Exploitation of a turbot (*Scophthalmus maximus* L.) immune-related expressed sequence tag (EST) database for microsatellite screening and validation

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Abstract

In this study, we identified and characterized 160 microsatellite loci from an expressed sequence tag (EST) database generated from immune-related organs of turbot (*Scophthalmus maximus*). A final set of 83 new polymorphic microsatellites were validated after the analysis of 40 individuals of Atlantic origin including both wild and farmed individuals. The allele number and the expected heterozygosity ranged from 2 to 18 and from 0.021 to 0.951, respectively. Evidences of null alleles at moderate–high frequencies were detected at six loci using population data. None of the analysed loci showed deviations from Mendelian segregation after the analysis of five full-sib families including approximately 92 individuals/family. The markers are used to consolidate the turbot genetic map, and because they are mostly EST-derived, they will be very useful for comparative genomic studies within flatfishes and with model fish species. Using an *in silico* approach, we detected significant homologies of microsatellite sequences with the EST databases of the flatfish species with highest genomic resources (Senegalese sole, Atlantic halibut, bastard halibut) in 31% of these turbot markers. The conservation of these microsatellites within Pleuronectiformes will pave the way for anchoring genetic maps of different species and identifying genomic regions related to productive traits.

Keywords: cross-species analysis, EST database, microsatellites, Pleuronectiformes, Scophthalmus maximus, turbot

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Introduction

Expressed sequence tags (ESTs) resources have increased during the last decade in aquaculture species including fish (Pardo *et al.* 2008; Sha *et al.* 2010; Bowman *et al.* 2011), shellfish (Hedgecock *et al.* 2005; Zhang & Guo 2010) and crustaceans (Du *et al.* 2010; Gorbach *et al.* 2010; Leu *et al.* 2011). These resources are essential to develop molecular markers [microsatellites and single nucleotide polymorphism (SNPs)] useful for constructing genetic maps and QTL identification and for population screening and parentage analysis (Canario *et al.* 2008).

The turbot (*Scophthalmus maximus*; Scophthalmidae; Pleuronectiformes) is a flatfish species for which human consumption has sharply risen in the last decade. In fact, a burgeoning industry is being developed mainly in Europe and lately in China, currently making turbot a promising aquaculture species. Genetic studies are being

Correspondence: Paulino Martínez, Fax: +34 982822428; E-mail: paulino.martinez@usc.es conducted to improve turbot production by optimizing broodstock organization and through genetic breeding programmes. The development of highly polymorphic, codominant and easily assayed molecular markers, such as microsatellites, is necessary to support breeding programmes.

Microsatellite loci have been characterized in turbot to evaluate the genetic resources (Coughlan *et al.* 1996; Estoup *et al.* 1998; Iyengar *et al.* 2000; Bouza *et al.* 2002) and to construct genetic linkage maps (Bouza *et al.* 2007, 2008; Pardo *et al.* 2007; Martínez *et al.* 2008; Ruan *et al.* 2010). Drawing medium- to high-density maps is a requirement for the screening of QTL related to productive traits and for their subsequent application in markerassisted selection (MAS) programmes. However, most microsatellites characterized to date have been isolated from genomic DNA libraries, and thus, anonymous microsatellites (type II markers) have been mainly used for genetic map construction in turbot (Bouza *et al.* 2007; Martínez *et al.* 2008; Ruan *et al.* 2010). As these markers are mostly associated with nonannotated sequences, they are less conserved than EST linked markers, and so they show lower cross-species amplification with related organisms hampering comparative genomic analyses. Accordingly, microsatellites linked to genes (type I markers) are useful to identify the syntenies by comparative mapping with model species or with other flatfish species where cross-amplification has proven to be feasible (Cerdá et al. 2010). A few EST-derived microsatellites have been described in turbot (Chen et al. 2007; Bouza et al. 2008), and to date, only 31 of them have been mapped in this species (Bouza et al. 2008). It is therefore necessary to characterize new EST-derived markers in order to consolidate the turbot map and to facilitate comparative genomic studies. Additionally, enlarging EST-linked microsatellite resources will be useful to search for adaptive variation in turbot populations (Vilas et al. 2010).

As in other economically important flatfish species-Senegalese sole (Solea senegalensis; Cerdà et al. 2008), bastard halibut (Paralichthys olivaceus; Aoki et al. 1999; Arma et al. 2005), winter flounder (Pseudopleuronectes americanus, Douglas et al. 1999), European flounder (Platichthys flesus; Williams et al. 2006) and Atlantic halibut (Hippoglossus hippoglossus; Douglas et al. 2007)-an EST database was developed in turbot from cDNA libraries of immune-related organs (Pardo et al. 2008). This database, which contains 6170 unique sequences, was updated with new ESTs from a nodavirus-infected head kidney library (Park et al. 2009), and from cDNA libraries of new immune organs (pyloric caeca and thymus) and pathogens (Enteromyxum scophthalmi) (Vera et al. 2011). Additionally, 4339 sequences from previously exploited microsatellite-enriched genomic libraries were included in the turbot database (Pardo et al. 2006).

In this work, we developed and technically validated a large set of mostly EST-associated microsatellite markers using the updated turbot EST database. The aim of our study was to increase EST-linked microsatellite resources for (i) consolidating the turbot genetic map; (ii) enhancing comparative genomic strategies; and (iii) searching for adaptive variation in natural populations. Additionally, these microsatellite-containing sequences were used to screen EST databases of other flatfish (Senegalese sole, Atlantic halibut and bastard halibut) in order to carry out a preliminary evaluation on their conservation and utility for comparative genomics within Pleuronectiformes.

Materials and methods

EST database and microsatellite screening

Microsatellite-bearing sequences were selected from the updated turbot database (Pardo *et al.* 2006; Vera *et al.* 2011). Mining of microsatellites was carried out using the SPUTNIK program (http://espressosoftware.com/ sputnik/index.html) while looking for dinucleotide motifs with more than five repeats, trinucleotide motifs with more than three repeats and tetranucleotide motifs with more than two repeats. Sequences from SPUTNIK output were selected according to the following criteria: (i) high-quality and enough flanking region for primer design; (ii) annotated ESTs preferentially; and (iii) appropriate technical parameters (product size between 100 and 300 pb; primer Tm 54–65 °C; primer %GC up to 50; Max self-complementarity = 5.00; Max 3' self-complementarity = 3.00; Max Poly-X = 5) for primer pair design using the PRIMER3 program (Rozen & Skaletsky 2000).

Microsatellite genotyping

Standard phenol-chloroform protocols (Sambrook et al. 1989) were used to extract the DNA from the caudal fin in a sample of turbot from natural and cultured populations. Each selected microsatellite was amplified at a range of annealing temperatures and MgCl₂ concentrations in four individuals and checked in 2% agarose gels. The microsatellites that showed appropriate amplification (discrete bands of expected size) were genotyped on an ABI 3100 DNA sequencer using the forward primer labelled for fluorescent detection. Each amplification reaction was carried out in a 15-µL reaction mixture containing 30 ng of DNA sample; 1.5-2 mM of MgCl₂; 10 mM Tris, pH = 8.3; 5 mм NH₄Cl; 50 mм KCl; 0.2 mм of each deoxyribonucleotide triphosphate; 5 pmol of both forward and reverse PCR primers; and 0.5 U Taq DNA polymerase for a initial denaturation at 94 °C for 10 min, 35 cycles of denaturation at 94 °C for 1 min, variable annealing temperature for 45 s, and extension at 72 °C for 45 s (for full details, see Table 1). A final extension step was performed at 72 °C for 10 min. Multiplex PCR from two to four microsatellites was carried out when temperature and MgCl₂ concentrations were similar and amplification size and/or label colour were compatible. Results were analysed using GeneMapper 3.7 software (Applied Biosystems).

Gene diversity and population analysis

Genetic diversity was evaluated on 40 turbot individuals, all of them of Atlantic origin: 22 coming from a natural population of NW Spain and 18 parents or grandparents from seven unrelated families (F1–F7) currently used for genetic mapping and QTL identification (Bouza *et al.* 2008; Martínez *et al.* 2009; Rodríguez-Ramilo *et al.* 2011; Sánchez-Molano *et al.* 2011). These families came from the 2nd generation of the genetic breeding programme of Stolt Sea Farm SA (SSF), and pedigree information was

 Table 1
 Summary of the 83 polymorphic microsatellite loci validated from the turbot (Scophthalmus maximus) expressed sequence tag database

	Locus	Primers 5'-2'	Annealing temp [MgCl2]	No. of	Repeated	Size	Expected	Hardy– Weinberg
Small F. CACATCGATCGACCATCAG 60°C 8 (CT)9(GA)16 242-274 0.562 0.190 Small F. ATTGCTCAGCGATCTTTGCAC 15 m 0°C 2 (AC)9 254-256 0.344 1.000 Small F. CTGCATCATCGCCAGAGCA 15 m 0°C 2 (AC)9 0.21 1.000 R. TAGGGAAGCCCCAACAGA 15 m 0°C 4 (CC)7 0.28 0.000 R. GAGAGGACCACACAGA 15 m 0°C 4 (CC)7 0.28 0.000 R. GAGATCGCTCTCCCCCCC 15 m 0°C 4 (CC)7 0.29 0.916 Smal-E7 F. GAGACACACACACACTCCCCCA 15 m 0°C 1000 0°C 0°	Locus	Timers 5 –5	[wige12]	alleles	moui	JIZE	neterozygosity	1-value
R-AGCAGTCAGCCCTTTIGAC 15 mM Ban-ESI F.ATGCATGCAGCGATTCATTCC 0°C 2 ACAP 1.000 R-TAGAGGAGCCCCACAGAC 15 mM (TAT) 305-307 0.021 1.000 Ban-ESI F.CAGAGAGCACCGGAATGACCGC 15 mM (TAT) 305-307 0.021 1.000 Sma-EGI F.CAGAGCACACCGGACTTACTCC 15 mM (GATTCCCTGGCTTACTCC 15 mA 0.622 0.157 Sma-EGI F.CAGATCCTCTCCCC 0°C 4 (CCCT) 139-12 0.622 0.157 Sma-ESI F.CAGATCACTCACTTCTCCC 15 mM (CCC)13 403-418 0.796 0.718 Sma-ESP F.AGCAGCTGTCATCTACTCG 5 % C 2 (CA)5 175-177 0.229 1.000 Sma-ESP F.AGCAGCTTCATCACTCG 5 S % C 2 (CA)5 175-177 0.229 1.000 Sma-ESP F.AGCAGCTTCATCACTCG 5 S % C 2 (CA)5 175-177 0.229 1.000 Sma-ESP F.CAGCAGCACTCTCTCTCTTCT 5 M (CT)9114	Sma-E50	F: CACATCGTTGGGACAATCAG	60 °C	8	(GT)9(GA)16	242–274	0.562	0.190
Sma E5 F. ATTGCTTCACGGATTGTTCC 60 °C 2 (AC)9 254-256 0.344 1.000 R: TAGAGGACCCACCACACA 15 mM Sma E51 F. CTCCATGTGTGCCAGAGA 56 °C 2 (TAT)4 305-307 0.021 1.000 Sma E51 F. ATGGGAATGAAATGGTCCG 54 °C 6 (TAT)7 640-657 0.628 0.000 R: GCATCCCTGCTTCTCGCTCC 15 °M 5 °C 4 (GCL)8 139-152 0.672 0.157 R: TCACACGCACTCCCCACAC 62 °C 1 (ACA)13 203-244 0.768 0.718 Sma-E52 F. CGGACACACACACGTCCCCAC 58 °C 2 (CA)13 203-244 0.768 0.718 Sma-E54 F. ACGGCTGTCTCTCTCCT 54 °C 4 (CCC)13 403-418 0.599 0.916 Sma-E57 F. CCACCCCACATCACGCAC 58 °C 2 (CA)5 175-177 0.229 1.000 Sma-E58 F. TGAACCGACTCCTCTCCTCC 58 °C 3 (CCT)4 113-119 0.650 0.835 Sma-E59 F. CCACCGCACTCTCTCTCCCCCA 51 °C 9 (TA)18 29-235		R: AGCAGTGAGCCTCTTTGGAC	1.5 mM					
R.TAGAGCACCCCACACACCA 15 mJ SmaE52 F.CCCATCATGATGCCCCACACGA 55 °C 2 (TAT)4 30°-307 0.021 1.000 SmaE51 F.AGGCATACACGCGACACGCA 15 mJ 6 (TA)7 640-657 0.628 0.000 SmaE72 F.GGAGACACACACGCCCCCC 15 mJ 139-152 0.672 0.157 SmaE72 F.GGACACACACACGCCCCCCC 2 °C 1 (ACA)13 20-424 0.768 SmaE72 F.GGACACACACACGCCCCCCC 15 mJ 0.768 0.718 SmaE74 F.ACCCCCCTGTCTCTCTCCC 5 °C 15 (GT)6-(AT)14 20-719 0.299 0.916 SmaE75 F.AGACACCACATCGACGCCGC 15 mJ (CCCC)13 40-318 0.794 0.766 SmaE79 F.GCACGACTTCGTCGTCTCC 15 mJ (CT)9-(TA) 7 0.29 0.805 SmaE80 F.GCACGACTTCGTCGCC 15 mJ (CT)9-(TA) 27-318 0.794 0.858 SmaE50 F.GCACGACTTCTCACACTCG 5 °C 15 mJ 0.794 0.806 0.800 </td <td>Sma-E51</td> <td>F: ATTGCTTCACGGATTGTTCC</td> <td>60 °C</td> <td>2</td> <td>(AC)9</td> <td>254–256</td> <td>0.344</td> <td>1.000</td>	Sma-E51	F: ATTGCTTCACGGATTGTTCC	60 °C	2	(AC)9	254–256	0.344	1.000
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Ref Accade Acc	Sma-E52	F: CTCGATGATGTGCCAGAAGA	56 °C	2	(TAT)4	305-307	0.021	1.000
Smallel F. ATGGETAATGAAATGGTCG 54 °C 6 (TA7) 640-657 0.628 0.000 Smallel F. GAGATGCTCGCCTTTCTCCC 15 mM 15 mM 139-152 0.672 0.157 Smallel F. GAGAGACACAGTGCCGCAC 62 °C 11 (ACA)13 203-244 0.768 0.718 Smallel F. GAGACACAGTGCCGAC 15 mM 15 mM 1000 1000 1000 Smallel F. AGCAGCTCTCCTCAGGTGCGGG 15 mM (CCC013 403-418 0.599 0.916 Smallel F. AGCAGCTTCCTCTCTCTC 58 °C 2 (CA)5 175-177 0.229 1.000 Smallel F. AGCAGCTGATCGACTGACT 58 °C 3 (CCT)4 13-119 0.650 0.835 Smallel F. TGCATCGATCTGTCTGTCACGG 15 mM GCT)4 113-119 0.650 0.835 Smallel F. TGCATCGATCATGCAGCAC 15 mM GCT)4 12 mallel 0.460 0.835 Smallel F. TGCATCGATCGTCTGCGCA 15 mM GCT)4 13-119 0.650 0.835 Smallel F. TGCAGCGAGATCTCGTGGTGGTGGTGG 5 mV		R: GAGAGAGAAGCGGAACAGGA	1.5 mM					
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Ringer and the second	Sma-E71	F: CAGATCGTCTTCTCGCTCCT	60 °C	4	(GCT)8	139–152	0.672	0.157
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Sma-E74 F. ACCGCGCTGTCTCTTCTC 54 °C 4 (CCG1)3 403-418 0.599 0.916 Sma-E78 F. AAGAACTGCAACGACG 15 mM (CT) 175-177 0.229 1.000 Sma-E78 F. CAGCGACTTGCTCTTTCTTT 58 °C 2 (CA) 175-177 0.229 1.000 Sma-E78 F. CAGCGACTTGCTCTTCTTTCTT 58 °C 15 (CT)-(AT)/T 113-119 0.650 0.835 Sma-E84 F. TGCACTCATCTGCTGTGTGGG 1.5 mM (GT)-(T,)/T 113-119 0.650 0.835 Sma-E84 F. CACGACGACACTGCACCGAC 1.5 mM (GT) 9 8-108 0.758 0.190 Sma-E86 F. CACGACGCACACCAC 1.5 mM 22 (TG) 186-188 0.461 0.060 R: ATTATCTCTCCACAAGCACACA 1.5 mM 3 (CT) 166-88 0.676 0.000 Sma-E97 F. CTACACACACCCTGACCT 5 °C 11 (CT)(GT)25 289-331 0.668 0.930 Sma-E97 F. CTAACAGACACCACCTGACC 1.5 mM 3 (TG)2 289-331 0.668 0.930 Sma-E108 <td></td> <td>R: CGTTCTCCTAAGTTGCAGCG</td> <td>1.5 mM</td> <td></td> <td></td> <td></td> <td></td> <td></td>		R: CGTTCTCCTAAGTTGCAGCG	1.5 mM					
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$ \begin{array}{cccc} {\rm R: } \operatorname{CGTGGTTTCCA1CAACTGG} & 1 \mathrm{mM} & & & & & & & & & & & & & & & & & & &$	Sma-E78	F: AAGAACTGCATCGACCGACT	58 °C	2	(CA)5	175–177	0.229	1.000
Sma-E9 F: CCACCCACTTCCTTCTTCT 58 °C 15 (GT)6-(AT)14 274-318 0.794 0.766 Sma-E82 F: TTGAACGGAACTTCTACACTCG 58 °C 3 (CCT)4 113-119 0.650 0.835 Sma-E84 F: TCGATCATTCTCTGCTGTGGCG 1.5 mM (CT)9 98-108 0.758 0.190 Sma-E84 F: TCGATCATACTCAGGCAGCA 1.5 mM 292-325 0.852 1.000 Sma-E91 F: CACGGACGATACCTCGTGAT 59 °C 2 (TG)5 186-188 0.461 0.060 Sma-E91 F: CACGGACGATACCTGCTGAT 59 °C 2 (TG)5 186-188 0.461 0.060 Sma-E97 F: CTGACTGCACCTGACGA 54 °C 9 (ACC)8 646-668 0.676 0.000 Sma-E97 F: CTAACAGACGCAAATCCACCT 56 °C 11 (CT)GT)2 289-331 0.668 0.930 Sma-E90 F: CCAGGCAAATCCACCTGT 15 mM 0.400 1.5 mM 0.400 0.817 0.400 0.817 0.400 0.417 0.417 0.417 0.417 0.410 0.410 0.410 0.417 </td <td></td> <td>R: CGTGTGTTTTCCATCAACTGG</td> <td>1 mM</td> <td></td> <td></td> <td></td> <td></td> <td></td>		R: CGTGTGTTTTCCATCAACTGG	1 mM					
R: GTCAGTTTGTGGTGTGTGGG 1.5 mM (GT)9-(TA)7 Sma-E82 F. TGCAGCGAACTTCTACACACTG 8 °C 3 (CC1)4 113-119 0.650 0.835 Sma-E84 F. TGCAGTTCGTGGTAGTGT 1.5 mM 1.5 mM 0.758 0.190 Sma-E84 F. TGCAGTTCATATCCGAGGCAC 1.5 mM 0.758 0.190 Sma-E86 F. CAGGAGGACTTCTCTGGCCAA 51 °C 9 (TA)18 292-325 0.852 1.000 Sma-E97 F. CAGGAGGACACCTCAACT 50 °C 2 (TG)5 186-188 0.461 0.060 R: ACACTCGCCTCGTTTCTCAT 1.5 mM 0.668 0.676 0.000 Sma-E96 F. TCTGCTGGGCTCAACCTAACGAC 54 °C 9 (ACA)8 289-331 0.668 0.930 Sma-E97 F. CTAACAGACGAAATGCACC 56 °C 11 (CT)(GT)25 289-331 0.668 0.930 Sma-E90 F. CAGGACACACACACCACGCACGA 54 °C 3 (TG)5 303-311 0.509 0.817 R: TACAGACAGATAACCACACTCAACG 1.5 mM 0.509 0.817 0.710 0.817 Sma-E100 F. CCAACACACACAC	Sma-E79	F: GCAGCGACTTGCTTCTTTCT	58 °C	15	(GT)6-(AT)14-	274–318	0.794	0.766
Sma-B2 F. TIGAACGGAACTICTACACICG 58 °C 3 (CC)14 113-119 0.650 0.835 R: GCGGTTICGTCGTGATGGTGA 47 °C 5 (GT)19 98-108 0.758 0.190 Sma-E84 F: TGCATCTATTCCTGTGGGA 47 °C 5 (GT)19 98-108 0.758 0.190 Sma-E84 F: CAGGAGACTTCTCGCCCAA 1.5 mM 7 9 (TA)18 292-325 0.852 1.000 Sma-E96 F: CAGGAGACCATCCTCCTCAA 59 °C 2 (TG)5 186-188 0.461 0.060 R: TATACTCGCGCTCACCTTAACA 59 °C 2 (TG)5 186-188 0.461 0.060 Sma-E97 F: CTACCAGCACTCATCGCC 1.5 mM 7 7 0.000 8: TACAGACACCACAACCCCCACTCGT 1.5 mM 7 0.371 0.694 0.817 Sma-E108 F: CCAAGCAAACACTCACCTCG 1.5 mM 7 0.313 0.509 0.817 R: CAGGATAACCACACTCAAACCACACTCAAGA 1.5 mM 7 0.330 1.000 8 1.000 8 1.000 8 1.000 8 1.0694 1.001 8		R: GTCAGTTTGTGGTGTGTGGG	1.5 mM		(GT)9-(TA)7			
R: GCGGTTICGTCGTTGGTGGT 1.5 mM Sma-E84 F: GCACTCATTCCTGGTGA 47 °C 5 (GT) 9 8-108 0.758 0.190 Sma-E86 F: CAGGAGGACTTCTCTGCGCA 1.5 mM 22-325 0.852 1.000 Sma-E91 F: GACGGACGATACCTGCTGCA 1.5 mM 22-325 0.852 1.000 Sma-E91 F: GACGGACGATACCTGCTGCA 1.5 mM 0.461 0.060 R: TTTACTCTCCCACAGGCAGCA 1.5 mM 0.461 0.060 Sma-E91 F: CTGCTGGCTCACCTTAACA 5 °C 2 (TC)(GT)2 289-331 0.668 0.930 Sma-E97 F: CTACAGACGCAAATGCACC 56 °C 11 (CT)(GT)2 289-331 0.668 0.930 Sma-E90 F: ACGACTTCTCCCAAAGCACATGCAC 56 °C 11 (CT)(GT)2 289-331 0.668 0.930 Sma-E91 F: CACACACCAACGCAAGCATA 1.5 mM 0.509 0.817 Sma-E91 F: CCAGACTAACCACTGCACCAT 54 °C 3 (TG)5 303-311 0.509 0.817 Sma-E101 F: CCAGACTCAACACACACACACACA 1.5 mM 0.500 0.817 0.71 <t< td=""><td>Sma-E82</td><td>F: TTGAACGGAACTTCTACACTCG</td><td>58 °C</td><td>3</td><td>(CCT)4</td><td>113–119</td><td>0.650</td><td>0.835</td></t<>	Sma-E82	F: TTGAACGGAACTTCTACACTCG	58 °C	3	(CCT)4	113–119	0.650	0.835
Sma-B4 F TGCATCTATTCCTGATCGAC 47 °C 5 (T)19 98-108 0.758 0.190 Sma-B84 F: CAGGAGGACTTCTCTGCACCA 1.5 mM 9 (TA)18 292-325 0.852 1.000 Sma-B86 F: CAGGAGGACTTCTCTGCACAA 51 °C 9 (TA)18 292-325 0.852 1.000 Sma-B91 F: GACGACAACCTGCGCGAC 1.5 mM 9 C 2 (TG)5 186-188 0.461 0.060 R: ACACTCGCCTCGCTCACCTTAACA 54 °C 9 (ACC)8 646-668 0.676 0.000 Sma-B97 F: CTAACAGACGACAAATGCACC 56 °C 11 (CT)(GT)25 289-331 0.668 0.930 Sma-B99 F: AACGACTCTCCCAAGCCAA 54 °C 3 (CAG)4 328-342 0.351 0.694 Sma-B10 F: CCAGCAAGCAGAAGGCACCT 1.5 mM 5 1.5 mM 5 0.59 <td< td=""><td></td><td>R: GCGGTTTCGTCGTTAGTGTT</td><td>1.5 mM</td><td>_</td><td>()</td><td></td><td></td><td></td></td<>		R: GCGGTTTCGTCGTTAGTGTT	1.5 mM	_	()			
Sima-Ease F: CAGGAGACTACTCGCGCAA 1 s mM 292-325 0.852 1.000 Sima-Ease F: CAGGAGCATACTCGCGCAA 1 s mM 200 200 1.000 Sima-Ease F: GACGACGACCATACCTCGCTGAT 59 °C 2 (TG)5 186-188 0.461 0.060 Sima-Ease F: CTCGCGCCACCTGATGCA 54 °C 9 (ACC)8 646-668 0.676 0.000 R: ATAGGGTCTGCACCTTAACA 54 °C 9 (ACC)8 646-668 0.676 0.000 Sima-Ease F: CTAACAGACGCAAATGCACC 56 °C 11 (CT)(GT)2 289-331 0.668 0.930 Sima-Ease F: CTAACAGACGCAATACCACTGT 1 s mM 303-311 0.509 0.817 Sima-Ease F: CTGAGCTAACCACTGACCT 54 °C 3 (CGA) 303-311 0.509 0.817 Sima-Ease F: CTAACAAACACACTGAAGATGACGCT 1 s mM 303-311 0.509 0.817 Sima-Ease F: CTCACAAACCACTGAACTAC 5 °C 3 (GT)2 275-29 0.200 1.000 Sima-Ease F: TTCACAAACCACTAACTGAC 1 s mM 300 0.711 </td <td>Sma-E84</td> <td>F: TGCATCTATTCCTGTTGGTGA</td> <td>47 °C</td> <td>5</td> <td>(GT)19</td> <td>98–108</td> <td>0.758</td> <td>0.190</td>	Sma-E84	F: TGCATCTATTCCTGTTGGTGA	47 °C	5	(GT)19	98–108	0.758	0.190
Sma-Ba6 F: CAGGAGGACTICTCTCGCCAA 51 °C 9 (1A)18 $292-325$ 0.852 1.000 R: TTTACTCTCCACAGGCACGA 1.5 mM Sma-E91 F: GACGACGATACCTGCTGAT $59 °C$ 2 (TG)5 $186-188$ 0.461 0.060 Sma-E90 F: TCTGCTGGCTCACCTTAACA $54 °C$ 9 (ACC)8 $646-668$ 0.676 0.000 Sma-E97 F: CTAACAGACGCAAATGCACC $56 °C$ 11 (CT)(GT)25 $289-331$ 0.668 0.930 Sma-E97 F: CTAACAGACGCACAATGCACC $56 °C$ 11 (CT)(GT)25 $289-331$ 0.668 0.930 Sma-E97 F: ACCAACACTCACACTGATG $1.5 mM$ $282-342$ 0.351 0.694 Sma-E108 F: CCAGCAAACACTCACCTGT $54 °C$ 3 (TG)3 $303-311$ 0.509 0.817 Sma-E108 F: TACAGAACAGATGAAGAT $1.5 mM$ $275-294$ 0.201 0.000 R: TGGAGCACTCAAACTGCACG $1.5 mM$ $275-294$ 0.721 0.373 Sma-E112 F: GCACAATCCAACTGCAGC $52 °C$ 8 (TA)2 $318-330$ 0.710 <		R: TGTTGGTTCATAACTGAGCGAC	1.5 mM					
R: TITACI CICCACAGGCAGCA 1.5 mM Sma-E91 F: GACGGACCATACCTGCTGAT 59 °C 2 (TG)5 186–188 0.461 0.060 R: ACACTCGCCTCGCTTTTCTCAT 1.5 mM 1.5 mM 0.461 0.000 Sma-E90 F: TCTGCTGGCTCACCTTAACA 54 °C 9 (ACC)8 646–668 0.676 0.000 Sma-E97 F: CTAACAGACGCAAATGCACC 56 °C 11 (CT)(GT)25 289–331 0.668 0.930 Sma-E98 F: CAACGACTCACACGGCCA 54 °C 3 (CAG)4 328–342 0.351 0.694 Sma-E10 F: CCGAGCAGAGAGAGGCGCCG 1.5 mM 7 303–311 0.509 0.817 Sma-E105 F: TCACAAACACACATGCACAGG 60 °C 3 (TG)5 303–311 0.509 0.817 Sma-E105 F: TCACAAACCACATCCAAGG 60 °C 3 (TG)5 286–302 0.200 1.000 R: TGGGACGAGGCCATAGTCATT 1.5 mM 7 7 7 0.712 0.373 0.710 0.247 Sma-E112 F: GCAGCAAACAGACAGAGC 57 °C 3 (CA99 318–330 0.710	Sma-E86	F: CAGGAGGACTICICIGCCAA	51 °C	9	(TA)18	292–325	0.852	1.000
Sma-E91 F: GACCGACGACIACCTGAT 15 9°C 2 (1G.)5 186–188 0.461 0.060 R: ACACTCGCCTCGTTTCTCAT 1.5 mM .5 mA .6 0.000 0.000 Sma-E97 F: TCTGCGCCCACCTTAACA 54°C 9 (ACC)8 646–668 0.676 0.000 Sma-E97 F: CTAACAGACCCCAAATGCACC 56°C 11 (CT)(GT)25 289–331 0.668 0.930 Sma-E97 F: CACGACTTCTCCAGAGCCA 54°C 3 (CAG)4 328–342 0.351 0.694 Sma-E90 F: ACCGACTTCACAGACGCAA 54°C 3 (TG)5 303–311 0.509 0.817 Sma-E108 F: CCGAGCAAACCAACAGGACAG 1.5 mM	0 504	R: TTTACICICCACAGGCAGCA	1.5 mM			104 100	0.474	0.070
R: ACACICGCCICGITICICAT 1.5 mM Sma-E96 F: TCTGCTGGGCTCACCTTAACA 54 °C 9 (ACC)8 646-668 0.676 0.000 Sma-E97 F: CTAACAGACGCAAATGCACC 56 °C 11 (CT)(GT)25 289-331 0.668 0.930 Sma-E97 F: CTAACAGACGCAAATGCACC 56 °C 11 (CT)(GT)25 289-331 0.668 0.930 Sma-E97 F: ACAGACAGATGACGCACGT 1.5 mM . <t< td=""><td>Sma-E91</td><td>F: GACGGACGATACCTGCTGAT</td><td>59 °C</td><td>2</td><td>(TG)5</td><td>186–188</td><td>0.461</td><td>0.060</td></t<>	Sma-E91	F: GACGGACGATACCTGCTGAT	59 °C	2	(TG)5	186–188	0.461	0.060
Sma-E96 F: TCTGCTGGCGTCACCTTAACA 54 °C 9 (ACC)8 646-668 0.676 0.000 R: ATAGGGTCTGCACTCATGGC 1.5 mM		R: ACACICGCCICGITICICAT	1.5 mM		(1.2.2)		a	
R: ATAGGGTCIGGACICGATGCACC 1.5 mM Sma-Eyp F: CTAACAGACGCAAATGCACC 56 °C 11 (CT)(GT)2 289–331 0.668 0.930 Sma-Eyp F: AACGACTTCTCCAGAGCCAA 54 °C 3 (CAG)4 328–342 0.351 0.694 Sma-Eyp F: AACGACAGATGACGGCTCG 1.5 mM	Sma-E96	F: TCIGCIGGCICACCITAACA	54 °C	9	(ACC)8	646–668	0.676	0.000
Sma-E97 F: CTAACAGACGCCAAAGCCACC 56 °C 11 (C1)(G1)25 289–331 0.668 0.930 R: CCATGCAAACACTCACCTGT 1.5 mM 1.5 mM 280–342 0.351 0.694 Sma-E99 F: ACAGACTTCTCCAGAGCCAA 54 °C 3 (CAG)4 328–342 0.351 0.694 Sma-E100 F: CCGAGCTAACCACTGACCT 54 °C 3 (TG)5 303–311 0.509 0.817 Sma-E105 F: TTCACAAACCACATCCAAGG 60 °C 3 (GT)6 286–302 0.200 1.000 Sma-E112 F: GGTGCAGGCCATAGTCATTT 59 °C 6 (TA)12 275–294 0.721 0.373 Sma-E113 F: CACACAGTCCACAGACATT 1.5 mM 7°C 3 (CA)9 321–333 0.520 0.000 Sma-E117 F: GCACAACGACAACACGC 57 °C 7 (TG)21 216–244 0.485 0.067 Sma-E118 F: TATTATGGAGGGATCGGCTG 52 °C 7 (TG)21 216–244 0.485 0.067 Sma-E120 F: TACTGGGTCTACTGCAGCC 52 °C 7 (TG)21 216–244 0.485 0.001 <td></td> <td>R: ATAGGGTCTGCACTCATGGC</td> <td>1.5 mM</td> <td></td> <td></td> <td></td> <td>0.440</td> <td>0.000</td>		R: ATAGGGTCTGCACTCATGGC	1.5 mM				0.440	0.000
Sma-Energy F: AACGACTTCTCCAGAGCCAA 54 °C 3 (CAG) 328–342 0.351 0.694 Sma-Energy F: AACGACAGATGACCGCTCG 1.5 mM	Sma-E97	F: CTAACAGACGCAAATGCACC	56 °C	11	(CT)(GT)25	289–331	0.668	0.930
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	C E00	R: CCAIGCAAACACICACCIGI	1.5 mM	2	$(C \land C) \land$	200 240	0.251	0.604
Sma-E100F: CCGAGCTAACCACTGACCGCTCG1.5 mM(TG)5303-3110.5090.817Sma-E105F: TTCACAAACCACTGACGATA1.5 mM(GT)6286-3020.2001.000R: TGGCACAAGCTCAAACTGAC1.5 mM(GT)6286-3020.2001.000Sma-E112F: GGTGCAGGCCATAGTCAATG59 °C6(TA)12275-2940.7210.373R: TGTGAGTGATTCGGCAACAG1.5 mM(GA)2318-3300.7100.247Sma-E113F: CACACATCCACAGACTCGCT52 °C8(TA)25318-3300.7100.247Sma-E117F: GCACAAACGACAAACAGCG1.5 mM(GA)9321-3330.5200.000R: TCAAATGCAACAATGACGTT1.5 mM(GA)9321-3330.5200.000Sma-E118F: TATTATGGAGGGATCGGCTG55 °C7(TG)21216-2440.4850.067Sma-E120F: TACTGGGTCATCTGGGTGCC52 °C4(AGG)4206-2160.3760.001R: CCGTCCGTTTCCTTCAAATA1.5 mM(GA)2216-2440.4850.0670.001Sma-E127F: TGAGATTTGCATGGATGTGG52 °C4(AGG)4206-2160.3760.001Sma-E128F: CTTCATCGCCATCTCATTT55 °C18(ATC)8270-3120.9170.156Sma-E128F: CTTCATCGCCATCTCCATTT55 °C18(ATC)8270-3120.9170.156Sma-E128F: CTTCATCGCCATCTCCATTT55 °C18(ATC)8270-3120.9170.156Sma-E128F: GGTCGGTCATCTCG	Sma-E99		54 °C	3	(CAG)4	328-342	0.351	0.694
Sma-E100 F: CCGAGCTAACCACIGACCT 54 °C 3 (1G)5 303-311 0.509 0.817 R: CGAGCACGCAGTAATGGATA 1.5 mM 0.000 1.000 Sma-E105 F: TTCACAAACCACATCCAAAG 60 °C 3 (GT)6 286-302 0.200 1.000 Sma-E112 F: GGTGCAGGCCATAGTCATTT 59 °C 6 (TA)12 275-294 0.721 0.373 Sma-E113 F: CACACATCCACAGACTCGCT 52 °C 8 (TA)25 318-330 0.710 0.247 Sma-E117 F: GGCACAAACGACAAGACCGC 1.5 mM Sma-E117 F: GCACAAACAGCACAGACAGC 1.5 mM 0.000 R: TCAAATGCAACCATGACGTT 1.5 mM 0.067 R: TCAACGTGATTGCGGTGG 55 °C 7 (TG)21 216-244 0.485 0.067 R: TCAACGTGATGTTGCCTTC 1.5 mM Sma-E120 F: TATTGGGGTCATCGGATG	C E100		1.5 mM	2		202 211	0 500	0.017
Sma-El10F: TCACAAACCACATCCAAGG60 °C3(GT)6286-3020.2001.000R: TGGCACAAACCATCCAAACTGAC1.5 mMSma-El12F: GGTGCAGGCCATAGTCATTT59 °C6(TA)12275-2940.7210.373Sma-El13F: CACACATCCACAGACTCGCT52 °C8(TA)25318-3300.7100.247Sma-El13F: CACACATCCACAGACTGGCG1.5 mMSma-El14F: GCACAAACAGACAAACAGC57 °C3(CA)9321-3330.5200.000R: AAACATTCCTCTCAGTGCCG1.5 mMSma-El18F: TATTATGGAGGGATCGGCTG55 °C7(TG)21216-2440.4850.067R: TCAACGTGATGTTTGCCTTC1.5 mMSma-El20F: TACTGGGTCTACTGGGTGCC52 °C4(AGG)4206-2160.3760.001<	Sma-E100		54 °C	3	(IG)5	303-311	0.509	0.817
Sma-E105 F: ITCACAAACCAACCATCCAACG 60 °C 3 (G1)6 286-302 0.200 1.000 R: TGGCACAAGCTCAAACTGAC 1.5 mM 1.000 1.000	C E10E		1.5 mM	2		201 202	0.200	1 000
Sma-E112F: GGTGCAGGCCATAGCTCAAACTGAC1.5 mMSma-E113F: GGTGCAGGCCATAGTCATTT $59 ^{\circ}$ C6(TA)12 $275-294$ 0.721 0.373 Sma-E113F: CACACATCCACAGACTGGCT $52 ^{\circ}$ C8(TA)25 $318-330$ 0.710 0.247 R: AAACATTCCTCTCAGTGCCG $1.5 ^{m}$ M77 0.373 0.710 0.247 Sma-E117F: GCACAAACAGACAAACACGC $57 ^{\circ}$ C3 $(CA)9$ $321-333$ 0.520 0.000 R: TCAAATGCAACCATGACGTT $1.5 ^{m}$ M77 0.762 0.247 Sma-E118F: TATTATGGAGGGATCGGCTG $55 ^{\circ}$ C7 $(TG)21$ $216-244$ 0.485 0.067 R: TCAACGTGATGTTGCCTTC $1.5 ^{m}$ M77 0.376 0.001 0.376 0.001 Sma-E120F: TACTGGGTCTACTGGGTGGC $52 ^{\circ}$ C4 $(AGG)4$ $206-216$ 0.376 0.001 Sma-E127F: TGAGAATTGCATGGATGGG $52 ^{\circ}$ C6 $(GT)10$ $78-102$ 0.791 0.304 Sma-E128F: CTTCATCGCCATCTCCATTT $55 ^{\circ}$ C18 $(ATC)8$ $270-312$ 0.917 0.156 Sma-E132F: GGTCGGTCATCTCGATAACA $1.5 ^{m}$ M7 0.669 0.000 Sma-E132F: GGTCGGTCATCTCGTAGCAT $59 ^{\circ}$ C7 $(GCC)6$ $350-370$ 0.669 0.000	Sma-E105		60 °C	3	(GI)6	286-302	0.200	1.000
Sma-E112F: GGTGGCAGGCCATAGTCATTT59 °C6(TA)12275-294 0.721 0.373 R: TGTGAGTGATTCGGCAACAG1.5 mM	C E110	K: IGGCACAAGCICAAACIGAC	1.5 mM	((TA)10	075 004	0 701	0.272
Sma-E113 F: CACACATCCACAGACTCGCT 52 °C 8 (TA)25 318–330 0.710 0.247 Sma-E117 F: GCACAAACAGACAAACAGGC 57 °C 3 (CA)9 321–333 0.520 0.000 R: TCAAATGCAACCATGACGTT 1.5 mM 7°C 3 (CA)9 321–333 0.520 0.000 R: TCAAATGCAACCATGACGTT 1.5 mM 7°C 7 (TG)21 216–244 0.485 0.067 R: TCAACGTGATGTTTGCCTTC 1.5 mM 7 (TG)21 216–244 0.485 0.001 Sma-E120 F: TACTGGGTCTACTGGGTGCC 52 °C 4 (AGG)4 206–216 0.376 0.001 Sma-E120 F: TACTGGGTCTACTGGGTGGC 52 °C 6 (GT)10 78–102 0.791 0.304 Sma-E127 F: TGAGATTTGCATGGATGTGG 52 °C 6 (GT)10 78–102 0.791 0.304 Sma-E128 F: CTTCATCGCCATCTCCATTT 55 °C 18 (ATC)8 270–312 0.917 0.156 Sma-E132 F: GGTCGGTCATCTCGATACA 1.5 mM 7 (GCC)6 350–370 0.669 0.000 <td>5ma-E112</td> <td></td> <td>59 °C</td> <td>6</td> <td>(1A)12</td> <td>275-294</td> <td>0.721</td> <td>0.373</td>	5ma-E112		59 °C	6	(1A)12	275-294	0.721	0.373
Sma-E113F: CACACATCCACAGACTCGCT $52 \cdot C$ 8 $(1A)25$ $518-350$ 0.710 0.247 R: AAACATTCCTCTCAGTGCCG 1.5 mM Sma-E117F: GCACAAACAGACAAACAGGC $57 \circ C$ 3 $(CA)9$ $321-333$ 0.520 0.000 R: TCAAAATGCAACCATGACGTT 1.5 mM Sma-E118F: TATTATGGAGGGATCGGCTG $55 \circ C$ 7(TG)21 $216-244$ 0.485 0.067 R: TCAACGTGATGTTTGCCTTC 1.5 mM Sma-E120F: TACTGGGTCTACTGGGTGGC $52 \circ C$ 4(AGG)4 $206-216$ 0.376 0.001 R: CCGTCCGTTTCCTTCAAATA 1.5 mM Sma-E127F: TGAGATTTGCATGGATGTGG $52 \circ C$ 4(AGG)4 $206-216$ 0.376 0.001 Sma-E128F: CTTCATCGCATGGATGTGG $52 \circ C$ 6(GT)10 $78-102$ 0.791 0.304 Sma-E128F: CTTCATCGCCATCTCCATTT $55 \circ C$ 18 (ATC)8 $270-312$ 0.917 0.156 Sma-E132F: GGTCGGTCATCTCGTAGCAT $59 \circ C$ 7(GCC)6 $350-370$ 0.669 0.000 R: $AAGCCCTGCCACTGCCACTGCAACTA$ 15 mM 15 mM	C E112		1.5 mivi	0	(TA))25	210 220	0.710	0.247
Sma-E117 F: GCACAAACAGACAAACACGC 57 °C 3 (CA)9 321–333 0.520 0.000 R: TCAAATGCAACCATGACGTT 1.5 mM 7°C 3 (CA)9 321–333 0.520 0.000 Sma-E118 F: TATTATGGAAGGGATCGGCTG 55 °C 7 (TG)21 216–244 0.485 0.067 R: TCAACGTGATGTTTGCCTTC 1.5 mM 7 (AGG)4 206–216 0.376 0.001 Sma-E120 F: TACTGGGTCTACTGGGTGCC 52 °C 4 (AGG)4 206–216 0.376 0.001 Sma-E127 F: TGAGATTTGCATGGATGTGG 52 °C 6 (GT)10 78–102 0.791 0.304 Sma-E128 F: CTTCATCGCCATCTCCATTT 1.5 mM 7 7 0.917 0.156 Sma-E128 F: GTTCATCGCCATCTCCATTT 55 °C 18 (ATC)8 270–312 0.917 0.156 Sma-E132 F: GGTCGGTCATCTCGTAACA 1.5 mM 7 (GCC)6 350–370 0.669 0.000	5ma-E115		52 C	0	(1A)25	516-550	0.710	0.247
Sind-E117F. GCACAAACAGACAAACAGC57C53 $(CA)^{g}$ $521-353$ 0.520 0.000 R: TCAAATGCAACCATGACGTT1.5 mM1.5 mM 1.5 mM 0.007 0.007 Sma-E118F. TATTATGGAGGGATCGGCTG $55 ^{\circ}$ C7 $(TG)21$ $216-244$ 0.485 0.067 R: TCAACGTGATGTTTGCCTTC1.5 mM 0.007 0.001 0.001 0.001 0.001 Sma-E120F. TACTGGGTCTACTGGGTGCC $52 ^{\circ}$ C4 $(AGG)4$ $206-216$ 0.376 0.001 Sma-E127F. TGAGATTTGCATGGATGTGG $52 ^{\circ}$ C6 $(GT)10$ $78-102$ 0.791 0.304 Sma-E128F. CTTCATCGCCATCTCCATTT $55 ^{\circ}$ C18 $(ATC)8$ $270-312$ 0.917 0.156 Sma-E132F. GGTCGGTCATCTCGTAGCAT $59 ^{\circ}$ C7 $(GCC)6$ $350-370$ 0.669 0.000 R: AAGCCCTGCCACTGCCACTGCAACTA $1.5 ^{\circ}$ M $1.5 ^{\circ}$ M $1.5 ^{\circ}$ M $1.5 ^{\circ}$ M	Sma E117		1.5 mivi	2	$(C \Lambda) 0$	201 222	0.520	0.000
Sma-E118F: TATTATGGAGGGATCGGCTG55 °C7(TG)21216–2440.4850.067R: TCAACGTGATGTTTGCCTTC1.5 mM	5111d-12117		57 C	3	(CA)9	321-333	0.320	0.000
Sina-E118F. TATTATGGAGGGATCGGCTC 55° C 7° $(16)/21^{\circ}$ $210-244^{\circ}$ 0.465° 0.007° Sma-E120F. TACTGGGTCTACTGGGTGCC 52° C4 $(AGG)4^{\circ}$ $206-216^{\circ}$ 0.376° 0.001° R: CCGTCCGTTTCCTTCAAATA 1.5 mM 1.5 mM 30° 0.304° 0.304° Sma-E127F. TGAGATTTGCATGGATGTGG 52° C6 $(GT)10^{\circ}$ $78-102^{\circ}$ 0.791° 0.304° Sma-E128F. CTTCATCGCCATCTCCATTT 55° C 18° $(ATC)8^{\circ}$ $270-312^{\circ}$ 0.917° 0.156° Sma-E132F. GGTCGGTCATCTCGTAGCAT 59° C 7° $(GCC)6^{\circ}$ $350-370^{\circ}$ 0.669° 0.000° R: AAGCCCTGCACATGCAAGCTA 1.5 mM	Sma-F118		1.5 mivi	7	(TC)21	216 244	0.485	0.067
Sma-E120 F: TACTGGGTCTACTGGGTGCC 52 °C 4 (AGG)4 206–216 0.376 0.001 R: CCGTCCGTTTCCTTCAAATA 1.5 mM 1.5 mM 0.304 0.304 Sma-E127 F: TGAGATTTGCATGGATGTGG 52 °C 6 (GT)10 78–102 0.791 0.304 Sma-E128 F: CTTCATCGCCATCTCCATTT 55 °C 18 (ATC)8 270–312 0.917 0.156 R: GGCCGAATACTCCGATAACA 1.5 mM 55 °C 7 (GCC)6 350–370 0.669 0.000 B: AAGCCCTGCACATGCAAGCAA 1.5 mM 55 °C 15 mM 550–370 0.669 0.000	Jilla-E110		15 mM	/	(10)21	210-244	0.405	0.007
Sina-E120R: CCGTCCGTTTCCTTCAAATA1.5 mM $(AGG)^4$ $200-210$ 0.570 0.001 Sma-E127F: TGAGATTTGCATGGATGTGG $52 ^{\circ}C$ 6 $(GT)10$ $78-102$ 0.791 0.304 Sma-E128F: CTTCATCGCCATCTCCATTT $55 ^{\circ}C$ 18 $(ATC)8$ $270-312$ 0.917 0.156 R: GACCCTGGATACCACATCTCGATAACA $1.5 ^{\circ}mM$ $Sma-E132$ F: GGTCGGTCATCTCGTAGCAT $59 ^{\circ}C$ 7 $(GCC)6$ $350-370$ 0.669 0.000	Sma-E120	F: TACTCCCTCTACTCCCTCCC	52 °C	4	(ACC)4	206_216	0 376	0.001
Sma-E127 F: TGAGATTTGCATGGATGTGG 52 °C 6 (GT)10 78–102 0.791 0.304 Sma-E128 F: CTTCATCGCCATCTCCATTT 55 °C 18 (ATC)8 270–312 0.917 0.156 R: GACCCTGCGCTCTCCGATAACA 1.5 mM 5 °C 7 (GCC)6 350–370 0.669 0.000 Sma-E132 F: GGTCGGCAATCTCGAAGCAT 59 °C 7 (GCC)6 350–370 0.669 0.000	5ma-1120	$R \cdot CCCTCCCTTTCCTTC \Delta \Delta \Delta T \Delta$	15 mM	4	(AGG)4	200-210	0.570	0.001
R: GACTCCTGGCTCCTTCT 1.5 mM Sma-E128 F: CTTCATCGCCATCTCCATTT 55 °C 18 (ATC)8 270–312 0.917 0.156 R: GGCCGAATACTCCGATAACA 1.5 mM 350 °C 7 (GCC)6 350–370 0.669 0.000 Sma-E132 F: GGTCGCACTCCCATCAGCAT 59 °C 7 (GCC)6 350–370 0.669 0.000	Sma-F127		52 °C	6	(CT)10	78_102	0 791	0 304
Sma-E128F: CTTCATCGCCATCTCCATTT 55 °C 18 (ATC)8 $270-312$ 0.917 0.156 R: GGCCGAATACTCCGATAACA 1.5 mM Sma-E132F: GGTCGGTCATCTCGTAGCAT 59 °C 7(GCC)6 $350-370$ 0.669 0.000 R: $\Delta AGCCCTGCACATGCAAGTA$ 1.5 mM	5111a-11127	R.CACTCCTCCTCCTCCTTCT	15 mM	0	(01)10	70-102	0.7 / 1	0.504
R: GGCCGAATACTCCGATAACA 1.5 mM Sma-E132 F: GGTCGGTCATCTCGTAGCAT 59 °C 7 (GCC)6 350–370 0.669 0.000 R: AAGCCCTGCACATGCAAGTA 1.5 mM	Sma-F128	F. CTTCATCCCCATCTCCATTT	55 °C	18	(ATC)8	270-312	0 917	0 156
Sma-E132 F: GGTCGGTCATCTCGTAGCAT 59 °C 7 (GCC)6 350–370 0.669 0.000 R: A A GCCCTGCA CATGCA A GTA 1.5 mM	5ma E120	R: GGCCGAATACTCCCATAACA	1.5 mM	10	(2110)0	2,0 012	5.717	0.100
R·AACCCCTCCACATCCAACTA 15 mM	Sma-E132	F: GGTCGGTCATCTCCTACCAT	59 °C	7	(GCC)	350-370	0.669	0.000
	511m E102	R: AAGCCCTGCACATGGAAGTA	1.5 mM	,	(600)	555 570		
Sma-E134 F: CGGCTTTCTCTCCTCCTGTT 48 °C 2 (TG)7 115–119 0.142 1.000	Sma-E134	F: CGGCTTTCTCTCCTCCTGTT	48 °C	2	(TG)7	115-119	0.142	1.000
R: AGCTCACGGCCAGATTAGAA 1.5 mM		R: AGCTCACGGCCAGATTAGAA	1.5 mM		();			

Table 1 (Continued)

Locus	Primers 5'–3'	Annealing temp [MgCl2]	No. of alleles	Repeated motif	Size	Expected heterozygosity	Hardy– Weinberg P-value
Sma-E136	F: ATGGAGACTCACACGGAGGT	51 °C 1 5 mM	3	(TGT)6	198–204	0.279	0.572
Sma-E137	F: CTGTGTCCCTTGGAGATGGT R: AAAGGGTCGTGCAGAAGCTA	57 °C 1 5 mM	6	(CGC)5	614–624	0.778	0.005
Sma-E139	F: GAACAATGACTTGCTGCTGG R: ACCTCAACACCCTATCCCAC	50 °C	4	(CTG)10	400-408	0.672	0.500
Sma-E142	F: TCCATCGCAATATCACAGGA	58 °C	9	(AGA)12	266–294	0.793	0.002
Sma-E144	F: CTTCTACAGCCAAACGAGGG R: CATTGATGCGCCTTTCCTAT	48 °C	7	(TA)10	292–306	0.793	0.020
Sma-E145	F: CTGTCTCCCTGTCCGTCTGT R: GAGAAGCTCGGGATGATGAC	58 °C 1 5 mM	3	(TC)6	430-440	0.395	1.000
Sma-E154	F: CTCTTCTCTGCGTTTCTGCC R: GAGTCTCGTGAACCTGGAGC	52 °C 1.5 mM	14	(TCC)4	560–588	0.919	0.000
Sma-E156	F: GTGATGAGGGTGATGAGGGT R: CCAGCCTCTCTTTGTTGCTC	54 °C 1.5 mM	4	(CTG)10	337–346	0.647	0.002
Sma-E158	F: GTCTCGCACTTCCTGTCTCC R: TGGAATCTGTCCGTCTGTTG	56 ℃ 1.5 mM	4	(TCT)9	309–336	0.452	1.000
Sma-E159	F: GATCAATGTGGTCCTCCACC R: CTCCTTCTCCAAGTCCACCA	52 °C 1.5 mM	3	(TC)7	156–161	0.357	0.072
Sma-E164	F: ATTCTCAGCCATCTGGAACC R: AGTGATGACCACGACCACAA	54 °C 1.5 mM	3	(TA)10	289–293	0.294	0.300
Sma-E167	F: TTACGTTTGTGAGTCGTCTGG R: CATCAGTCCACATCCGTCTG	63 °C 1.5 mM	2	(CA)6	86-88	0.221	0.540
Sma-E168	F: CGTCTTTGTACGCGAAGCTC R: GATTTCAAAGTCAAGGCCCA	63 °C 1.5 mM	3	(AC)9	117–129	0.451	0.842
Sma-E170	F: TTCACCATGAAGCCATGAAA R: TGACGTAACAAGACGGAGGA	54 °C 1.5 mM	9	(AC)18	328–346	0.796	0.812
Sma-E174	F: CCCAGATGAGACATGGACAA R: ACAGTATGTGGGGCCTTTCAG	58 °C 1.5 mM	3	(CA)12	82–86	0.563	0.055
Sma-E180	F: AGAGCAATGTAAGCGCCTTT R: CTTGGTACAGCATTCACGATG	58 °C 1.5 mM	3	(AC)12	218–222	0.485	0.403
Sma-E183	F: GAAACAGGAAGGGAACAGCA R: CTTTGGTCCTTGCCAACACT	58 °C 1.5 mM	2	(TTG)5	289–292	0.145	1.000
Sma-E184	F: AGGACGACACAACCATCACA R: AACCTCCTCTCTCTGGAGCC	58 °C 1.5 mM	6	(GGA)9	241–266	0.754	0.001
Sma-E187	F: GTTCGTGTCGCTGAAGATGA R: ACAGACGGAACAGCAGTGAG	50 °C 1.5 mM	2	(CTC)9	279–288	0.099	1.000
Sma-E189	F: CGACTGACCTCTGCATCGTA R: GCCTCCTGAAGACGCTATTG	56 °C 1.5 mM	4	(TG)8	271–279	0.552	0.390
Sma-E191	F: GGAGGGCGAAGAAGAAGAAG R: GCTGCTCCAGTCTGCGTT	58 °C 1.5 mM	6	(CGA)4	267–282	0.670	0.247
Sma-E194	F: CCACACGTTGCTATACACGG R: ACGGTAAGAGAGGAGACGCA	52 °C 1.5 mM	6	(TC)10	116–126	0.669	0.905
Sma-E195	F: CGCCTGAGAGTTTCTCTTCC R: AACAAACAAAGCTCCGCAGT	52 °C 1.5 mM	11	(TG)23	313–344	0.820	0.000
Sma-E197	F: AGCTCTGTTGGAGGAACACG R: GTAGCAGAGGAGCTGGATGG	58 °C 1.5 mM	2	(GAC)4	378–380	0.082	1.000
Sma-E205	F: GTCCCGGTGAGGAGTACAGA R: TCAGCCGGATAGGGAAGATA	54 °C 1.5 mM	2	(AGG)6	235–237	0.498	0.400
Sma-E215	F: TGTTGCATTCCGAGAAACTG R: GACCATGCCCTTGATTTGTT	57 °C 1.5 mM	2	(AGA)11	410–419	0.569	0.444

Table 1 (Continued)	Table
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Locus	Primers 5'-3'	Annealing temp [MgCl2]	No. of alleles	Repeated motif	Size	Expected heterozygosity	Hardy– Weinberg P-value
Sma-E218	F: GGATTGGCTTCTGAAATGGA	49 °C	3	(AAAC)6	169–177	0.413	0.900
Sma-E220	R: GAGGETGGACACCAAGACTG F: CAGGATTGAGGAGGAGCTTG	1.5 mM 55 °C	2	(AC)5	212–214	0.175	1.000
Sma-E224	R: ACCACAGAGAGAGGAGCCIIGG F: GCTCAGAGAGAGAGAGAGCGG	1.5 mM	4	(AACA)7	248–267	0.746	0.078
Sma-E225	F: GCCAAAGGAATGTCGGTAAA	1.5 mM	3	(GT)12	284–288	0.604	0.620
Sma-E227	R: CACACACACACACICACCCA F: GAAGGCGGTAATCATCCAGA R: CCTTCACACCTCCTCTTTCA	1.5 mW 58 ℃ 1.5 mM	2	(CT)12	315–319	0.474	0.216
Sma-E231	F: CAGTTGTGGGTGTGAGGTTG R: CGTCACGAGAGAAATGAGGC	57 ℃ 1.5 mM	5	(TCC)6	302–308	0.490	0.512
Sma-E244	F: TCCATGCAAAGCAGACACAT R: CACACCGTGCATTCAAGTTC	54 ℃ 1.5 mM	3	(CA)9	303–307	0.417	0.140
Sma-E248	F: TGGGACTTAATGGGACAAGG R: GAATACCCACCCAAATGCAC	58 ℃ 1.5 mM	3	(TG)18	271–287	0.764	0.786
Sma-E254	F: ATGCCGTCCCACTACAGTTC R: CCACATTCTACTGGCGAGGT	54 °C 1.5 mM	3	(GCT)8	188–241	0.648	0.797
Sma-E255	F: TCTATGGAGCCCACAAGTCC R: TCAACCTGGTGAAGAAAGGC	56 °C 1.5 mM	3	(CA)6	314–324	0.509	0.000
Sma-E261	F: CTGGAAGGAGGAAAGAACCC R: GCTGAGCGGAGAGAGAGAGAGA	51 °C 1.5 mM	8	(CT)7	90–95	0.532	0.786
Sma-E270	F: TGACACCATTTCTGGGAACA R: GAGGCACGCGACTACTTCAC	57 ℃ 1.5 mM	2	(TTGA)3	319–332	0.474	0.444
Sma-E272	F: TGCAACTAGCCGATTTAACCA R: GTTGAGGACAAAGCCGAGAG	47 ℃ 1.5 mM	3	(CTT)4	207–212	0.192	0.068
Sma-E276	F: CTCAATCACGCTCTCACACG R: CCGAGGGACGGAGATACATA	63 °C 2 mM	4	(CA)12	115–145	0.475	0.236
Sma-E277	F: AGACACAAGCGCACACAGAC R: TCCAGAGCTGAACATCACCA	58 °C 2 mM	2	(TC)9	322-330	0.743	0.276
Sma-E279	F: TGTTATAGCCGACAGCAGCA R: TCACTCCCGGTCTGATGTTT	54 °C 2 mM	3	(TCC)9	297–313	0.687	0.756
Sma-E283	F: TCACAGCTTGGGCCTTATTT R: AGTTACAGCAGCAGGCAACA	54 °C 2 mM	8	(AC)15	283–293	0.672	0.222
Sma-E284	F: ACTTCATCCGCTTTGACTGC R: GGGCGAAGGAGTTGTGTTTA	57 °C 2 mM	5	(GT)9	319–323	0.444	0.457
Sma-E286	F: ACGACAGCGACACACACACT R: TACATTCGGTGACGATGCTG	54 °C 0.5 mM	7	(GT)12	243–257	0.756	0.756
Sma-E289	F: CAATGAGGACTGATGCTTCG R: GTTCAGCGACAGGAAGTGCT	54 °C 0.5 mM	7	(GT)30	240–366	0.951	0.324
Sma-E290	F: GAGACCCACAGACCTCGTGTA R: TGTTCTTTGGTCCCTTGCTC	53 °C 2 mM	4	(TG)16	336–354	0.762	0.548
Sma-E293	F: CTGTAGCAGCCTCCTCCCT R: GGAGAACAAAGTCCGTCCAC	59 ℃ 1.5 mM	7	(CGT)7	215–221	0.506	0.901
Sma-E294	F: GCATCGTGAAACACTGGAGA R: GAACGAACCAAACCACGACT	58 °C 2 mM	2	(TAA)6	218–224	0.221	0.067
Sma-E302	F: TCTTTGTCCAGAACAGTCGG R: CATGTGAAATTGGCAGCATC	57 °C 2 mM	2	(AGC)7	313–331	0.270	0.149
Sma-E305	F: GATTTGTGTGGAAACTGCCAT R: CCATGCAAACACTCACCTGT	56 °C 2 mM	3	(TG)16	211–215	0.302	0.006
Sma-E310	F: CGCTCCTGCACATCTACACT R: GGCTCCCTCAACACACAAAT	55 °C 2 mM	5	(AT)10	215–225	0.547	0.545
Sma-E315	F: CCGTTCAGAATACCTGCTCC R: TGTCGCTCTCTGCTGGTCTA	50 °C 2 mM	2	(TTA)8	187–190	0.379	0.720

Locus	Primers 5'-3'	Annealing temp [MgCl2]	No. of alleles	Repeated	Size	Expected	Hardy– Weinberg P-value
Locus		[1416C12]	uncles	moui	ULL	neterozygosity	i value
Sma-E316	F: GAATGGAAATGGATGCAGTGT	56 °C	5	(AC)9	282–292	0.697	0.836
	R: TGTAACAACCGTGTGTCTGTC	1.5 mM					
Sma-E317	F: GTGACCCTCTGACCTTTGCT	58 °C	3	(GT)8	80-84	0.557	0.440
	R: ACACACCTCAGTGCAGAACG	1.5 mM					
Sma-E318	F: CCTGAACACTGGAACCTTCA	56 °C	3	(GT)14	247-257	0.579	0.332
	R: AATAACTCACCTAGCACTCACG	1.5 mM					

Table I (Continued)	Table	1	(Continued)
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available for all of them. However, because some parents or grandparents had died when offspring were collected, DNA was only available for 18 of them. Genotyping information from these families is highly valuable for further mapping analysis. The GENEPOP 4.0 program (Raymond & Rousset 1995; Rousset 2008) was used to estimate the genetic diversity [expected heterozygosity (He) and number of alleles (A)] and to check for conformance to Hardy-Weinberg (HW) expectations. To manage an appropriate sample size for these analyses, we decided to include the 18 individuals from SSF families because of their Atlantic origin and the low genetic structure reported for turbot populations from this area (Vilas et al. 2010). However, caution was taken especially when analysing genetic disequilibria by reason of divergence after two generations of selection. Micro-Checker 2.2.3 was used to investigate the causes of HW deviations (Van Oosterhout et al. 2004) and CERVUS 3.0.3 (Kalinowski et al. 2007) to estimate the frequency of null alleles and combined exclusion probabilities for parentage assignment.

Conformance to Mendelian segregation

Progenies ranging between 85 and 96 offspring in five of the aforementioned families (F1–F5) were finally used to evaluate the conformance to Mendelian segregation of polymorphic loci. Chi-square tests were applied to check the null hypothesis, using the progeny from at least one of the families. Segregation distortion was tested at each locus, adjusting the significance level for multiple tests within each type of segregation (1:1, 1:1:1:1 or 1:2:1) using Bonferroni correction (Rice 1989).

Microsatellite conservation analysis within Pleuronectiformes

We were interested in analysing the degree of evolutionary conservation of microsatellites, but especially within Pleuronectiformes, given the low cross-species amplification previously reported in this order (Bouza *et al.* 2002; Castro et al. 2006). The in silico information obtained would also be of practical interest to develop new microsatellites in other species and to obtain shared microsatellites useful for comparative genomics between flatfish species. The increase in EST resources in several Pleuronectiformes pertaining to different families made this analysis possible. Flanking regions of all polymorphic and monomorphic turbot microsatellites were BLASTed using BLASTn (Altschul et al. 1990) against EST resources of the three flatfish species with highest genomic information: bastard halibut (13 869 sequences), Atlantic halibut (20 886 sequences) and Senegalese sole (5208 sequences). All sequences were retrieved from the NCBI-EST database (http://www.ncbi.nlm.nih.gov/dbEST/) as they were of December 2010, except for S. senegalensis sequences, which were extracted from the Pleurogene project database (Cerdà *et al.* 2008). Best hits at $<10^{-5}$ E-value cut-off and >80 bp were considered. Alignments of significant hits were performed using ClustalX (Thompson et al. 1997) followed by manual adjustment. As the homologies found between sequences were based on flanking regions, we considered the microsatellite motif, the number of repeats and presence/absence status of the microsatellite to evaluate the degree of conservation. Accordingly, we established three different categories: (i) monomorphic microsatellites in turbot, with either identical or different repeat number in other flatfish species; (ii) polymorphic microsatellites in turbot with either identical or different repeat number in other species; and (iii) microsatellite-containing sequences in turbot lacking microsatellite in other species.

Annotation

For putative function determination and annotation, EST-bearing microsatellite sequences were BLASTed against several model fish genomes (http://genome.ucsc. edu/index.html) and the GenBank nucleotide collection (http://www.ncbi.nlm.nih.gov/genbank/) by means of BLASTx (*E*-value cut-off: <10⁻³) or BLASTn (*E*-value cut-off: <10⁻⁵) when no BLASTx hits were found.

Results

Microsatellite selection and amplification conditions

Among the 16 255 high-quality ESTs (>100 bp; PHRED >20) sequences included in the turbot database, we identified 298 microsatellite-containing sequences according to the established criteria and with long enough flanking regions to design primers. In 228 of them, a band with the expected size was found when the PCR products were run in agarose gels. In another 13 cases, the amplicons exceeded the expected size (with bands from 400 to 600 bp), which may indicate the presence of intron(s) in the genomic DNA. All the 241 primer pairs were tested in the automatic sequencer.

Gene diversity and population analysis

From the 241 technically selected microsatellites, 81 (33.6%) showed poor resolution, while the remaining 160 yielded unambiguous genotyping in the automatic sequencer. These 160 sequences were deposited in Gen-Bank and given a corresponding accession number (Table S1). Among them, 77 (48.1%) corresponded to monomorphic loci and 83 (51.9%) resulted polymorphic. Genetic diversity was estimated in a sample of 40 individuals of Atlantic origin for all polymorphic loci. The number of alleles per locus ranged from 2 to 18 (mean = 4.9 ± 0.354) and expected heterozygosity between 0.021 and 0.951 (mean = 0.540 ± 0.039) (Table 1). Among the 83 polymorphic loci, six (Sma-E145, Sma-E225, Sma-E227, Sma-E231, Sma-E276 and Sma-E277) belonged to sequences reanalysed from partial genomic DNA libraries (Pardo et al. 2006) incorporated to the ESTenriched database. Seventy-six of the 83 polymorphic loci conformed to HW expectations after Bonferroni correction. The remaining 7 loci showed significant deviation owing to heterozygote excess (Sma-E154) or deficit (Sma-E61, Sma-E96, Sma-E117, Sma-E132, Sma-E195 and Sma-E255; Table 1), suggesting the presence of null alleles in the last group. Null allele frequencies were estimated using CERVUS 3.0.3: Sma-E61 (0.482), Sma-E96 (0.367), Sma-E117 (0.786), Sma-E132 (0.438), Sma-E195 (0.182)

and Sma-E225 (0.149). The presence of null alleles was confirmed by using the Micro-Checker 2.2.3 software, which also suggested genotyping errors owing to stuttering at Sma-E61, Sma-E96, Sma-E117 and Sma-E132. No evidence for allele dropout was found at any locus (P > 0.05). Eight of 13 loci with amplicons longer than expected were polymorphic and three of them (Sma-E61, Sma-E96 and Sma-E154) deviated from HW expectations (Table 1). The probabilities to exclude a false parent for paternity inference ranged from 0.005 (Sma-E187) to 0.787 (Sma-E289) for the first parent (Excl1) and from 0.069 (Sma-E187) to 0.881 (Sma-E289) for the second parent (Excl2). Combined exclusion probabilities for parentage assignment using the 83 polymorphic loci were virtually 1 for both Excl1 and Excl2.

Family analysis

All but three microsatellites (Sma-E134, Sma-E145 and Sma-E293) could be tested for Mendelian segregation in the families. Eight of the 80 tests performed deviated from Mendelian expectations (Table S2), but none of them after Bonferroni correction.

Microsatellite conservation within flatfishes

In order to analyse the conservation of turbot microsatellite sequences in other flatfish species, we explored EST databases of three representative Pleuronectiformes: bastard halibut, Atlantic halibut and Senegalese sole, each belonging to different families (Paralichthyidae, Pleuronectidae and Soleidae, respectively). Of 160 sequences tested, including both polymorphic and monomorphic microsatellites, 49 (approximately 31% of the total) showed a significant match (*E*-value $<10^{-5}$) with any of the EST databases explored (Table 2). Of these, 10 (approximately 6%) showed a match with all species analysed, while 24 (approximately 15%) and 15 (approximately 9%) showed match with one or two species, respectively. We found a higher number of matches with the bastard halibut EST database (39) than with the other species: halibut (26) and sole (22). The

Table 2 Significant hits of the 160 turbot microsatellite-containing sequences with other flatfish species: Senegalese sole (Solea senegalensis), bastard halibut (Paralichthys olivaceus) and Atlantic halibut (Hippoglossus hippoglossus)

	Hits (%)	B. halibut	S. sole	Halibut	B. halibut/S. sole	B. halibut/A. halibut	S. sole/A. halibut
Turbot-specific microsatellites	111 (69.4)	_	_	_	_	_	_
Match with one species	24 (15)	15	3	6	-	-	-
Match with two species	15 (9.4)	_	_	-	6	7	2
Match with all species	10 (6.2)	-	-	-	-	-	-

S. sole, Senegalese sole; A. halibut, Atlantic halibut; B. halibut, bastard halibut.

remaining 111 sequences (approximately 69%) were turbot-specific and displayed no match with the EST databases explored (Table 2). On the other hand, variable sequence conservation was observed when comparing turbot microsatellite-containing sequences against the same flatfish databases (Table 3). Of the 49 homologous microsatellites found in the other flatfish databases, 26 were monomorphic and 23 polymorphic in turbot. All of them showed the same microsatellite motif in the other species where a significant match was detected, although with imperfections in some cases. Among monomorphic loci, nine showed the same repeat number in the other species, 13 showed different repeat numbers, and three lacked microsatellite. Among polymorphic ones, five microsatellites showed the same repeat number as a certain turbot allele, six differed in repeat number, and 10 lacked microsatellite. Approximately in half of the cases the homologous repetitive motif was imperfect. Despite the conservation of one of the flanking regions, no data could be obtained for three microsatellites because the corresponding EST sequences were incomplete in flatfish databases, and no information existed for the repetitive region (Table 3).

EST annotations

After BLASTing polymorphic microsatellite-containing sequences against different protein and nucleotide databases, most polymorphic loci showed significant sequence similarity with annotated genes and, in three cases (Sma-E254, Sma-E283 and Sma-E317), with genomic DNA or mRNA clones of fish model species (Table S3). Among the sequences belonging to partial genomic libraries, there were even three that showed significant homology with gene-related sequences (Sma-E145, Sma-E225 and Sma-E231). The other three could also be considered informative markers because they showed homology with specific unidentified cDNA sequences or with clones of genomic DNA regions of other fish species (Table S3).

Discussion

Genomic resources have greatly increased in aquaculture species especially after the arrival of new generation sequencing (NGS) technologies, and several transcriptomes and whole-genome sequencing projects are underway in different fish species (Davidson et al. 2010; Kuhl et al. 2010). Exploitation of these resources requires their organization in databases with appropriate bioinformatic tools for sequence edition, clustering, annotation and functional classification among others. EST databases have proven to be a valuable source of molecular markers such as microsatellites (Bouza et al. 2008; Cerdá et al. 2010) and SNPs (Pardo et al. 2008; Vera et al. 2011). Within economic important flatfish species such as H. hippoglossus (Douglas et al. 2007), P. olivaceus (Liu et al. 2006) and Cynoglossus semilaevis (Liu et al. 2007), EST sequences have been used to identify microsatellite loci mostly to be used for linkage map construction. In turbot, a screening of the EST database v1.0 (Pardo et al. 2008) yielded a set of 31 type I markers useful for genetic mapping and population genomics (Bouza et al. 2008; Vilas et al. 2010).

In this study, we characterized 83 new polymorphic microsatellites from the updated turbot EST database (Pardo et al. 2008; Vera et al. 2011). Genetic diversity of these 83 loci showed allele number and expected heterozygosity in the range previously observed in other EST microsatellites characterized in flatfish (Liu et al. 2006, 2007; Chen et al. 2007; Douglas et al. 2007), including turbot (Bouza et al. 2008). Deviation from HW expectations was found in seven loci (8.4%), mostly attributable to the presence of null alleles. The 83 microsatellites characterized in this study, together with the previous ones and the 77 SNPs characterized by Vera et al. (2011), constitute a suitable set of EST-linked markers to consolidate the turbot genetic map mostly based on anonymous markers (Bouza et al. 2007, 2008; Ruan et al. 2010). Also, these markers will be essential for comparative genomics strategies and to extend analysis on adaptive variation in turbot populations (Vilas et al. 2010).

status	
	Same motif

Table 3 Classification of microsatellite sequence showing significant hits according to their motif, repeat number and presence/absence

Turbot loci	Same repeat number	Different repeat number	Lacking microsatellite	No data	Total
Monomorphic	9 (3)	13 (6)	4	1	26
Polymorphic	5 (2)	6 (3)	12	2	23
Total	14 (5)	19 (9)	16	3	49

All turbot microsatellites considered in the analysis were perfect. In parentheses, the number of imperfect repeats is given. When comparing polymorphic microsatellites, we considered the same repeat number if a match with any of turbot alleles was observed at that locus.

There are several flatfish species of great commercial value, and relevant genomic advances have been made in four of them (bastard halibut, Senegalese sole, Atlantic halibut and turbot). Cross-checking this information is then crucial to obtain insights on flatfish evolution and to identify the genomic regions and/or candidate genes related to productive traits. To achieve this goal, it is essential to construct a flatfish database, which would be additionally useful to identify the common genetic markers to anchor genetic and physical maps between species. In our study, we carried out a first approach by comparing the conservation of turbot microsatellites with the existing EST flatfish databases. This comparison provided data on microsatellite evolution and also useful information to identify common sets of microsatellites between different flatfish species. We could detect significant homology of 31% of turbot microsatellite flanking sequences in the other EST flatfish databases, which demonstrates a certain conservation of turbot microsatellites in the genomes of related species, as reported for other fish groups (Rico et al. 1996; DeWoody & Avise 2000). Higher similarities were detected with the bastard halibut EST database (39 matches) than with the other species [Atlantic halibut (26 matches); Senegalese sole (22 matches)]. This agrees with phylogenetic relationships of Pleuronectiformes, which place the family Scophthalmidae closer to Paralichthyidae and Pleuronectidae than to Soleidae (Pardo et al. 2005). Also, the lower similarity observed with Senegalese sole is in accordance with the low cross-species amplification previously reported (Castro et al. 2006). In our comparison, a notable proportion of perfect turbot microsatellites (43%) turned out to be imperfect in the other flatfish species analysed, and all data indicate that microsatellite conservation was higher at monomorphic loci, suggesting a higher evolutionary rate for polymorphic ones. Moreover, dramatic changes, involving the complete loss of the microsatellite, were observed at 27% of loci despite the fact that flanking regions were mostly conserved. Our study is preliminary and we cannot rule out a certain bias in these figures because of the incompleteness of the databases explored. In fact, the Atlantic halibut database is the best represented one with one and a half times more sequences than that of bastard halibut, both databases showing a similar average sequence size (around 600 bp). On the other hand, the Senegalese sole database contains only 25% sequences of the halibut database, but with higher average size (715 bp).

Microsatellite markers characterized in this study are being integrated together with a SNP panel (Vera *et al.* 2011) in the updated version of turbot genetic map (C. Bouza , M. Hermida, B. G. Pardo, M. Vera, C. Fernández, R. de la Herrán, R. Navajas, J.A. Álvarez-Dios, A. Gómez-Tato and P. Martínez, submitted) and in a new Senegalese sole map (M. J. Molina-Luzón, M. Hermida, J. I. Navas, F. Robles, R. Navajas-Pérez, P. Martínez, C. Ruiz-Rejón and R. de la Herrán, unpublished data). They are mostly annotated and have exhibited significant homologies with other flatfish species highly valuable for comparative mapping to look for candidate genes related to adaptive variation or productive characters.

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Data Accessibility

DNA sequences: GenBank accessions (see Table S1 or Dryad repository).

Data deposited in the Dryad repository: doi:10.5061/ dryad.q2c86hb2.

Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Comparative analysis of turbot microsatellites with Senegalese sole (*Solea senegalensis*), bastard halibut

(*Paralichthys olivaceus*) and Atlantic halibut (*Hippoglossus hippoglossus*).

Table S2 Segregation analyses of 83 EST-derived micro-satellites in five turbot families.

Table S3 Annotation of EST-derived microsatellites using BLASTx and BLASTn against several model fish genomes and the nucleotide collection of GenBank.

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