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PERMANENT GENETIC RESOURCES

Isolation and characterization of 12 dinucleotide microsatellites in the European eel, *Anguilla anguilla* L., and tests of amplification in other species of eels

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Abstract

Twelve polymorphic dinucelotide microsatellites in the freshwater eel *Anguilla anguilla* L. were isolated and characterized. Genetic diversity was assessed in eels from Lake Constance, Germany. Allele numbers ranged from five to 26 per locus with observed heterozygosities between 0.125 and 0.875. A portion of locus AangCT77 aligns with a transcribed region of the zebrafish gene crystallin beta B2. Cross-species amplification of most markers was possible for nine other *Anguilla* eel species. The newly developed primer pairs will facilitate population and conservation genetic studies in order to refine the understanding of the subtle population genetic structure typical of eels, and to identify interspecies admixture due to global trade.

Keywords: *Anguilla anguilla*, catadromous fish, conservation biology, cross-species amplification, microsatellite, population genetics

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The European eel, *Anguilla anguilla* L., has been studied genetically for more than 20 years (e.g. Williams *et al*. 1984; Avise *et al*. 1986, 1990; Wirth & Bernatchez 2001, 2003). Using small sets of microsatellite markers (Daemen *et al*. 1997; Wirth & Bernatchez 2001), there is evidence for subtle population genetic structure in the European eel either following a statistically significant pattern of isolation by distance (Wirth & Bernatchez 2001) or isolation by time (Maes *et al*. 2006). Moreover, in a recent effort, 12 microsatellite loci have been isolated and described for the Japanese freshwater eel (*Anguilla japonica*, Ishikawa *et al*. 2001). Our objective was to increase the current set of oligonucleotide markers available for anguillid eels. A larger set of markers should facilitate examination of the subtle population genetic structure typical of eels, as well as identification of interspecies admixture due to global trade.

Short tandem repeats (STR) were identified from partial genomic libraries enriched for CA or CT repeats and isolated with magnetic beads following the protocol of Tenzer *et al*. (1999), including modifications by Garner *et al*. (2000). Total gDNA was extracted from eel blood following standard protocols of proteinase K digestion (Sambrook *et al*. 1989). Enriched DNA was ligated into the pCRII-TOPO cloning vector and transformed into chemically competent *Escherichia coli* TOP10 cells supplied with the TOPO TA Cloning kit (Invitrogen), following the manufacturer's protocol. After plating, the cells were grown overnight on 1× Luria-Bertani agar, containing 50 μg/mL of ampicillin and 80 μg/mL of X-Gal. Single colonies were picked and re-grown for 14 h in a 96-well plate format in 150 μL liquid 1× Luria-Bertani medium, containing 50 μg/mL of ampicillin. Bacterial cells were disrupted using a 5-min heat shock at 94 °C and lysates were used directly as polymerase chain reaction (PCR) templates. Inserts were identified and screened for short tandem repeats using forward and reverse primers targeting the SP6 and T7 sites in the vector used, respectively. Sequencing reactions were performed with PerkinElmer's recommended protocol for BigDye version 3.1 sequencing chemistry on a 3100 Genetic Analyser (ABI-Hitachi).

Screening of inserts revealed a total of 26 positive clones, two of which contained more than one repetitive region.

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Table 1 Characterization of polymorphic microsatellite loci from European freshwater eel, *Anguilla anguilla* L., tested for an eel stock from Lake Constance, Germany (n = 24)

Locus	Core motif (N_x)	GenBank Accession no.	Primer sequences (5′–3′) (including label descriptors)	T_m/T_a (°C)	A	Size range (base pair)	Null genotypes (percentage)	H_E	H_O	F_{IS}	P	MICRO-CHECKER performance
AangCT53	$(CT)_{17}$	EU310488	H-AGGTGACACACAGTCTCTTTGG ACAATGCATGTGCCTGAATG	59/55 60/55	8	74–94	2.5	0.72	0.63	0.15	0.100	+
AangCT59	$(CT)_{18}$	EU310487	H-GCAACCCTTTCTCACTCCAC CTCACTGCGCAAACAAGAAG	60/55 60/55	12	70–91	4.0	0.81	0.74	0.11	0.477	+
AangCT67	$(TG)_6N_8(TG)_5TA$ $(TG)_4(AG)_2(TG)_7$	EU310489	H-GACAGACGGACAGACAATGC GGTGGTGAATTTTGGTCCTG	59/55 60/55	19	124–236	4.0	0.91	0.65	0.31	0.001	null
AangCT68	$(AG)_{22}$	EU310490	F-CCAGGCAATTGCTTTCTCAC TCATTGTGTTTGGCACTTCC	61/55 60/55	11	169–195	0	0.86	0.83	0.06	0.873	+
AangCT76	$(TC)_{17}(AC)_{13}$	EU310480	F-CTTCAGCTTGGAGGTGTTCC CTGTGCAGGAGTCACGTTTC	60/55 59/55	10	196–232	4.0	0.80	0.74	0.09	0.389	+
AangCT77	$(CT)_{46}GT(CT)_3$	EU310481	F-CCTGATGTTTTCAGCGTTTG GAAAGTGGGCTCAGTTCTGG	60/55 59/55	10	101–117	0	0.70	0.50	0.31	0.000	null
AangCT82	$(GA)_{17}$	EU310479	N-CCACTCTAGCGACACAACACTC GCATTTTAACCTTGTCCCTGTC	60/55 60/55	12	188–214	0	0.87	0.83	0.06	0.312	+
AangCT87	$(GA)_{11}GG(GA)_2$ $GG(GA)_7$	EU310483	N-CGATGAAGCCGAAAATTAGC TGGCTTTAAAGTGGCGATG	60/55 60/55	12	104–166	0	0.83	0.71	0.17	0.003*	+
AangCT89	$(CT)_{15}(TC)_3(CT)_4$	EU310484	H-AACCAGCGAGATGATGATTG AGAGCGTGAAGCCTTTTGAC	59/55 60/55	11	198–220	16.6	0.86	0.85	0.04	0.326	+
AangCA55	$(TG)_5AG(TG)_3$ $TA\ (TG)_5$	EU310486	N-TCTGTACGGCGCTTCAGAC CAGGTGCTTTAGTCCAGTTACATC	60/55 59/55	5	114–132	0	0.16	0.13	0.23	0.126	+
AangCA58	$(CA)_8TA(CA)_8N_{32}$ $(CA)_9$	EU310485	N-CAGTCAGACGTCAGCCACTG GAGGTCTCTCTCACTGCGAAC	61/55 59/55	26	164–286	0	0.95	0.88	0.10	0.127	+
AangCA80	$(TG)_4CG(TG)_{14}$	EU310482	F-TTCCTCTGGTCTTTCACACG AGCTGGAGGACACGGATG	59/55 60/55	13	74–110	0	0.89	0.79	0.13	0.193	+

F-, 6-FAM, H-, HEX, N-, NED; A, number of alleles; T_m, melting temperature; T_a, amplification temperature; H_E, expected heterozygosity; H_O, observed heterozygosity; F_{IS}, inbreeding coefficient; P, value of probability for Hardy–Weinberg equilibrium exact test (α = 0.05); +, flawless allele scoring; null, null alleles expected; N_x, deviating dinucleotide repeat within the core motif of length x; underlined P values indicate significant violation from Hardy–Weinberg expectations (α = 0.05); *violation from HWE in the absence of allele-bound typing defects.

However, only one STR per positive clone was considered for specific primer pair design using the PRIMER 3 software (Rozen & Skaletsky 2000). Thirteen loci could be successfully amplified using the same PCR protocol for all loci on a GeneAmp PCR System 9700 (PerkinElmer-ABI): a 12.5-μL total reaction contained 1× of Genaxxon's Reaction Buffer S (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100); 200 nM of each dNTP; 0.8 U of Red*Taq* (Genaxxon); 200 nM of each primer and 20 ng of gDNA. An initial 5-min hot start at 94 °C was followed by 35 cycles of denaturation for 35 s at 94 °C, annealing for 35 s at 55 °C and elongation for 45 s at 72 °C. PCR amplification was terminated with a post-elongation step of 10 min at 72 °C. Forward primers with an attached fluorescent label at the 5′-end permitted multiplexing of differently coloured amplicons. Alleles were run against the internal size standard Genescan-500 ROX (ABI), analysed with GENESCAN and scored in GENOTYPER (ABI software version 3.7 NT). One marker (AangCA75, not listed) could not be scored consistently due to elongated stutter peaks and was removed from the submitted set of markers (Table 1).

Single loci were tested for Hardy–Weinberg equilibrium and pairs of loci for linkage disequilibrium in a sample (n = 24) of an eel stock from Lake Constance, Germany, using GENEPOP version 3.4 (Raymond & Rousset 1995) with 10 000 dememorization steps, 10 000 batches and 1000 iterations. Sequential Bonferroni tests (Dunn–Sidák method) were conducted to correct for errors in multiple comparisons among means (Sokal & Rohlf 1995). None of the loci showed significant linkage disequilibrium after Bonferroni correction. Three of the 12 loci violated Hardy–Weinberg expectations (Table 1). According to the MICRO-CHECKER program (van Oosterhout *et al*. 2004), two of those markers, AangCT67 and AangCT77, are expected to bear null alleles with a frequency of 0.144 and 0.134, respectively, when compared to 10 000 randomly generated genotypes. Blasting locus AangCT77 revealed a 132-bp long partial overlap (*e*-value = 7.0e^{-42}) with the reversed sequence of crystallin beta B2 transcripts from several teleost fish species (*Danio rerio* and *Tetraodon* spp.). Thus, because of its tight linkage to a gene, the influence of selection is one probable explanation for the violation of Hardy–Weinberg expectations at this locus (Table 1). However, this marker may be useful in future studies on expressed sequence tags (EST)-linked microsatellites in the eel.

Cross-species amplification was tested with nine different anguillid eel species (Table 2). Species status was verified by sequencing a portion of the 16S rRNA gene (Aoyama

Table 2 Cross-species amplification with microsatellite primers developed for *Anguilla anguilla* using PCR conditions optimized for this species. Alleles of each genotype are separated by commas

Taxon (individual no.)	AangCT53	AangCT59	AangCT67	AangCT68	AangCT76	AangCT77	AangCT82	AangCT87	AangCT89	AangCA55	AangCA58	AangCA80
A. australis (1)	72, 192	70, 74	97, 97	169, 171	X	127, 145	192, 192	X	208, 212	X	X	78, 84
A. australis (2)	72, 190	66, 74	97, 97	173, 191	X	109, 111	190, 192	X	210, 212	X	261, 261	78, 78
A. australis (3)	74, 78	68, 68	97, 97	179, 191	X	109, 111	192, 192	X	206, 214	X	164, 164	78, 80
A. bicolor bicolor (1)	72, 72	72, 72	97, 97	157, 169	202, 202	113, 121	196, 200	100, 160	204, 204	X	192, 232	74, 80
A. bicolor bicolor (2)	72, 72	72, 72	97, 97	161, 169	202, 202	113, 119	198, 200	138, 138	204, 204	X	202, 230	72, 80
A. dieffenbachi (1)	74, 74	X	X	175, 175	200, 208	143, 145	194, 196	94, 140	208, 214	X	X	76, 82
A. dieffenbachi (2)	74, 74	X	X	175, 177	206, 241	141, 143	196, 196	94, 94	208, 208	X	X	82, 82
A. japonica (1)	72, 90	176, 176	97, 97	175, 177	206, 212	129, 145	226, 232	122, 164	204, 206	124, 124	218, 218	74, 80
A. japonica (2)	70, 72	172, 176	227, 227	171, 175	204, 204	101, 121	206, 208	154, 154	208, 208	124, 124	236, 236	80, 80
A. mossambica	72, 72	168, 176	X	167, 175	212, 220	101, 101	190, 206	90, 100	204, 206	X	X	82, 82
A. malgumora	74, 74	168, 172	97, 97	169, 173	214, 214	101, 101	208, 214	132, 132	204, 214	X	178, 178	72, 78
A. marmorata	72, 72	172, 172	97, 97	173, 173	216, 218	131, 139	210, 214	124, 130	204, 204	X	182, 198	80, 80
A. reinhardtii (1)	74, 80	X	X	163, 195	214, 230	91, 101	202, 206	92, 136	210, 214	X	202, 202	80, 88
A. reinhardtii (2)	78, 112	64, 64	182, 186	175, 181	218, 222	105, 115	196, 198	128, 142	204, 212	124, 124	180, 180	82, 84
A. rostrata (1)	78, 78	64, 64	180, 190	167, 171	208, 218	101, 123	196, 200	122, 138	202, 206	124, 124	174, 202	78, 92
A. rostrata (2)	78, 78	80, 84	174, 190	173, 185	200, 206	109, 165	196, 198	114, 162	204, 212	124, 124	196, 214	96, 96

X, no amplification.

et al. 2001). Six of the 12 loci were successfully amplified for all specimens. Five of the remaining markers failed for one or the other eel species, mainly including native species of New Zealand and Australia. Only one locus, AangCA55, failed for the majority of eel specimens, and appeared monomorphic in species other than *A. anguilla*. These results suggest that the novel microsatellite markers can be useful for population and conservation genetics studies in other anguillid species as well.

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