FAS Fisheries and Aquatic Sciences

Original Article
Fish Aquat Sci 16(4), 303-309, 2013

Development of Polymorphic Microsatellite Markers Suitable for Genetic Linkage Mapping of Olive Flounder *Paralichthys olivaceus*

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Abstract

Microsatellite markers are important for gene mapping and for marker-assisted selection. Sixty-five polymorphic microsatellite markers were developed with an enriched partial genomic library from olive flounder *Paralichthys olivaceus* an important commercial fish species in Korea. The variability of these markers was tested in 30 individuals collected from the East Sea (Korea). The number of alleles for each locus ranged from 2 to 33 (mean, 17.1). Observed and expected heterozygosity as well as polymorphism information content varied from 0.313 to 1.000 (mean, 0.788), from 0.323 to 0.977 (mean, 0.820), and from 0.277 to 0.960 (mean, 0.787), respectively. Nine loci showed significant deviation from the Hardy-Weinberg equilibrium after sequential Bonferroni correction. Analysis with MICROCHECKER suggested the presence of null alleles at five of these loci with estimated null allele frequencies of 0.126-0.285. These new microsatellite markers from genomic libraries will be useful for constructing a *P. olivaceus* linkage map.

Key words: *Paralichthys olivaceus*, Olive flounder, Microsatellite markers, Linkage map

Introduction

Olive flounder *Paralichthys olivaceus* is one of the most important fishery and aquaculture species with a selective breeding program in Korea. Stock-enhancement programs for olive flounder have been carried out for several years, and hatchery-reared offspring are released into the wild as a way to increase the biomass of depleted fishery stocks. To ensure responsible stock-enhancement programs, the genetic diversity of both wild populations and hatchery strains should be scrutinized using molecular markers. The Genetic and Breeding Research Center (Geoje, Korea) runs a breeding program to increase olive flounder aquaculture production, and a family characterized by fast growth and disease resistance has been created. Traits such as weight, shape, and disease are controlled by more than one locus (O'Connel and Wright, 1997). The development of a genetic linkage map is a prerequisite for mapping quantitative trait loci and for marker-assisted selection (Cho et al., 1994).

Because microsatellite markers have high levels of polymorphism, co-dominant inheritance, genome-wide distribution, and high reproducibility, they are the most popular and powerful molecular markers in population genetics and can be used to construct genetic linkage maps (Liu and Cordes, 2004). In recent years, microsatellite markers have become one of the most commonly used molecular markers in population and evolutionary biology research, and are applied widely in studies of biological breeding, genetic linkage maps, ge-

http://dx.doi.org/10.5657/FAS.2013.0303

Received 11 July 2013; **Revised** 30 August 2013
Accepted 05 September 2013

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netic diversity, and phylogeny (Goldstein and Pollock, 1997). Seventy-nine microsatellite markers have been developed previously for *P. olivaceus* (Kim et al., 2003, 2009). However, further *P. olivaceus* polymorphic microsatellite markers are required to facilitate genome-mapping studies. In this study, we identified 65 new microsatellite loci isolated from a *P. olivaceus* genomic library.

Materials and Methods

Isolation of microsatellites

A partial genomic library enriched with GT repeats was constructed using a slight modification of the procedures described by Hamilton et al. (1999). Genomic DNA was extracted from *P. olivaceus* muscle tissue using the TNES-urea buffer method (Asahida et al., 1996). DNA was digested with the enzymes *Alu*I, *Rsa*I, and *Hae*III (New England Biolabs, Ipswich, MA, USA), and DNA fragments of 300-800 bp were isolated and ligated to SNX/SNX rev linker sequences. Linker-ligated DNA was polymerase chain reaction (PCR) amplified using SNX as the primer, and PCR products were hybridized to biotinylated $(GT)_{10}$ probes attached to streptavidin-coated magnetic beads (Promega, Madison, WI, USA). Then, the enriched fragments were amplified again. The products were digested with *Nhe*I and ligated into the *Xba*I-digested pUC18 vector (Pharmacia, Uppsala, Sweden), followed by transformation into *E. coli* DH5α-competent cells. Positive clones with repeats were identified by PCR with $(GT)_{10}$ and M13 primers. A negative control with no template was included in each PCR. The PCR products were analyzed in 1.5% agarose gels, and clones producing two or more bands were considered to contain a microsatellite locus. Plasmid DNA from the positive clones was purified using Acroprep 96-well filter plates (Pall Co., Port Washington, NY, USA). All positive colonies were sequenced using the M13 forward or reverse primer with a BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI 3130*xl* automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Primer design and genotyping

Primers were designed from the unique sequences flanking microsatellite motifs using OLIGO 5.0 software (National Biosciences, Plumouth, MN, USA). PCR conditions were initially optimized using DNA samples originally used for microsatellite isolation to establish whether the desired size product was amplified by changing the annealing temperature, the primers, and $MgCl_2$ concentrations as well as the amplification profiles. Suitable microsatellite loci were genotyped to test the level of genetic polymorphism using 30 *P. olivaceus* individuals collected from the East Sea in Korea. The PCR reactions were performed in 10-μL volumes containing 10-ng genomic DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 3 pmol of each primer, 0.5× Band Doctor, and 0.5 U of f-Taq DNA polymerase (Solgent, Solon, OH, USA). The forward primers were end-labeled commercially with the dyes 6-FAM, NED, or HEX (Applied Biosystems). The reactions were amplified using a PTC-200 thermocycler (MJ Research, Waltham, MA, USA) with an initial denaturation at 95°C for 15 min, followed by 35 cycles of 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C, and a final 30-min extension at 72°C. The lengths of the PCR products were determined with an ABI 3130*xl* Genetic Analyzer (Applied Biosystems) using the GeneScan-400HD (ROX) size standard (Applied Biosystems).

Data analysis

The number of alleles per locus, polymorphism information content (PIC), and observed and expected heterozygosity at each locus were calculated using CERVUS 3.03 (Marshall et al., 1998). Deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were estimated using GENEPOP 4.0 (Raymond and Rousset, 1995), and adjusted *P*-values for both analyses were obtained using a sequential Bonferroni test for multiple comparisons (Rice, 1989). We also estimated F_{IS} values (Weir and Cockerham, 1984) that are used to determine HWE departures within a population. The presence of null alleles was examined using MICRO-CHECKER 2.2.3 (Van Oosterhout et al., 2004).

Results and Discussion

In total, 800 white colonies with inserts were randomly selected and screened for the repeat using PCR, which yielded 425 (53.1%) true positive clones. These were sequenced producing 330 (41.3%) sequences containing simple sequence repeats, of which 184 (23%) were eliminated because they possessed no flanking sequence. A total of 146 (18.3%) sequences containing microsatellites were obtained, and primers were designed to amplify microsatellite-containing regions of the genome. Only 98 of the 146 primer pairs successfully amplified the target region, and the remaining pairs either failed to amplify or produced nonspecific bands. Finally, we chose 70 primer sets because they produced clear and reliable bands at high temperatures, and we tested polymorphisms in 30 *P. olivaceus* individuals collected from the East Sea in Korea. Sixty-five loci were polymorphic (Table 1), and another five loci were monomorphic. The repeat motif, product size, and annealing temperatures at each of the 65 microsatellite loci are presented in Table 1. Conventional protocols for isolating microsatellites are cost, time, and labor intensive, and the efficiency of microsatellite isolation is low, ranging from 0.045% to 12% (Zane et al., 2002). Several enrichment techniques have been developed to overcome these challenges (Zane et

Table 1. Characterization of 65 polymorphic loci in *Paralichthys olivaceus*

Locus	Repeat motif	Primer sequence (5′→3′) (forward/reverse)	*Ta* (°C)	*Na*	Allele size range (bp)	PIC	H_O	H_E	P	F_{IS}	Frequency of null allele	GenBank accession no.
KOP100	$(AC)_{11}ATACAT(AC)_{11}$	GGTAGAGCTGAGCAGGGAGTTC AGGTGAAGAAGAGGAAAACGACA	58	18	130-172	0.892	0.871	0.915	0.262	0.049	0.015	KC847407
KOP101	$(GT)_{24}TT(GT)_7$	CTACACCGCATCACCTAAAAGAGT CGTGTGTCCTGTACGTCCTG	58	21	101-181	0.868	0.844	0.892	0.221	0.055	0.017	KC847408
KOP102	$(GT)_{11}$	TGTCATGTCGTCCATCTGTATTTAC CCAGTGTTACATCACAGCCTTTAG	58	11	69-97	0.862	0.969	0.857	0.904	−0.133	−0.079	KC847409
KOP103	$(GT)_{19}$	CGTAAGCCTAGAAAGAACTGCTCT AGTTTCTGCTACAGTAGGACCACAT	58	14	76-120	0.792	0.844	0.821	0.388	−0.028	−0.034	KC847410
KOP104	$(GT)_6AT(GT)_6$	GATGCTGAGTCAGGCGGTCTGTG TCATGCTGGAAAGGGTCGGGTAC	58	3	73-79	0.277	0.387	0.323	0.626	−0.202	−0.213	KC847411
KOP105	$(AC)_{18}$	GAATGGCATCTGTCCACTGTGT ATTTCTCACATGCATCACTCCATC	58	2	62-118	0.327	0.581	0.419	0.035	−0.395	−0.352	KC847412
KOP106	$(GT)_{15}$	TGTGTTGGAGTTTGTTTGAGAATGT CAGAGAGCAAAGCACAAGGTCTAA	58	23	89-149	0.928	0.938	0.946	0.291	0.010	−0.001	KC847413
KOP107	$(GT)_8GA(GT)_{12}$	CGTGCTGTCTGTATGAATCTGT GATGATGCTACTGAACAAATGAAG	58	20	134-184	0.936	0.926	0.957	0.148	0.033	0.008	KC847414
KOP108	$(GT)_{20}$	TGGAATAGCAGAGTTTGGAGTAAG GGGAGTTTGTGGGTGATTTT	58	22	130-188	0.890	0.844	0.912*	0.000	0.076	0.030	KC847415
KOP109	$(AC)_{23}GC(AC)_4$	AAAATGGCTCAATGTAAGGGATA GAGAAAGTGAAACGATGTGTAATGA	58	22	222-278	0.921	0.906	0.940	0.006	0.036	0.008	KC847416
KOP111	$(GT)_{15}AT(GT)_{14}$	CGCAACAATATAACAAAACAATGAT GCTGCTGCCAACTGTATGAC	58	14	196-260	0.683	0.704	0.714	0.447	0.014	−0.027	KC847417
KOP112	$(GT)_{23}$	GCCAAGCACAAAAACCAACCAGAGT ACCGTCCTCCACCGTCATCGT	58	28	230-346	0.943	0.871	0.961	0.014	0.096	0.038	KC847418
KOP113	$(GT)_{13}CT(GT)_4T(GT)_6$	GTGAGCGTAAGTTCATCAAACAAC CCCAAGCTAACCTGTACACAAAC	58	29	122-266	0.945	0.406	0.963*	0.000	0.582	0.285	KC847419
KOP114	$(AC)_{10}$	AGGCTGCGTTTCGATTTATCC TGAGGGGTCAACTATGATTAGATGG	58	8	87-101	0.814	0.719	0.849	0.053	0.155	0.072	KC847420
KOP115	$(GT)_4GC(GT)_{11}$	TGGAATAGCAGAGTTTGGAGTAAGG AAGGGAGTTTGTGGGTGATTTTTA	58	22	132-190	0.896	0.906	0.917	0.009	0.012	0.001	KC847421
KOP116	$(GT)_{34}$	GCACTAATTTGTCTCTGTGTCCATA AAACAAGGTCACTCCCCGTAT	58	20	210-292	0.930	0.719	0.949	0.003	0.246	0.113	KC847422
KOP117	$(AC)_{18}$	GAGAGCAATGATGCATGGAGGAGA CCCCGGGCATGTACACGAGTA	58	13	151-183	0.767	0.900	0.809	0.348	−0.115	−0.073	KC847423
KOP118	$(GT)_{47}$	AGATGTCACGTTCACATGAACAGGG GCTGACACCAAACACCTGCTCTG	58	31	167-255	0.948	0.938	0.965	0.137	0.029	0.008	KC847424
KOP119	$(AC)_{37}$	TAAACTTGTCTGGCAGCACAGT TCGTGGTAATGTAGGAGGATAGAAA	58	26	182-266	0.947	1.000	0.965	1.000	−0.037	−0.027	KC847425
KOP120	$(GT)_{10}$	TTTCCATTCTCCTCCCTGTT GTTTTTGTCCTATCAGCACACATAC	58	12	65-103	0.775	0.833	0.808	0.311	−0.032	−0.027	KC847426

Table 1. continued

Locus	Repeat motif	Primer sequence (5′→3′) (forward/reverse)	*Ta* (°C)	*Na*	Allele size range (bp)	PIC	H_O	H_E	P	F_{IS}	Frequency of null allele	GenBank accession no.
KOP121	$(GT)_{33}$	AGAGGAGAACTGGTCTGGATTGAT ACATGGCTCTTGGCTTTACTCAC	58	24	229-303	0.932	0.552	0.952*	0.000	0.425	0.203	KC847427
KOP122	$(AC)_{12}AT(AC)_6$	AGATATGAGCCTGCTACACAGACT TCGGTGAAAACAGCCTCTTA	58	22	205-281	0.919	0.906	0.939	0.197	0.035	0.008	KC847428
KOP123	$(AC)_{18}$	GCGGTCTGAATGCCACTCATC CAGACATGCTCAATCACCTCCAAG	58	3	229-249	0.383	0.781	0.496*	0.000	−0.591	−0.516	KC847429
KOP125	$(AC)_8AT(AC)_{21}$	AATTTAAAGTCACAAGTTGCTGGTT GAAGAGGCATGTCTGAGGTGT	58	23	152-228	0.933	0.625	0.952*	0.000	0.347	0.166	KC847430
KOP126	$(AC)_{12}$	GATCCCCATCCATATGAGAAGTAAT AACCGCGTGAAGGTATTTTAATATC	58	7	57-73	0.717	0.562	0.770	0.006	0.273	0.131	KC847431
KOP127	$(GT)_9TT(GT)_{21}$	TTGATGTTGGCAGGCGAGTG TCTTCTTCTTGGACGATGTTCCCTC	58	25	115-175	0.937	0.938	0.955	0.366	0.019	0.002	KC847432
KOP128	$(AC)_{12}$	CTCAGGCTCCACATCCCAACA TCGTAATCAGCCCCCATCTCTGTA	58	29	51-151	0.937	0.969	0.955	0.675	−0.015	−0.017	KC847433
KOP129	$(AC)_{13}$	CGTTTCGTGTTTTAGTGACCTCTC TCTCCACCAGCTCATAATTGATG	58	25	214-286	0.930	0.938	0.949	0.069	0.012	−0.002	KC847434
KOP130	$(AC)_9AG(AC)_{12}$	CCATAATGCACAGGTGAGACAG ACTGAACAGAGAAGAGGAGCAACT	58	27	143-225	0.944	1.000	0.962	0.581	−0.040	−0.029	KC847435
KOP131	$(AC)_5AG(AC)_{10}$	GCATGTCGCAAGAACCTCCA TTGTGTCCTTTATGATCGCTGTCTG	58	19	65-109	0.917	0.906	0.937	0.035	0.033	0.008	KC847436
KOP132	$(AC)_{11}AT(AC)_{18}$	GCCTCTGCAAGGTTAAAACTCTCCA GGTGCATGATGATTAATCGAGCAAG	58	21	182-262	0.907	0.875	0.928	0.133	0.058	0.017	KC847437
KOP133	$(AC)_{14}$	CCTCTTTCTGCTGCTGCTGA GGGGCTTTCTGATATTAACGACAC	58	8	139-155	0.658	0.656	0.701	0.087	0.065	0.021	KC847438
KOP134	$(GT)_9$	ATATACTAGCAGCATGCGAATGCG TCTTTCCTCCCCAACAGCCTC	58	10	77-111	0.744	0.563	0.790	0.048	0.291	0.135	KC847439
KOP135	$(GT)_4CT(GT)_9$	TTGTTTCTCTGCGTGGTTTTATCTC GCAGGGGCTGATGATTTACTTCT	58	5	93-131	0.403	0.438	0.451	0.033	0.030	0.020	KC847440
KOP136	$(GT)_8CT(GT)_{12}(GA)_2(GT)_7$	ACAAACCTGCCATAGAAAACACTGC CTGAGATCTGCCACCTTCACAAAG	58	2	228-232	0.283	0.313	0.347	0.612	0.101	0.041	KC847441
KOP137	$(GT)_{10}$	GACGGCTCATCACTCCTGTTTATG CCGTCTCCTCCCAACTCACAC	58	33	155-243	0.958	0.969	0.975	0.613	0.006	−0.005	KC847442
KOP138	$(GT)_{30}$	GGGGAATATTTACACCATCACAGG ACCGGGGCAGTTCTTCAAC	58	22	196-264	0.927	0.967	0.947	0.685	−0.021	−0.020	KC847443
KOP139	$(AC)_8AG(AC)_9$	TGACAGCCCCTACACAAACACA GCGTCCAGGCACAATGAAAC	58	28	167-271	0.932	1.000	0.950	0.685	−0.054	−0.036	KC847444
KOP140	$(AC)_6GC(AC)_9$	CTGGCGGACTGGAGGTTGAC AGGAGGCGAGACAGACACGAAC	58	10	237-265	0.756	0.793	0.799	0.415	0.007	−0.000	KC847445

Table 1. continued

Locus	Repeat motif	Primer sequence (5′→3′) (forward/reverse)	*Ta* (°C)	*Na*	Allele size range (bp)	PIC	*Ho*	*HE*	P	*FIS*	Frequency of null allele	GenBank accession no.
KOP141	$(GT)_{22}$	TGTTTCTGTATATGATGGTTGTCCG TGAAAAATGGCTAAAGCGTGTCT	58	19	66-122	0.903	0.867	0.924	0.463	0.063	0.026	KC847446
KOP142	$(AC)_5AT(AC)_{10}AT(AC)_7$	TCGTGCTGCACAGTAACACAGACC CCACGCTGCTCGTTCCCTC	58	31	154-238	0.954	1.000	0.971	0.911	−0.030	−0.024	KC847447
KOP143	$(AC)_{10}$	ACCAGGAGCGTTTCATCACAG TGTCCGTGTTGTCCAAGACTATGT	58	23	75-159	0.924	0.906	0.943	0.948	0.040	0.011	KC847448
KOP144	$(AC)_7TC(AC)_2$	TCGAGTTGCGCCTCCTTACCTTTT CACTTCCCACTGCGATGTGACCT	58	2	106-110	0.349	0.313	0.458	0.113	0.322	0.140	KC847449
KOP145	$(AC)_{16}$	AACTCCTAACCCCTCTATTCAACA CCTTCTGACCCAACGATACTTT	58	19	161-211	0.910	0.906	0.930	0.889	0.026	0.005	KC847450
KOP146	$(GT)_{11}CT(GT)_{15}$	AATGTCCTGGCCTCTTTTCTCTG TCAGTTGAATGAAGAAGCAAACACA	58	25	75-167	0.933	0.813	0.951*	0.000	0.148	0.126	KC847451
KOP147	$(GT)_{16}GA(GT)_{41}$	CTGAGACACGACGGAGGACATCAT CCCTCCTCATTCTGCTATTCATCCC	58	6	206-254	0.384	0.563	0.442	0.596	−0.280	−0.313	KC847452
KOP148	$(AC)_{25}$	GCTCGCACTCTCCTGGGGTCAC GACATTCAGGGTAGCGTGCGTGTG	58	2	61-85	0.375	1.000	0.508*	0.000	−1.000	−1.000	KC847453
KOP149	$(AC)_{21}$	GATGAGCGGACCTGTCAATG TATTTGTGCACATGAGCCCATAC	58	20	132-232	0.903	0.844	0.924	0.317	0.088	0.036	KC847454
KOP150	$(AC)_3AAGTGTGC(AC)_6$	GGAACGACTTGCCTCTGAG GCTAAATTCCTCTGCCATCTCT	58	5	146-158	0.515	0.969	0.607*	0.000	−0.612	−0.411	KC847455
KOP151	$(GT)_{11}$	TATGTAATTGCAGATGGGGATGTG TTGCCAAATACTGAAAGGGTGTG	58	12	132-196	0.732	0.710	0.767	0.113	0.076	0.015	KC847456
KOP152	$(AC)_{29}$	CTCGAGCACTTTTTGGTGACTTT CGGAGAATACCATCACTGCTACTTT	58	25	113-177	0.938	0.844	0.956	0.047	0.119	0.053	KC847457
KOP153	$(GT)_{16}$	CAGCAGAGCCAATCAGAGAGC GCACAAGCACAAGAAGACCAAGTA	58	28	204-292	0.942	0.969	0.960	0.539	−0.010	−0.014	KC847458
KOP154	$(GT)_{31}$	ACAAATGGAAAGGGGTAGCAT ATCGCTTGGGGAAAATAGTAATC	58	21	156-222	0.933	0.969	0.952	0.295	−0.018	−0.017	KC847459
KOP155	$(GT)_{33}$	GACAGGAGACAATACATGTGACTGA CCCCTCCTCCTCATATCTCAA	58	15	199-235	0.880	0.793	0.904	0.005	0.124	0.053	KC847460
KOP156	$(GA)_9(GT)_7$	CAGGAAGTCCAGGCTGGTGTA CTCCCACTTTTAACAACTGGTGAGA	58	11	149-193	0.808	0.906	0.842	0.011	−0.077	−0.054	KC847461
KOP157	$(GA)_{10}$	TGTAGATAAGCCCGAGAACCAGTAA CAATGCCAAAGTCTCGTCCTC	58	10	58-90	0.762	0.781	0.802	0.456	0.026	0.006	KC847462
KOP158	$(AC)_{12}(AG)_{10}$	TGGCAAAGTTGTGTTGATACAGAG AATATTCCCCCTCATCTACAGTGG	58	36	241-395	0.960	0.906	0.977	0.063	0.073	0.028	KC847463
KOP159	$(AC)_4AG(AC)_{19}TC(AC)_6$	GTGAATATGCTCCTGTTGCGACTC CTGGTCTTTAGGGGCAGTGTGTC	58	18	193-253	0.795	0.750	0.818	0.691	0.084	0.026	KC847464

Table 1. continued

Locus	Repeat motif	Primer sequence (5′→3′) (forward/reverse)	*Ta* (°C)	*Na*	Allele size range (bp)	PIC	*Ho*	H_E	P	F_{IS}	Frequency of null allele	GenBank accession no.
KOP160	$(CT)_9TT(CT)_7$	ATTGATTGGACTCTGGGGCTCTCA GGATACTCAGCAGCAGCCATGTCTC	58	7	101-119	0.524	0.438	0.563	0.017	0.226	0.107	KC847465
KOP162	$(GA)_{20}$	CAATGGGTTGCTAGATATGGTTGC TCTCCATGACGACGCTTGTTC	58	23	204-268	0.925	0.906	0.944	0.015	0.040	0.016	KC847466
KOP163	$(GT)_{11}CT(GT)_4$	GCATCGCTTCACCCGTTCAGA GCACCACCCGTCCTCTTCCTC	58	12	97-129	0.818	0.875	0.850	0.804	−0.030	−0.022	KC847467
KOP164	$(GA)_{22}$	AGTGAAGCTGGATGTTGTGTCTGAG CTCTGCACTGTTTTCACTGGGTCT	58	19	102-170	0.914	0.531	0.934*	0.000	0.435	0.209	KC847468
KOP166	$(GA)_8AA(GA)_9$	TTCACACATATCCTGCGACGAG GTCAGTATGGCTGTGAGGTATCCAA	58	6	232-254	0.537	0.656	0.599	0.652	−0.097	−0.055	KC847469
KOP167	$(GA)_4GC(GA)_4$	GACGCGTCATTGCTCACTAC CTCCATATGGCTTGATGATGTCTA	58	7	118-140	0.319	0.344	0.336	0.402	−0.023	−0.015	KC847470
KOP169	$(AC)_{16}$	CGCATGCATAACAAAGCCTGGAG GAAAGGGACTCGTGTGTCTGTCAG	58	8	64-86	0.759	0.781	0.803	0.897	0.028	0.007	KC847471
Mean				17.1		0.787	0.788	0.820				

Significant deviations from HWE after sequential Bonferroni correction ($P < 0.0008$) are indicated by asterisks.
Ta, optimal annealing temperature; *Na*, number of alleles; PIC, polymorphic information content; H_O, observed heterozygosity; H_E, expected heterozygosity; P, probability of deviation from Hardy-Weinberg equilibrium (HWE); F_{IS}, inbreeding coefficient.

al., 2002). We constructed a microsatellite enrichment library for olive flounder using $(GT)_{10}$ biotin-labeled probes, and 78% (330/425) of the positive clones contained microsatellite repeats. This efficiency is lower than that in tilapia (96%) (Carleton et al., 2002) but higher than that in cutlassfish (48%) (An et al., 2010).

The 65 new polymorphic microsatellite loci developed in *P. olivaceus* varied widely in their degree of polymorphism (Table 1). The number of alleles observed per locus ranged from 2 to 33 (mean, 17.1). Observed heterozygosity ranged from 0.313 to 1.000 (mean, 0.788), expected heterozygosity (H_E) was 0.323-0.977 (mean, 0.820), and PIC was 0.277-0.960 (mean, 0.787). Heterozygosity, also referred to as gene diversity, is a suitable parameter for investigating genetic variation. For a marker to be useful for measuring genetic variation, it should have a heterozygosity of at least 0.3 (Takezaki and Nei, 1996). The H_E range of the markers analyzed here was between 0.323 and 0.977; thus, the markers were appropriate for measuring genetic variation. The PIC value is related to the availability and utilization efficiency of a marker; the higher the PIC value of a marker is in a population, the higher the heterozygote frequency is and the more genetic information it provides (Arora et al., 2004). Genetic markers showing PIC values >0.5 are normally considered informative for population genetic analyses (Botstein et al., 1980). In this study, all 65 microsatellite loci were highly polymorphic. The mean PIC value across all loci was >0.5, which could provide sufficient information to assess of genetic diversity and construct genetic maps.

Nine loci (KOP108, KOP113, KOP121, KOP123, KOP125, KOP146, KOP148, KOP150, and KOP164) deviated from HWE in the tested population after sequential Bonferroni correction ($P < 0.0008$) (Table 1). Six of these loci (except KOP123, KOP148, and KOP150) exhibited a significant deficiency of heterozygotes. Analysis with MICROCHECKER indicated the possible occurrence of null alleles at six of the loci (KOP113, KOP121, KOP125, KOP134, KOP146, and KOP164). In addition to the loci with deviations from HWE, null alleles were detected in three loci (KOP116, KOP126, and KOP134). In all cases, evidence for the presence of null alleles was relatively weak and, thus, insufficient to confirm a significant departure from HWE following Bonferroni correction. The estimated null allele frequencies ranged from 0.126 (KOP146) to 0.285 (KOP113). Moreover, four of the loci (KOP113, KOP121, KOP125, and KOP164) showed high estimated null allele frequency together with a highly significant positive F_{IS} (heterozygote deficiency), strongly suggesting a causative relationship. Furthermore, no significant linkage disequilibrium between loci pairs was detected after Bonferroni correction ($P < 0.0008$), except in two pairs (KOP102-KOP157 and KOP107-KOP112). These markers will be useful for population genetics, parentage analysis, association studies, and construction of a *P. olivaceus* linkage map.

Acknowledgements

This work was supported by grants from the National Fisheries Research and Development Institute (RP-2013-BT-077).

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