3	CCBP003	JX684021	CCBP003AF	TAG GCG TTC CCT GTG ATT GT	159	58
			CCBP003AR	GCC CAA TAG CCA CAT GAA CT		
4	CCBP004	JX684022	CCBP004AF	AAG ACG TGA TCC CTG TGG AC	155	58
			CCBP004AR	TGG AGG CTG TTG AAA GAG GT		
5	CCBP005	JX684023	CCBP005AF	TAA AGC CAC GTG AGT GTT CG	252	58
			CCBP005AR	AGC GCC TAG CTC TAT GGT CA		
6	CCBP006	JX684024	CCBP006AF	TAG CAA GGC AAG GCC TCT AA	264	58
			CCBP006AR	CGG TCA AAC ACA CAC AGG AC		
7	CCBP007	JX684025	CCBP007AF	GTG GTC TCG TGG GTG TTG TT	202	58
			CCBP007AR	AGC CAG TCT CAG CAG AGG AA		
8	CCBP008	JX684026	CCBP008AF	GGT TGC CAA AGA AAC TGC TC	213	58
			CCBP008AR	TCC TCC AGG TCT TGC TCT GT		
9	CCBP009	JX684027	CCBP009AF	GAG AAG GGT TCA TTG GTG GA	242	58
			CCBP009AR	ACG GCC AGT TTA ATG TCA GC		
10	CCBH001	JX684028	CCBH001AF	GGA CAC AGC CAG GTT TCA GT	225	58
			CCBH001AR	CCC CCG ACC ACC TAA ATT AT		
11	CCBH002	JX684029	CCBH002AF	TCC CTG CCT CTC TGT TCA CT	272	58
			CCBH002AR	CAT TTC CCC CTC CTT CTC TC		
12	CCBH003	JX684030	CCBH003AF	GGG ACA GGT GAG GCT AAC AT	234	58
		31323334	CCBH003AR	CAG GGT CAG TTC ACC GTT TT		
13	CCBH008	JX684035	CCBH008AF	CCA CAA AGA AAA GCC TGG AC	288	58
			CCBH008AR	GTA ACA CTC GCC ACA CAT CG		
14	CCBH009	JX684036	CCBH009AF	ACC GTG TCC ATC CCT TTA TG	249	58
			CCBH009AR	GCT TCA CAA TGA GCC ACA GA		
15	CCBH010	JX684037	CCBH010AF	CCC TAT TGG CCT CAC TTT CA	291	58
			CCBH010AR	ACT TCC GTT GTC CCA CTG TC		
16	CCBH011	JX684038	CCBH011AF	CCA GTG CCC AAA CCT AGA AG	228	58
			CCBH011AR	TGG TCT CCA GAG CTG AGG TT		
17	CCBH012	JX684039	CCBH012AF	GAC ATG GTC ACA CCA ACA GC	210	58
			CCBH012AR	GGC GAA CTT ATT GGA CCG TA		
18	CCBH013	JX684040	CCBH013AF	CTG GCT CCC TGT AAA ATC CA	276	58
			CCBH013AR	GGA GAG CCC TGA TAG CTG AA		
19	CCBH014	JX684041	CCBH014AF	CTT TCC TTA CAG GCC CAT CA	114	58
			CCBH014AR	GTG CCC CAC CAT CAA ATA TC		
20	CCBH015	JX684042	CCBH015AF	CAA AAA CCG CTA GAG GTC CA	245	58
			CCBH015AR	AAC CAG GAT CTC GAT TGC AG		
21	CCBS001	JX684044	CCBS001AF	GAC GTC AGC ACT CGG AAG TT	204	58
			CCBS001AR	CGA GAG TCC CAT TCC GAC TA		
22	CCBS002	JX684044	CCBS002AF	GAC GGA GCA GTG AGT GAC AA	344	58
			CCBS002AR	GCG TGT TTC CAT CCA CTA CA		
23	CCBS004	JX684046	CCBS004AF	TGG TTC TTC CCA TAG GCA AC	312	58
			CCBS004AR	CGG GAC CAG ATT TTA CGA GA		
24	CCBS005	JX684047	CCBS005AF	GGC TCA AGC GTC AGA TCT TC	209	58
			CCBS005AR	ACT TTC TGT GCA GCA TGT CG		
25	CCEB001	JX684048	CCEB001AF	GTG TGA ACA TGC CCT GAT TG	305	58
			CCEB001AR	CCG ATG TGC TGG AGT ACA GA		
26	CCEB002	JX684049	CCEB002AF	GAA ACA CGA GCA GAG CTG AA	319	58
			CCEB002AR	CGG GTC TCA TCC AAG AGA AA		
27	CCEB003	JX684050	CCEB003AF	TAA GCG AGG GGC ATC TAT TG	214	58
			CCEB003AR	CTG TTT GAT GGG ACA CAT GC		
28	CCEB004	JX684051	CCEB004AF	GCC GAG AGT AAC ACG GAA AA	309	58
			CCEB004AR	GCT AAC ATG GGT GGG ACA TT		
29	CCEB005	JX684052	CCEB005AF	CTC CCT GGC TAG CAC GTT TA	216	58
			CCEB005AR	TTG CTG AGT GGC GTG TAA TC		
30	CCEB006	JX684053	CCEB006AF	AAA GGC TAT GCG AGA CTG GA	225	58

			CCEB006AR	CGC AAG GGT AAA CAG GTG AT		
31	CCEB008	JX684055	CCEB008AF	CAT GCT CCT GAT GTG CCT TA	380	58
			CCEB008AR	TAT GTC AGC CAT GTC CGT GT		
32	CCEH002	JX684057	CCEH002AF	GAG GCG GTT TTG ACT GTG AT	222	58
			CCEH002AR	AAT CGA CCG TCC ACT TTG TC		
33	CCEH003	JX684058	CCEH003AF	GTG GTG TGT GAG GTG ATC CT	338	58
			CCEH003AR	TGA CCG CAC AAA CTA AGC TG		
34	CCEH004	JX684059	CCEH004AF	TGA GTG CTT GGA CTT TGT GC	389	58
			CCEH004AR	GGG CTC TCA ACC TGA ATC AA		
35	CCSP001	JX684060	CCSP001AF	TTT GCT GCC ACA CTT GAG AC	255	58
			CCSP001AR	CCC CAT CAG GCA ATG TTT AG		
36	CCSP004	JX684063	CCSP004AF	ATT TCC AGA CTG GGT GTT GC	284	58
			CCSP004AR	TGG AGC ATA GCT GTC ACC AG		
37	CCSP005	JX684064	CCSP005AF	TAG TTG TTG ACG GGC CTC TT	249	58
			CCSP005AR	CCT GGC CAT TCT TAT CGA TG		
38	CCSP006	JX684065	CCSP006AF	TTC AGC CCG TCT CAG ACT TT	298	58
			CCSP006AR	TCT CCC CTC AGC TTA CAT GG		
39	CCSP007	JX684066	CCSP007AF	ACC AGG AAG CCC AGA AAA T	286	58
			CCSP007AR	GGG AGT GCT TAG CAG AAT CG		
40	CCHX002	JX684067	CCHX002AF	GCT ATG AAC GCA GGT CAA CA	230	58
			CCHX002AR	CCT GCT GTA CGT GTC ATG CT		
41	CCXP001	JX684068	CCXP001AF	ACT GGT GTT CAT CCG TAG CC	252	58
			CCXP001AR	AAG GGG CCA ATT TAG TAC CC		

Table 4: Intra-specific genetic profiling of *E. corallicola* broodstock collected from the wild. '1' indicates the presence of a PCR amplicon of the expected product size, '0' indicate the absence of a PCR amplicon.

No	Genomic Locus	Observed Product Size (bp)	CC001 Control	CC 002	CC 003	CC 004	CC 005	CC 006	CC 007	CC 008	CC 009	CC 010
1	CCBP001	144	1	0	1	1	0	1	0	1	0	0
2	CCBP002	178	1	0	1	0	0	1	0	0	0	0
3	CCBP003	159	1	1	1	1	0	1	0	0	1	0
4	CCBP004	155	1	0	1	1	0	1	0	0	1	0
5	CCBP005	252	1	0	0	0	1	0	0	0	0	0
6	CCBP006	264	1	1	0	0	0	1	0	0	1	0
7	CCBP007	202	1	0	0	0	0	1	0	0	1	0
8	CCBP008	213	1	0	1	0	0	0	0	0	1	0
9	CCBP009	242	1	0	1	0	0	0	0	1	1	0
10	CCBH001	225	1	1	1	1	0	0	0	0	0	0
11	CCBH002	272	1	1	0	0	0	1	0	1	1	1
12	CCBH003	234	1	0	1	0	0	0	0	1	1	0
13	CCBH008	288	1	0	0	0	0	0	0	1	0	0
14	CCBH009	249	1	0	0	0	0	1	0	1	1	0
15	CCBH010	291	1	0	0	0	0	0	1	0	0	0
16	CCBH011	228	1	0	1	0	0	1	1	1	1	1
17	CCBH012	210	1	0	1	0	0	0	0	0	1	0
18	CCBH013	276	1	0	1	1	0	0	0	0	1	1
19	CCBH014	114	1	0	1	1	0	1	1	0	1	0
20	CCBH015	245	1	0	0	0	0	0	0	0	1	0
21	CCBS001	204	1	0	0	0	0	1	0	0	1	0
22	CCBS002	344	1	0	0	0	0	1	0	1	0	0
23	CCBS004	312	1	0	0	0	0	0	0	1	0	0
24	CCBS005	209	1	0	0	0	0	0	0	1	1	0
25	CCEB001	305	1	0	0	0	0	0	0	1	1	1
26	CCEB002	319	1	0	0	0	0	0	0	1	0	0
27	CCEB003	214	1	0	0	0	0	1	1	1	1	1
28	CCEB004	309	1	0	1	0	0	1	1	1	1	0
29	CCEB005	216	1	1	1	1	1	1	1	0	1	0
30	CCEB006	225	1	0	0	0	0	0	0	1	1	0
31	CCEB008	380	1	1	1	1	0	1	0	1	1	0
32	CCEH002	222	1	1	1	1	1	1	0	1	1	0
33	CCEH003	338	1	1	1	0	0	0	0	1	1	1
34	CCEH004	389	1	1	1	0	0	0	0	0	0	1
35	CCSP001	255	1	1	1	1	1	1	1	0	1	1
36	CCSP004	284	1	1	1	1	0	1	0	0	1	1
37	CCSP005	249	1	1	1	1	0	1	1	0	1	1
38	CCSP006	298	1	1	1	1	0	1	0	0	0	1
39	CCSP007	286	1	0	0	0	0	0	0	0	1	0
40	CCHX002	230	1	0	0	0	0	1	0	0	0	0
41	CCHXP001	252	1	0	1	0	0	1	0	0	0	0
		TOTAL	41/41	13/41	23/41	13/41	3/41	23/41	8/41	18/41	28/41	11/41
	Similarity	%	100	32	56	32	7	56	20	44	68	27

4. Discussion

4.1 Single Locus DNA Markers for *Epinephelus fuscoguttatus*

The tiger grouper, *Epinephelus fuscoguttatus* is highly sought after by fish breeders as it adapts well to commercial aquaculture and mariculture systems. The genetic profile indicated a high degree of similarity with 31 primers amplifying consistently across the 12 samples and 15 primers exhibiting differential profiles. This implies that the breeding population is not as genetically diverse as compared to *Epinephelus corallicola* and may require the acquisition and screening of additional recruits from the wild. Earlier reports [20] based on genotyping using microsatellites have arrived at similar conclusions regarding the abundance and diversity of wild populations of *Epinephelus fuscoguttatus*. Single locus DNA markers can be tested for Mendelian inheritance and applied for marker assisted selection of intraspecific grouper hybrids.

4.2 Single Locus DNA Markers for *Epinephelus corallicola*

These are the first reported genomic molecular markers for *Epinephelus corallicola*. The amplification profiles revealed a high level of intraspecific genetic diversity as the ten individuals tested exhibited unique genetic fingerprints implying that the wild population is genetically diverse. A similar study conducted using microsatellites in a closely related species *Plectropomus maculatus* led to the discovery of highly polymorphic loci which have potential applications in broodstock management [21]. Single locus DNA markers can be applied to develop linkage maps as in the case of *Epinephelus aeneus* where 222 microsatellite loci were utilized to construct a linkage map representing 24 chromosomes [22]. Advances in next generation DNA sequencing technologies have led to the development of novel markers such as SNPs as reported in *Epinephelus coioides* [23] and microsatellites with intra specific applications [24]. The development of a database of markers is essential for the development of management strategies, however markers which require extensive technical expertise and complex analysis will not be relevant to aquaculture as there is no cost benefit advantage. Under these circumstances single locus markers offer an economical alternative to SNPs.

4.3 Implications for broodstock management

Grouper breeders rely on wild germplasm in order to develop inbred lines. The selection of seed is done on a random basis and not on the basis of genetic diversity. There are two primary reasons for this, the first is the lack of suitable species specific markers for genetic profiling and the second is the lack of technical expertise to interpret the data derived from highly informative microsatellite DNA loci and SNPs. The objective of this study was to develop molecular markers which could be applied for diagnostic testing in a simple laboratory setup comprising a thermal cycler, gel electrophoresis system and imaging systems which are can be accessed by fish breeders. The markers which have been developed can be scored directly purely on the basis of amplification or non-amplification. Previous studies [25] that have attempted to develop molecular markers for groupers have utilized Random Amplified Polymorphic DNA (RAPD) which are difficult to reproduce due to the low degree of species specificity and high number of PCR artefacts. Furthermore, the ability of the markers to resolve intraspecific genetic polymorphism makes them ideal for population diversity studies and the construction of linkage maps for mapping quantitative traits.

5. Conclusion

This study demonstrated that species specific single locus DNA markers can be applied for the genotyping of groupers recruited from the wild for the purpose of breeding. We developed 42 and 41 novel species specific DNA markers for *Epinephelus fuscoguttatus* and *Epinephelus corallicola* respectively and applied them to resolve intraspecific diversity. These markers will be of assistance to fish breeders during the process of broodstock selection and will facilitate the process of developing genetically diverse breeding populations.

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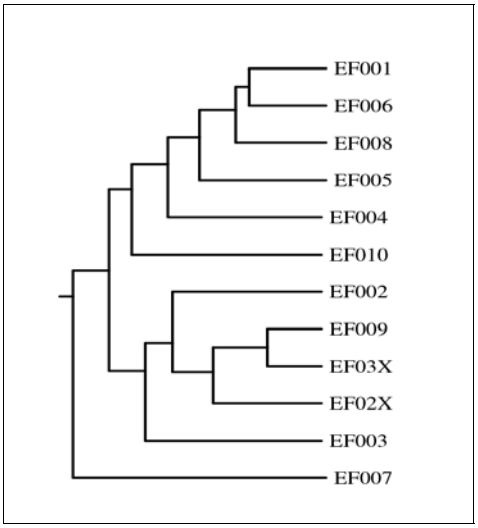

Fig 1: Phenogram of 12 *Epinephelus fuscoguttatus* OTU's resulting from the UPGMA cluster analysis of the OTU x OTU correlation matrix. Cluster 1 comprises EF001, EF006, EF008, EF005, EF004 and EF010. Cluster 2 consists of EF002, EF009, EF003X, EF002X and EF003. Cluster 3 is composed on only one individual EF007.

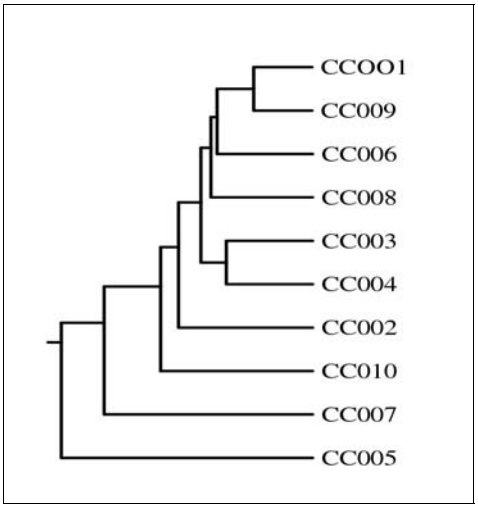

Fig 2: Phenogram of 10 *Epinephelus corallicola* OTU's resulting from the UPGMA cluster analysis of the OTU x OTU correlation matrix. The high degree of intraspecific genetic diversity is evident from the distribution of the nodes.

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