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MICROSATELLITE LETTERS

Characterization of thirteen new microsatellite markers for allis shad (*Alosa alosa*) and twaite shad (*Alosa fallax*)

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Abstract Allis shad *Alosa alosa* and twaite shad *Alosa fallax* are two related anadromous European clupeid species which have undergone a sharp decline in the past decades. We describe the development of 13 microsatellite markers for both species, based on 454 pyrosequencing technology. The number of alleles per locus ranged from 2 to 11 in *A. alosa* and from 4 to 13 in *A. fallax*. Gene diversity ranged from 0.364 to 0.800 for *A. alosa* and 0.226 to 0.849 for *A. fallax*. These markers will help in conservation genetics studies such as assessing the extent of population decline, defining appropriate conservation units and monitoring reintroduction programs.

Keywords *Alosa alosa* · *Alosa fallax* · Microsatellites · Genetic structure · Conservation genetics

Allis shad (*Alosa alosa*) and twaite shad (*Alosa fallax*) are two closely related European anadromous clupeids which can hybridize with varying levels of introgression (Aprahamian et al. 2002). Their historic geographic range overlapped on the Atlantic coast from Morocco to the Baltic Sea and Scandinavia, as far north as Iceland for *A. fallax,* which is found also in the Mediterranean area (Aprahamian et al. 2002). However, both species have undergone steep decline for several decades due to habitat degradation, dam construction, poor water quality, loss of spawning grounds and overharvesting. Consequently, their distribution area is now restricted to a few large rivers in France, Portugal and UK. They are both listed as priority species in Annex II and V of the Habitats Directive and listed as vulnerable in France in the IUCN Red List. In the Rhine River, allis shad, a major fishery resource in the early 1900s, is now considered extinct, and a large-scale reintroduction program is underway (Beeck et al. 2008). Microsatellites markers will help monitor the rate of population decline in the light of current gene flow, assess the extent of hybridization and its adaptive consequences, define appropriate conservation units and monitor the success of reintroduction attempts (parentage assignment). Only eight markers specific to *A. alosa* or *A. fallax* have been published (Faria et al. 2004). Here we describe the development of 13 new microsatellites markers for conservation genetics studies in both species.

Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) from finclips of three *A. alosa* and three *A. fallax* sampled in France, and pooled to make a single DNA library. This library was processed by the Genoscreen company (Lille, France) using a procedure combining DNA enrichment and high throughput pyrosequencing (GS-FLX, Roche Diagnostics; Malausa et al. 2011). Microsatellite sequences were selected and primer pairs were designed using the QDD program. A total of 710 microsatellites were identified.

PCR conditions were optimized for thirteen markers, selected based on successful amplification in both species, and their polymorphism was assessed on 30 *A. alosa* from the Vilaine River (France) and 37 *A. fallax* from the Rhone River (France) (Table 1). Genomic DNA was extracted

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Table 1 Characteristics of thirteen microsatellite markers developed for *Alosa alosa* and *Alosa fallax*

Locus/ GenBank accession	Primer sequence (5′-3′)	Repeat motif	Fluorescent label/multiplex panel	Allele size range (bp)	*A. alosa* ($n = 30$)			*A. fallax* ($n = 35$)		
					Na	*Ho*	*He*	*Na*	*Ho*	*He*
Alo1/ KM268818	F: M13-CCTTGAGTTTTACCATGAGCC R: TTCTCTGGAGTGGAGAGGGA	$(CA)_{17}$	NED/3	115–151	11	0.767	0.751	10	0.595	0.722
Alo6/ KM268819	F: M13-CCAAACACTCACTCTTCTGGG R: TCAGAACTGTAGGACTGGGGA	$(AC)_{17}$	PET/4	124–154	5	0.767	0.650	9	0.649	0.74
Alo7/ KM268820	F: M13-ACATGAAATTCCAAGCCTGC R: TCCTATGATGCGTTGGCATA	$(AC)_{15}$	VIC/2	123–160	5	0.333	0.425	6	0.432	0.419
Alo9/ KM268821	F: M13-ACCCACTGTGAGGTGACACA R: GTCATGTCGTCTGCATGGAT	$(AAC)_{20}$	NED/1	111–153	7	0.821	0.800	13	0.743	0.849
Alo15/ KM268822	F: M13-CATGGACTTTTGGAAATGGG R: GCATGCCTAGGCTACTCTTTG	$(AC)_{17}$	VIC/3	135-165	6	0.759	0.672	6	0.571	0.616
Alo16/ KM268823	F: M13-CAGCCACCTTGGTCACAGTA R: TTTTGTCATTTTCTATGCACTGG	$(CA)_{13}$	6FAM/2	150–158	2	0.2	0.364	4	0.243	0.226
Alo17/ KM268824	F: M13-TCTCCAGGAAATGGGAACAG R: GCTTGTGCTGCTGTGCTTTA	$(CA)_{14}$	6FAM/1	143–168	3	0.267	0.377	6	0.382	0.385
Alo26/ KM268826	F: M13-CTGGCTTTGGCAACACTGTA R: GTGTCCATCTATGTGCACCG	$(AG)_{17}$	PET/1	181–196	6	0.621	0.646	7	0.611	0.655
Alo29/ KM268827	F: M13-ATTTGTTGTCCTTGCCAACC R: TGCTAGGACAGTGCCAGTGA	$(AC)_{13}$	NED/1	198–218	5	0.517	0.523	6	0.500	0.601
Alo32/ KM268828	F: M13-TGTTACAGAGCTGGGATTGCT R: TGTCGTCTATCAAAAGGCCA	$(TGAT)_{12}$	VIC/4	209–237	4	0.633	0.607	4	0.595	0.545
Alo33/ KM268829	F: M13-AGATTCAGTGCACAGCAAGG R: AAAGGGGTAGGTAGGTTTTGG	$(ATCT)_{16}$	NED/2	194–230	7	0.923	0.792	7	0.611	0.745
Alo43/ KM268830	F: M13-ATGGTCTTGAGACATTGCCC R: TCATGGGCCTACTGTAAGGT	$(GATA)_{12}$	VIC/2	254–290	7	0.724	0.709	8	0.838	0.794
Alo45/ KM268831	F: M13-CCTGGTTTTGTGGCTGAGAT R: GTACCCATGAGGGTCAGAGC	$(AC)_{14}$	NED/3	282–302	7	0.724	0.725	5	0.444	0.467

M13 sequence: 5′-CACGACGTTGTAAAACGAC-3′

The annealing temperature is the same for all loci (see the main text)

n, number of sample; *Na*, number of alleles; *Ho*, observed heterozygosity; *He*, expected heterozygosity

from fin clips with a one-tube procedure using Chelex® (Sigma-Aldrich). Marker amplification followed the M13 method developed by Schuelke (2000). PCR was performed in a 6 µl total volume containing approximately 15 ng DNA, 0.06 U *Taq* DNA polymerase, 5X PCR buffer (*GoTaq*, Promega), 208 µM each dNTP, 2.17 mM MgCl2, 0.055 µM forward primer with a tail extented M13, 0.55 µM reverse primer and 0.57 µM M13 primer labeled with a fluorochrome (6-FAM, VIC, NED or PET). A Touchdown PCR program was applied: initial denaturation of 4 min at 94 °C, then 20 cycles including 30 s denaturation at 94 °C, 30 s annealing starting at 65 °C then decreasing of 0.5 °C per cycle and 1 min extension at 72 °C; then, 10 cycles with 30 s denaturation at 94 °C, 30 s annealing at 55 °C and 1 min extension at 72 °C; and a final extension step of 5 min at 72 °C. PCR products from different markers were pooled into four multiplex panels (Table 1). Fragments were size fractionated on an ABI Prism 3130xl Genetic Analyzer using GeneScan 500 LIZ as size standard. Allele sizes were determined with GeneMapper 3.7 (Applied Biosystems).

For each locus, the number of alleles (*Na*) and observed and expected heterozygosities (*Ho* and *He*) were calculated using Genetix 4.05.2. All loci were polymorphic (Table 1) and the number of alleles per locus ranged from 2 to 11 for *A. alosa* (mean 5.7) and 4 to 13 for *A. fallax* (mean 7). *He* ranged from 0.364 to 0.800 for *A. alosa* and 0.226 to 0.849 for *A. fallax*. No null alleles or genotyping errors were found using Micro-checker 2.2.3. Deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium between loci were tested using Genepop 4.1. No linkage disequilibrium was found and no deviation from HWE was detected after sequential Bonferroni correction. These loci can be amplified in both species and will be useful for conservation genetics projects.

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