Development of 30 microsatellite markers for dab ([u]limanda limanda L.[/u]): a key UK marine biomonitoring species

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Development of 30 microsatellite markers for dab (*Limanda limanda* L.): a key UK marine biomonitoring species

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Abstract

Dab (*Limanda limanda*) are the principal target fish species in offshore biomonitoring programmes in the UK; however, detailed knowledge of genetic structure and connectivity among sampling locations is unavailable. Here, the isolation and characterization of 30 polymorphic microsatellite loci for dab is described. The number of alleles per locus ranged from 2 to 42, with observed heterozygosities ranging from 0.089 to 1. These loci will enable high resolution of genetic population structure and dynamics of dab around the British Isles.

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

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Flatfishes are ideal indicator species for assessing the biological effects of contaminants in the marine environment, and in the UK, the dab (*Limanda limanda*) is studied in annual monitoring programmes (CEFAS 2005). Although an extensive database exists on the assessment of individual consequences of pollution exposure (Lyons *et al*. 2000), information on the genetic structure of dab and population connectivity is limited, both of which are important to correctly interpret biomonitoring data. Furthermore, the potential evolutionary processes in populations displaying elevated levels of disease, or exposed to high levels of pollutants, have not been addressed. Here we describe the isolation and characterization of 30 novel polymorphic microsatellite loci for dab which can be used to analyse the genetic structure of dab populations.

A microsatellite-enriched genomic library was constructed following a subtractive hybridization protocol (T.C. Glenn, personal communication; www.uga.edu/srel/DNA_Lab/Msat_Easy_Isolation_2000.rtf). Dab for library development were collected from the Irish Sea, and fin clips stored in 100% ethanol until processed. Genomic DNA was extracted using a phenol-chloroform protocol, and 2 μg of DNA was then simultaneously digested with *Rsa*I restriction enzyme (NEB) and ligated to double-stranded SNX linkers (SNX-f: 5′CTAAGGCCTTGCTAGCAGAAGC and SNX-r: 5′pGCTTCTGCTAGCAAGGCCTTAGAAAA). Success of the ligation reaction was checked by polymerase chain reaction (PCR) using single-stranded SNX linkers as primers.

Four biotin-labelled microsatellite motif probes $(AG)_{12}$, $(AC)_{13}$, $(ACAG)_6$ and $(AGAT)_8$ were hybridized to the PCR products. Streptavidin Dynabeads (Invitrogen) were used to capture the microsatellite-containing DNA fragments which were eluted in TLE (10 mM Tris, 0.1 mM EDTA, pH 8.0), amplified by PCR, and (polyethylene glycol) PEG-precipitated. Subsequently, amplicons were A-tailed and ligated into pCR TOPO Vectors (Invitrogen) and transformed into One Shot TOP10 competent cells (Invitrogen). Recombinant colonies were identified on Luria-Bertani agar plates by ampicillin resistance and disruption of the β-galactosidase gene. Microsatellite presence was evaluated on 960 colonies by PCR amplification with M13-F primer and a mixture of non-biotinylated microsatellite probes. Two hundred fifty-one positive amplicons were sequenced by Macrogen Inc. (Korea), and the ensuing sequences edited,

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analysed and checked for duplicates with BioEdit (Hall 1999). Enrichment efficiency was high at 87.5%. Primers were designed on either side of 58 putative microsatellites using Primer 3 (Rozen & Skaletsky 2000), and tested for successful amplification at several annealing temperatures on 3% TBE agarose gels. Forward primers of pairs reliably amplifying on several individuals were then ordered M13-tailed at the 5′-end (Schuelke 2000). Nested PCRs with forward-tailed 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 $MgCl_2$, 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 the forward primer annealing temperature (see Table 1), 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 dab from two locations, North Sea (55°17′59.96″N, 2°53′45.81″E) and Irish Sea (54°30′42.48″N, 3°47′37.68″E), were genotyped on an ABI 3130xl Genetic Analyser (Applied Biosystems) with an internal size standard (GeneScan LIZ-600). Allele sizes were scored with GeneMapper Software 4.0. Thirty primers produced polymorphic bands at the expected sizes (Table 1). Genotypes were analysed with Genetix (Belkhir *et al*. 1996–2004) and GenePop version 4.0 (Raymond 2008), yielding results where polymorphism varied from 2 to 42 alleles with an average of 15 alleles per locus. Observed heterozygosity ranged from 0.083 to 1. Significant deviations from the Hardy–Weinberg expectations in the form of heterozygote deficiencies were found in DAC4–34, DAC5–70, DAG2–15, DAG2–22, and DAG4–91 in either or both populations, suggesting the presence of null alleles in these markers. Marker DAC5–21 was highly similar/homologous to the *Hippoglossus hippoglossus* microsatellite Hhi61IMB (GenBank Accession no. EF569094), and parts of the sequence of DAC2–15 were highly similar to the potassium chloride transporter gene (BC136157.1; BLASTN value = $2e^{-11}$). Although significant linkage disequilibrium was found in one of two populations between several loci, no pair of loci was significantly linked for both populations, suggesting that linkage is likely to be an artefact of small sample sizes. Cross-species amplification on 11 European flatfish species was tested using the same parameters as for dab (Table 2). These markers will prove invaluable for the description of genetic population structure, connectivity and demographics of dab around the British Isles. Furthermore, they will enable more accurate interpretation of biomonitoring data, and provide a neutral genetic background with which adaptive genetic markers can be compared.

Table 1 Characterization of 30 microsatellite loci isolated from *Limanda limanda* in two populations. Motif, repeat sequence of the isolated clone; *Ta*, 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

Locus name	GenBank Accession no.	Motif	Primer sequence (5′–3′) F, forward; R, reverse	Overall				North Sea				Irish Sea			
				T_a (°C)	*N*	N_a	Range (bp)	N_a	H_O	H_E	*P*	N_a	H_O	H_E	*P*
DAC1–35	EU982372	$(AC)_{40}$	F: GAAGTCTCCAGGAACGACTACA R: TCAAGAACACAGACGTCAGGA	60	47	26	302–372	22	0.913	0.932	0.051	22	0.875	0.933	0.065
DAC1–55	EU982373	$(AC)_{20}$	F: AAAGTGGGGATTGAGGAAGG R: ACACCACACACCCACACAAT	60	48	10	242–268	9	0.792	0.745	0.558	8	0.667	0.802	0.070
DAC1–6	EU982374	$(AC)_{38}$	F: GTCAGAACCACCCCCACA R: TGAGACAGTTTGACCCTGATTTTT	55	45	42	144–346	28	0.917	0.946	0.040	27	0.905	0.955	0.084
DAC1–90	EU982375	$(AC)_{24}$	F: TGGCTCCTATCAAATACATA R: CTCTGTTTCTTTTCAGGACTC	60	48	21	102–146	18	0.958	0.918	0.241	19	0.917	0.925	0.185
DAC2–15	EU982376	$(AC)_{24}$	F: CTCAGAGATGCCCAGAGGTC R: GACAAGAACGCACGCACAC	60	48	11	174–216	8	0.625	0.681	0.072	8	0.708	0.723	0.313
DAC2–28	EU982377	$(AC)_{10}C(AC)_5$	F: GTGTTTCCGCTTGGCTTG R: GCCTGGCAGACACCTACACT	60	48	12	110–150	10	0.875	0.807	0.715	9	0.875	0.767	0.283
DAC2–36	EU982378	$(AC)_{18}$	F: GTTTGTTGCTCAGGTGCAGA R: TGGGGAAGACACGTGTAAGA	52	47	34	215–327	21	0.913	0.791	1.000	23	0.958	0.894	0.933
DAC2–37	EU982379	$(AC)_{11}$	F: GGTATGTGCTTTGCCCTCAG R: TGTTTGGTTGTCCGTTATGG	58	48	4	240–248	3	0.292	0.254	1.000	3	0.083	0.081	1.000

Table 1 *Continued*

Locus name	GenBank Accession no.	Motif	Primer sequence (5′–3′) F, forward; R, reverse	Overall				North Sea				Irish Sea			
				T_a (°C)	N	N_a	Range (bp)	N_a	H_O	H_E	P	N_a	H_O	H_E	P
DAC2–82	EU982380	$(AC)_{50}$	F: ATGAAGCCTGTGTGCCTTTC R: TTATGACCCTGGTTCCCTCA	55	45	34	335–434	24	0.913	0.946	0.031	28	0.909	0.953	0.239
DAC3–12	EU982381	$(AC)_{15}GC(AC)_{12}$	F: CTGCTTGTTTTGGTGACACA R: TAGGCGTGTGTGCATATGTT	55	47	18	103–141	15	1.000	0.903	0.902	14	0.750	0.897	0.029
DAC3–14	EU982382	$(AC)_{12}$	F: CTGTCAACTCGACTCTGGAGGA R: GCAAGAACACACATATTCAGCTACA	60	48	8	160–174	6	0.542	0.726	0.006	6	0.500	0.697	0.043
DAC3–86	EU982383	$(AC)_{15}$	F: GACCCCTCATGTGACTCCAG R: CCTCTGAGGGCCCTTGTC	55	48	8	221–241	6	0.458	0.550	0.318	6	0.458	0.418	0.763
DAC4–20	EU982384	$(AC)_{30}$	F: GTTTCCACGCTGCCTTCTT R: TTCATCAATTTAACATAAAAAGAGAGA	55	45	30	123–181	25	0.917	0.932	0.633	22	0.955	0.944	0.698
DAC4–34	EU982385	$(AC)_{15}$	F: TCCGGAGAGGTGAGGAGTTA R: CATCGAATGAAAATGGAGGAG	55	46	31	179–241	21	0.591	0.930	< **0.001**	22	0.625	0.939	< **0.001**
DAC4–40	EU982386	$(AC)_{21}$	F: TAGATAATGGGGCCCACAGG R: TTAGCCGTTGTGGTTGACAG	60	47	20	320–365	15	0.957	0.872	0.795	17	0.875	0.881	0.260
DAC5–21	EU982387	$(AC)_{11}(AGACAC)_5$	F: AAATGTGACGTAGGTTAGGTTTCTG R: CGAAGGCAGCTTTCTTCTCT	58	48	26	96–160	23	0.875	0.944	0.014	20	0.958	0.928	0.951
DAC5–5	EU982388	$(AC)_{11}AT(AC)_6$	F: TGCTTGAAGGCATTGTTGAC R: CGTAGCTGCCTCTGAGTATTTG	60	48	5	117–127	5	0.250	0.264	0.295	3	0.208	0.223	0.113
DAC5–70	EU982389	$(AC)_{13}$	F: CAGACATGTTTGTGTTTTCTCTCTG R: AGGCACGAAAGCATGAATGA	58	48	28	112–188	14	0.750	0.875	< **0.001**	24	0.750	0.942	0.004
DAC5–77	EU982390	$(AC)_{10}$	F: TCAATGGGGCAAAAGACAAT R: CTTTCAATCGTGCATTCTTCA	60	48	8	104–122	6	0.333	0.330	0.419	7	0.500	0.495	0.030
DAC5–78	EU982391	$(AC)_{18}$	F: AGGAATGAATCGTCCTGTGG R: CAAACCACCAGGGGAATAAA	55	47	36	100–186	23	0.870	0.930	0.053	27	0.875	0.952	0.011
DAG1–14	EU982392	$(AC)_{13}$	F: AAGGGATGATTGCACACACA R: TGCAAAGGTTTGTTGAAGAACT	52	48	8	175–193	6	0.71	0.62	0.95	7	0.38	0.45	0.030
DAG2–15	EU982393	$(AG)_{16}$	F: GACATGGCATCAGCTCTTGA R: TCCCACAAGTAAAAGAAATTCCA	52	46	13	145–169	10	0.61	0.88	< **0.001**	12	0.57	0.88	< **0.001**
DAG2–22	EU982394	$(AG)_{17}$	F: CGTTTACATGTGGTATCTGTCTG R: AGATGGACAGATAGATGGATTGA	55	48	20	122–166	15	0.667	0.892	< **0.001**	14	0.458	0.883	< **0.001**
DAG2–90	EU982395	$(AG)_{11}AT(AG)_5$	F: AGGCAAGGATTTGGAAGGTT R: TCACCCCTTAATCTGGAATTG	60	48	14	158–186	14	0.875	0.897	0.724	12	0.958	0.880	0.824
DAG4–64	EU982396	$(AG)_5GGG(AG)_{16}$	F: TGCACGTTGTGTGTCTCTCTC R: GGGAAAAAGGAGGGGAAATA	60	48	23	143–191	20	1.000	0.933	0.741	18	0.958	0.919	0.539
DAG4–91	EU982397	$(AG)_{24}CG(AG)_{10}$	F: CTGCCGATGAAGGAGTTTTC R: TGTGTGGTAGCAGACAGTGGA	60	45	30	209–345	23	0.583	0.920	< **0.001**	19	0.524	0.931	< **0.001**
DAG5–12	EU982398	$(ATCT)_{23}$	F: CCCCAATTCATTATCTATGAACG R: CCGGCAATCCAGGTTACTTA	55	47	21	132–256	18	0.958	0.887	0.229	13	0.957	0.843	0.780
DAG5–17	EU982399	$(AG)_{29}$	F: ACCTGTCTGCAGGAAGAGGA R: TCTGATGTGCTGCTGTTTCC	60	47	31	170–242	23	1.000	0.928	0.799	25	1.000	0.946	0.707
DAG5–45	EU982400	$(AGAT)_{39}$	F: AAATAAGACTGGAATAAATATGCAC R: AATATACCGGCTGCTATGAC	55	47	25	207–275	19	1.000	0.924	0.473	21	0.958	0.926	0.634
DAG5–88	EU982401	$(AG)_{10}AA(AG)_9$	F: TTTTCCCGAAAGTCCCTCTT R: AGCCGGGATTTCATTATTC	58	48	2	187–189	2	0.688	0.313	0.357	2	0.750	0.250	1.000

Table 2 Results of cross-species amplification of 30 microsatellite loci developed from dab on other European flatfish species (n = 1/spp.). Amplification conditions are identical to those described for dab. Presence of microsatellite-like products is indicated with allele sizes, absence with a '—'. Ll, *Limanda limanda*; Pf, *Platichthys flesus*; Pp, *Pleuronectes platessa*; Lw, *Lepidorhombus whiffiagonis*; Hp, *Hippoglossoides platessoides*; Sr, *Scophthalmus rhombus*; Pm, *Psetta maxima*; Mv, *Microchirus variegatus*; Mk, *Microstomus kitt*; Ss, *Solea solea*; Pl, *Pegusa lascaris*; Bl, *Buglossidium luteum*

Locus name	Ll	Pf	Pp	Lw	Hp	Sr	Pm	Mv	Mk	Ss	Pl	Bl
DAC1–35	342/356	—	—	—	—	—	—	—	—	—	—	—
DAC1–55	254/258	—	—	—	—	—	—	—	—	—	—	—
DAC1–6	249/249	144/170	—	144/144	196/196	158/160	162/162	152/154	—	—	—	185/189
DAC1–90	122/132	—	—	—	—	—	—	—	—	—	—	—
DAC2–15	202/204	—	—	—	—	—	—	—	—	—	—	—
DAC2–28	130/130	118/118	118/130	—	134/134	116/118	116/118	—	—	118/128	—	—
DAC2–36	231/271	—	—	—	—	—	—	—	—	—	—	
DAC2–37	244/248	240/240	240/240	—	242/242	238/238	232/232	—	—	—	—	—
DAC2–82	349/437	345/361	—	—	—	—	359/389	—	—	—	—	—
DAC3–12	117/125	103/109	107/125	101/109	113/113	99/99	89/125	85/93	—	93/101	—	—
DAC3–14	164/172	—	—	—	—	—	—	—	—	—	—	—
DAC3–86	223/229	—	—	—	—	—	—	—	—	—	—	—
DAC4–20	136/160	—	—	—	160/166	—	—	—	—	—	—	—
DAC4–34	235/235	—	—	—	239/239	—	—	—	—	203/203	—	—
DAC4–40	334/351	—	324/324	—	—	—	—	—	—	—	—	—
DAC5–21	109/119	—	—	—	126/126	—	134/134	—	—	—	—	—
DAC5–5	121/121	119/121	115/121	—	111/121	121/121	121/121	—	—	109/115	121/121	121/121
DAC5–70	146/154	—	—	—	—	—	—	—	—	—	—	—
DAC5–77	108/112	—	106/106	102/138	102/138	102/138	102/138	—	—	102/106	102/106	102/138
DAC5–78	131/153	—	161/161	—	—	101/103	114/124	—	—	133/139	—	—
DAG1–14	183/183	—	—	—	181/187	—	181/181	—	178/181	181/209	—	—
DAG2–15	155/155	—	—	—	—	—	—	—	—	—	—	—
DAG2–22	138/138	108/156	—	124/138	138/138	—	156/156	—	—	136/264	—	122/134
DAG2–90	162/176	178/186	—	—	170/182	156/176	152/156	—	—	—	—	—
DAG4–64	147/159	—	—	—	—	—	—	—	—	—	—	—
DAG4–91	260/260	—	—	—	—	—	—	—	—	—	—	—
DAG5–12	223/267	241/341	—	—	137/145	—	203/211	—	—	—	—	
DAG5–17	200/208	—	—	—	168/182	—	174/212	—	—	—	—	—
DAG5–45	233/271	—	—	—	—	—	—	226/226	—	—	192/192	—
DAG5–88	187/189	—	—	—	—	—	—	—	—	—	—	—

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