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PERMANENT GENETIC RESOURCES NOTE

Isolation and characterization of 28 new microsatellite markers for European flounder (*Platichthys flesus* L.)

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Abstract

The European flounder (*Platichthys flesus* L.) is used in ecotoxicological studies to provide detailed information on the effects of pollution on individual fish. Data on population and evolutionary level effects are, however, limited. Here, the isolation and characterization of 28 novel species specific microsatellite loci are presented. The number of alleles ranged from 8 to 38, and observed heterozygosity from 0.542 to 1.

Keywords: biomonitoring, ecotoxicology, flatfish, flounder, microsatellites, population genetics

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The European flounder (*Platichthys flesus* L.) is used as a model species in ecotoxicology to examine the ecosystem impacts of anthropogenic pollution. For example, recent studies have examined the effects of toxicants on DNA disruption, gene expression, transcriptomics and tumorigenesis (Stentiford *et al.* 2003; Marchand *et al.* 2006; Williams *et al.* 2008). Although some microsatellite markers have been used on European flounder (Casas *et al.* 2005; Hemmer-Hansen *et al.* 2007), only six non-EST-linked species-specific loci have been published to date. Increasing this number will extend the range of applications, such as population assignment, localized impacts of toxicant exposure, and ultimately contribute towards the production of linkage maps. Here we describe the development and characterization of 28 novel microsatellite markers developed from European flounder.

European flounder ($n = 2$) were collected from the Irish Sea (53°18'56.45"N; 3°53'59.64"W) and fin clips stored in 100% ethanol until processing. A microsatellite-enriched genomic library was constructed following the protocol of Tysklind *et al.* (2009). From this library, 1344 colonies were screened for microsatellites by polymerase chain reaction (PCR) amplification with M13 Forward primer and a

mixture of non-biotinylated microsatellite probes. One hundred and ninety-six positive amplicons were sequenced (Macrogen Inc.), edited, analysed and checked for duplicates using BioEdit (Hall 1999). Enrichment efficiency was high at 87.8%. Primers were designed either side of 47 putative microsatellites using Primer 3 (Rozen & Skaletsky 2000), and tested for successful amplification at several annealing temperatures on 3% TBE agarose gels. M13-tailed forward primers were then ordered (Schuelke 2000). Nested PCRs with tailed forward primer, reverse primer, and FAM-labelled M13-tail oligos were used for genotyping. PCR cocktails of 10 µL final volume contained around 20 ng of DNA, 1× GoTaq Flexi buffer (Promega), 1.5 mM MgCl₂, 125 µM dNTP, 0.1 µM forward-tailed primer, 0.5 µM of reverse primer, 0.5 µM of FAM-labelled M13 tails, and 0.5 U GoTaq DNA polymerase (Promega). PCRs were carried on a Bio-Rad Tetrad2 Peltier Thermal Cyclers and the thermocycling programmes were as follows: an initial denaturation phase of 3 min at 95 °C, followed by 13 cycles of 30 s at 95 °C, 45 s at 55 °C, 60 s at 72 °C, then 31 cycles of 30 s at 94 °C, 45 s at 50 °C, 60 s at 72 °C, and finishing with a 30-min extension phase at 72 °C.

Two samples of 24 individuals collected from the Mersey Estuary (53°19'28.70"N, 2°54'39.74"W) and the Tyne Estuary (54°59'10.21"N, 1°27'49.47"W) were genotyped on an ABI 3130xl Genetic Analyser (Applied Biosystems) with an

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Table 1 Characterization of 28 microsatellite loci isolated from *Platichthys flesus* in two estuarine populations. Motif, repeat sequence of the isolated clone; T_a , annealing temperature; N , number of individuals successfully amplified (out of 48). N_a , number of alleles; range, allele size range; H_O , observed heterozygosity; H_E , expected heterozygosity; P , associated probability value of conformation with Hardy–Weinberg equilibrium (HWE). **Bold** P values indicate significant deviation from HWE after Bonferroni correction

Name	Accession no.	Motif	Forward primer	Reverse primer	Overall				Mersey Estuary				Tyne Estuary			
					T_a	N	N_a	Range	N_a	H_O	H_E	P	N_a	H_O	H_E	P
FLAC1–32	FJ360492	(AC) ₁₉	GAGAAACCTCCCACAGGTGA	GGGAAATAACGCTGATACGAA	55	48	13	156–184	9	0.833	0.829	0.381	11	0.958	0.881	0.070
FLAC1–47	FJ360493	(AC) ₇ + (AC) ₁₆	ACGCACATACGAAGCCGTAG	ATTTCTGCCAGGGATTACC	55	47	10	242–270	7	0.542	0.680	0.044	8	0.652	0.700	0.500
FLAC2–16	FJ360494	(AC) ₁₅	CCATCTACCCCCAGAAAACA	CTCCGGGGAAACTTAAGAG	55	48	20	156–202	17	0.917	0.869	0.431	16	0.833	0.904	0.022
FLAC2–18	FJ360495	(AC) ₂₀	AAAAGCCAATGACCTGTTGC	GCATGCCAGTGAGAACAAATG	55	48	20	216–266	16	0.958	0.910	0.041	18	0.833	0.901	0.074
FLAC2–46	FJ360496	(AC) ₁₆	TGCATGGTTTTAAATACGACAA	GAACAGCAGCCTTTGTTTTTG	55	45	16	167–209	13	0.875	0.868	0.316	14	0.810	0.892	0.129
FLAC3–19	FJ360497	(AC) ₅ AC(AC) ₁₇	GAAACCCAGGGGCTCTTTTAG	TCTTAGATCCCCGAATGTG	55	48	11	221–279	8	0.792	0.813	0.579	11	0.833	0.858	0.537
FLAC4–60	FJ360498	(AC) ₂₂ TC(AC) ₅	CGCCTCTTGACACACAGAGA	AGCCCTTCTGTTTCGAGTTCA	55	48	18	179–257	13	0.833	0.859	0.877	15	0.792	0.893	0.001
FLAC4–67	FJ360499	(AC) ₂₇	ACCTGGACCAAACACACACA	CCCCACCATGTGAGAACTTA	55	48	27	175–296	19	1.000	0.914	0.613	22	0.875	0.931	0.206
FLAC4–69	FJ360500	(AC) ₅ + (AC) ₁₄	GGGAGAACCTGTCAAACCTG	AACGCAACACGCATACAAAC	55	47	23	228–300	20	0.875	0.934	0.161	19	1.000	0.919	0.995
FLAC4–7	FJ360501	(AC) ₅ AT(AC) ₃ GC(AC) ₂₃	CGATGGCCTTCTCTTTTTC	TTTTGATGCCAATTGTCATGT	55	47	12	169–197	12	0.913	0.837	0.969	10	0.792	0.806	0.470
FLAC4–81	FJ360502	(AC) ₁₆ AG(AC) ₄	CAAGGCTCCTGTGACAGCTT	CCAGTAAGAGATCAAACACG	55	48	14	174–208	12	0.708	0.867	0.020	12	0.625	0.850	0.006
FLAG1–11	FJ360503	(AG) ₁₈	GCGAGAGAGGGAGAAAGAAA	TTCTTATCTGGTTCAGTCCCTTCTT	55	48	23	235–337	18	0.833	0.914	0.021	18	0.917	0.919	0.622
FLAG1–81	FJ360504	(AG) ₂₀ GGAGGG(AG) ₈	AAAACCTGGTGGCATATGGT	GCTTTTCATTTCCAGGTCAG	55	47	20	196–236	17	1.000	0.899	0.688	14	0.833	0.892	0.068
FLAG2–76	FJ360505	(AG) ₆ CG(AG) ₂₁	ACCTTTTCGACCACCTGTCTG	TCTCATGTGCTGCTGTTTCC	55	43	25	156–226	17	0.800	0.918	0.146	21	0.870	0.928	0.034
FLAG3–55	FJ360506	(AG) ₁₆	GCCAGCTTCATGACACACAC	GCCGATGGCATGTAGAGAAT	55	48	13	228–266	8	0.833	0.686	0.791	11	0.833	0.797	0.064
FLAG3–73	FJ360507	(AG) ₁₆	TGATGGACAGCTTCAGCATC	GAACACCATCAGGTATCTTCATCA	55	48	23	257–325	16	0.917	0.903	0.115	18	0.875	0.909	0.025
FLAG4–25	FJ360508	(AC) ₁₅ A(AC) ₁₉	CGGGGTACAGTTTAACACA	TGTTTCATGTGGTTCATTTG	55	46	37	120–314	25	0.957	0.947	0.670	22	0.870	0.908	0.039
FLAG4–65	FJ360509	(AGAT) ₁₈ + (AG) ₁₁	TGTGTGTGAGTGTATGTTTACTTGG	CAGTTGCGCAAGCTAATGTC	55	48	24	232–302	19	0.833	0.926	0.024	22	0.958	0.941	0.506
FLAG4–71	FJ360510	(AG) ₁₇ AA(AG) ₅	TGAAAAGGGATAAGAGGGAGA	TTCTAGCTGGACTCAAGGGTAA	55	46	30	183–247	24	0.917	0.947	0.290	20	0.955	0.934	0.546
FLAG5–83	FJ360511	(AGAT) ₁₆	CCAGTGCAGAGGAGTTTTCAG	TTGAGTTCACTCCTGCACCA	55	41	38	200–340	28	1.000	0.957	1.000	24	0.895	0.950	0.076
FLAG5–87	FJ360512	(AG) ₉ GG(AG) ₁₆	TCTTCTCGCTGCATGAACAC	CCGTTTCCTTTGTCCAACAT	55	48	23	159–263	18	1.000	0.916	0.818	20	1.000	0.917	0.017
FLAG6–14	FJ360513	(AG) ₁₈ AT(AG) ₈	CAGTAGCAGGGTGTTTTTCCTT	CCAGTAATGACCAAAACCCAAA	55	44	31	226–364	21	0.857	0.925	0.026	21	0.870	0.936	0.009
FLAG6–77	FJ360514	(AGAT) ₃₃ (AT) ₃ (AGAT) ₉	TAAGATAGATAGCTGCATG	CATGTTTCTTTACAAATTA	55	48	29	136–274	23	0.833	0.944	0.043	22	0.958	0.940	0.726
FLAG7–17	FJ360515	(AG) ₁₈	GCAACGAGCTGCTAATTAAGG	CGACCAACACAAACCACTTG	55	48	8	170–188	6	0.917	0.759	0.251	7	0.875	0.747	0.864
FLAG8–19	FJ360516	(AG) ₁₁ GG(AG) ₁₃	ACCTCGGCCAGCACTTAATA	AAAGGGGGCAGATGATTAGG	55	48	20	165–209	16	0.875	0.901	0.555	18	0.917	0.932	0.344
FLAG8–37	FJ360517	(GT) ₁₀ G(AG) ₈ CG(AG) ₃₀	GAATCCTGTCTGCTGCTCTC	CCGTTCATCGCTCTCTGAGG	55	47	24	213–275	16	0.826	0.872	0.299	23	0.875	0.930	0.248
FLAG8–41	FJ360518	(AG) ₁₉	AAATCCAGATCAGGTCACA	GAGGCTCTGGCTGTTTGTTC	55	48	13	221–255	11	0.917	0.783	0.068	12	0.792	0.832	0.243
FLAG8–89	FJ360519	(AC) ₁₂ + (AG) ₂₃	CCCATACAGACAGCTGGTGA	TTTCCCACGATGGAGGAG	55	47	23	173–223	17	1.000	0.919	0.909	20	0.875	0.915	0.402

Table 2 Results of cross-species amplification of 28 microsatellite loci developed from European flounder on other European flatfish species ($n = 1/\text{spp.}$). Amplification conditions identical to those described for European flounder. Presence of microsatellite-like products is indicated with allele sizes, absence with a '—'. Pf, *Platichthys flesus*; Ll, *Limanda limanda*; Pp, *Pleuronectes platessa*; Hp, *Hippoglossoides platessoides*; Lw, *Lepidorhombus whiffiagonis*; Sr, *Scophthalmus rhombus*; Pm, *Psetta maxima*; Bl, *Buglossidium luteum*; Ss, *Solea solea*; Pl, *Pegusa lascaris*; Mv, *Microchirus variegatus*; Mk, *Microstomus kitt*

Locus	Pf	Ll	Pp	Hp	Lw	Sr	Pm	Bl	Ss	Pl	Mv	Mk
FLAC1-32	176/176	168/170	166/170	166/166	—	166/178	—	—	160/162	—	154/166	—
FLAC1-47	262/262	—	—	—	—	—	—	—	—	—	—	—
FLAC2-16	178/196	170/172	192/196	164/172	160/160	164/166	164/166	158/192	176/180	158/258	—	—
FLAC2-18	242/246	256/260	250/256	226/226	—	—	246/248	—	266/270	280/280	302/308	296/296
FLAC2-46	187/205	179/191	181/209	—	—	—	—	—	195/207	—	—	—
FLAC3-19	221/273	—	249/249	259/263	—	249/265	253/265	—	251/251	267/267	237/263	267/279
FLAC4-60	179/183	—	—	—	—	—	—	—	—	—	—	—
FLAC4-67	192/199	—	—	—	—	—	—	—	—	—	—	—
FLAC4-69	276/285	—	—	290/292	—	—	—	—	254/264	242/242	—	246/280
FLAC4-7	187/189	—	193/193	—	—	—	—	—	—	—	—	—
FLAC4-81	174/192	—	192/198	176/188	—	—	—	—	—	—	186/194	182/192
FLAG1-11	249/249	—	—	—	—	—	—	—	—	—	—	—
FLAG1-81	204/210	—	—	222/222	—	184/190	—	—	—	—	—	—
FLAG2-76	170/170	—	—	—	—	—	—	—	—	—	—	—
FLAG3-55	256/258	—	230/236	—	—	—	—	—	228/252	—	—	—
FLAG3-73	277/295	—	—	—	—	—	—	—	—	—	—	—
FLAG4-25	254/284	—	—	—	—	—	—	—	—	—	—	—
FLAG4-65	240/286	—	180/188	—	264/264	—	160/198	158/194	182/224	166/206	154/190	164/202
FLAG4-71	197/211	—	185/195	—	—	—	187/205	—	—	211/227	—	—
FLAG5-83	314/314	—	—	—	—	—	—	—	—	—	—	—
FLAG5-87	167/177	—	—	—	—	—	—	—	—	189/189	—	187/199
FLAG6-14	304/324	—	273/277	—	256/266	—	228/244	—	—	—	264/282	—
FLAG6-77	196/220	—	148/194	—	—	—	124/160	—	168/176	—	120/156	126/164
FLAG7-17	178/180	168/168	172/180	178/190	172/174	176/176	176/176	174/182	176/180	—	—	168/174
FLAG8-19	171/183	190/205	187/195	—	—	167/199	—	193/197	185/190	181/185	167/185	179/183
FLAG8-37	235/237	—	—	202/202	—	—	—	—	—	—	—	—
FLAG8-41	239/239	—	237/237	—	—	—	—	—	241/248	221/221	245/248	253/267
FLAG8-89	195/209	—	207/225	195/195	—	221/221	219/219	—	171/183	185/197	181/189	—

internal size standard (GeneScan[®] LIZ-600). Twenty-eight primer pairs produced polymorphic bands at the expected sizes. Allele sizes were scored with GeneMapper Software 4.0. Genotypes were analysed with Genetix (Belkhir *et al.* 1996–2004) and GenePop version 4.0 (Rousset 2008), where polymorphism varied from 8 to 38 alleles with an average of 16 alleles per locus per population (Table 1). Observed heterozygosity ranged from 0.542 to 1, and only one marker (FLAC4–60) significantly deviated from Hardy–Weinberg expectations in the Tyne sample after Bonferroni correction. FLAC2–18 was similar to *Sciaenops ocellatus* microsatellite Scoc76 (GenBank Accession no. EU727070.1). Markers FLAC1–32 and FLAG2–76 appear to be in linkage disequilibrium in both populations and overall, suggesting that both markers are gametically linked. Cross-species amplification on 11 European flatfish species was tested using the same parameters as for flounder (Table 2).

These novel nuclear markers will contribute to the library of available markers and will provide tools to enhance our understanding of the genetic structure of flounder throughout its range, and increase the power of detecting signals of

evolutionary processes imposed on populations under contaminant and other environmental stress.

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