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A combined AFLP and microsatellite linkage map and pilot comparative genomic analysis of European sea bass *Dicentrarchus labrax* L.

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Summary

European sea bass (*Dicentrarchus labrax* L., Moronidae, Teleostei) sustains a regional fishery and is commonly farmed in the Mediterranean basin, but has not undergone much long-term genetic improvement. An updated genetic linkage map of the European sea bass was constructed using 190 microsatellites, 176 amplified fragment length polymorphisms and two single nucleotide polymorphisms. From the 45 new microsatellite markers (including 31 type I markers) reported in this study, 28 were mapped. A total of 368 markers were assembled into 35 linkage groups. Among these markers, 28 represented type I (coding) markers, including those located within the *peptide Y*, *SOX10*, *PXN1*, *ERA* and *TCRB* genes (linkage groups 1, 7, 16, 17 and 27 respectively). The sex-averaged map spanned 1373.1 centimorgans (cM) of the genome. The female map measured 1380.0 cM, whereas the male map measured 1046.9 cM, leading to a female-to-male (F:M) recombination rate ratio of 1.32:1. The intermarker spacing of the second-generation linkage map of the European sea bass was 3.67 cM, which is smaller than that of the first-generation linkage map (5.03 cM). Comparative mapping of microsatellite flanking regions was performed with five model teleosts and this revealed a high percentage (33.6%) of evolutionarily conserved regions with the three-spined stickleback.

Keywords amplified fragment length polymorphism, comparative map, *Dicentrarchus labrax*, genomics, microsatellite, single nucleotide polymorphism, synteny.

Introduction

Among vertebrates, fishes are evolutionarily and ecologically a highly diverse and successful group. They offer key answers to questions on human evolution and are a main source of animal protein through fisheries and aquaculture (Boffelli *et al.* 2004). Although the role of aquaculture is increasing annually, most species of fish have a very short domestication history. There is an obvious lack of genetic knowledge on fishes and their populations, but the steadily growing access to genomic resources provides great opportunities for enhanced genetic improvement. Increased production can be envisaged through selective breeding for a range of traits (such as growth, oxygen stress, disease resistance and production traits), breeding of sex-controlled populations, hybridization, chromosome and gene manipulation (Hulata 2001). A high-density linkage map greatly benefits the location of molecular markers for traits suitable for selective breeding. Moreover, high-density genetic maps were a prerequisite for the location of a growing number of quantitative trait loci (QTL) in fish (Liu 2003; Rogers & Bernatchez 2005; Shirak *et al.* 2006).

The comparison of teleost genomes facilitates the identification of evolutionarily conserved chromosomal fragments (Chen *et al.* 2004). The genomes of the model species medaka, zebrafish, fugu and spotted green pufferfish are widely used for such comparisons (Woods *et al.* 2000;

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Rexroad *et al.* 2005; Kasahara *et al.* 2007). Comparisons of genomes within fish families, such as for the Salmonidae (Gharbi *et al.* 2006), have also been made. Major findings include evidence for an ancestral vertebrate karyotype of 12 chromosomes and high levels of conservation of synteny, although this decreases with increasing phylogenetic distance (Chen *et al.* 2004; Jaillon *et al.* 2004). Once conservation of synteny is established, the identification of heritable traits of interest will be facilitated. This can be achieved either by a genome scan or by comparative QTL analyses directed in a targeted way by synteny conservation and associated gene content information.

Over the past few years, European sea bass *Dicentrarchus labrax* L. (Moronidae, Perciformes, Teleostei) has become one of the most intensively studied fish species because of its importance as a food source and because of some unique characteristics such as temperature-dependent sex change (Pavlidis *et al.* 2000; Piferrer *et al.* 2005), adaptive plasticity to a range of habitats (Nebel *et al.* 2005) and feeds (Kaushik *et al.* 2004) and its compact genome size (Peruzzi *et al.* 2005). Genomic resources have expanded at a steady pace over the past 10 years (for a review, see Volckaert *et al.* 2008). Several kinds of polymorphic genetic markers were developed, including microsatellites (García de León *et al.* 1995; Castilho & McAndrew 1998; Ciftci *et al.* 2002; Tsigenopoulos *et al.* 2003; Chistiakov *et al.* 2004, 2005), random amplified polymorphic DNA (Caccone *et al.* 1997), mitochondrial DNA markers (Patarnello *et al.* 1993; Cesaroni *et al.* 1997), amplified fragment length polymorphisms (AFLPs, this study) and single nucleotide polymorphisms (SNPs) (Chistiakov *et al.* 2007). Except for AFLPs, all markers were successfully used in studies of the population structure of sea bass and particularly microsatellites (Naciri *et al.* 1999; Bahri-Sfar *et al.* 2000; Lemaire *et al.* 2000, 2005; Castilho & Ciftci 2005).

In sea bass, the first exploratory mapping experiment was based on 23 microsatellites and resulted in a genetic map of eight linkage groups (Chistiakov *et al.* 2004). Then, a first-generation linkage map comprised 162 microsatellite markers (including two genes) assembled into 25 linkage groups; the length of the sex-average map was 815 centimorgans (cM); the map based on female meioses covered 906 cM and was 1.6-fold longer than the male map, which encompassed 567 cM (Chistiakov *et al.* 2005).

Microsatellites are very useful for building solid map frameworks, which could be later enriched with other types of markers, as for example, the case with the linkage maps of rainbow trout (Nichols *et al.* 2003), brown trout (Gharbi *et al.* 2006) and zebrafish (Woods *et al.* 2000). A limited number of AFLP maps were made in animals to provide quick and high-density genome coverage (Liao *et al.* 2007). The dominant nature of the marker complicates the integration of an AFLP map into any other inter- or intraspecific genetic map that is based on codominant markers. More recently, SNPs have become increasingly popular because of their common occurrence in the genome (on average every 90 bp in sea bass; E. Souche, personal communication), uniqueness, ease of scoring and, above all, reproducibility (Liu & Cordes 2004).

Here, an updated linkage map of the European sea bass is presented. It is significantly denser than a first-generation map (Chistiakov *et al.* 2005) and consists of a higher number of microsatellites and additional types of polymorphic markers such as AFLPs and SNPs. The effectiveness of this map was assessed in a pilot comparative analysis of microsatellite flanking regions between the European sea bass and five model ray-finned fish species. A high level of conservation of synteny in comparison with the three-spined stickleback was established.

Materials and methods

Isolation of polymorphic markers

Microsatellites were isolated from microsatellite-enriched genomic libraries as described by Tsigenopoulos *et al.* (2003) and Chistiakov *et al.* (2004). Microsatellites from genes and mRNA of *D. labrax*, expression sequence tags (ESTs) from a liver cDNA library (Chini *et al.* 2006) available in the GenBank database and ESTs from a brain cDNA library were developed as reported by Chistiakov *et al.* (2005). Characteristics of new polymorphic microsatellite markers are shown in Table S1.

AFLP markers were developed using a total of 64 combinations of each of EcoRI-specific (EcoRI-ACC and EcoRI-ACG) primers with each of MseI-specific primers. The AFLP procedure was performed essentially as described by Vos *et al.* (1995). The Venezia Fbis family consisting of both parents and 50 full-sib progeny (biparental diploids) (Chistiakov *et al.* 2004) was fingerprinted using the LI-COR 4200 DNA sequencer (LI-COR BioSciences).

SNPs were developed by resequencing several genes and cDNAs of *D. labrax*, whose sequences were available in the GenBank database, in 10 unrelated sea bass individuals, using the ABI 3130 Genetic Analyzer (Applied Biosystems). A PCR-RFLP approach was used to develop new SNPs. SNP characteristics are summarized in Table 1.

Linkage analysis

For linkage analysis, the Venezia Fbis family was genotyped using the newly developed microsatellite, AFLP and SNP markers. Using the CRI-MAP program v3.0 (Green *et al.* 1990), the analysis was performed as described by Chistiakov *et al.* (2005). The microsatellite-based linkage map of Chistiakov *et al.* (2005) was used as a framework to incorporate new markers mainly represented by AFLPs. Sex-averaged and sex-specific linkage distances were estimated for each linkage group assuming the Kosambi (1944) mapping function.

Table 1 Characteristics of newly developed SNPs of the European sea bass, including location (gene and SNP position), PCR primer sequences and conditions (annealing temperature, Mg^{2+} concentration and number of PCR cycles), restriction enzyme digestion information (name of enzymes, definitions of alleles and lengths of digestion products) and allelic frequencies.

Gene	SNP location	PCR primers (5′–3′)	T_A (°C)	Mg^{2+} (mM)	No. PCR cycles	Restriction enzyme to digest PCR product	Definition of alleles and lengths of digestion products (bp)	Allelic frequency (no. individuals tested, *n*)
ACTB	c.*575T>C	F: CATGGTTGTAGTATCGCTTGTA R: GCACCCCATGTTACCCCTTG	60	2.0	40	AatII	C: 178+20 T: 198	C/T = 0.675/0.325 (20)
ERB1	c.*147C>T	F: GACACACACATTTTTAGAACGGAA R: GGCACATGAAGTCGTTCATTATC	54	2.0	35	EcoRI	C: 222 T: 220+20	C/T = 0.864/0.136 (22)
IL1B	c.76+52C>T	F: TTCTGGAGCAGATTTTAATCA R: CATCCGACAGAATAGATCTG	54	2.0	40	PagI	T: 129+19 C: 148	C/T = 0.952/0.048 (21)
IL1B	c.76+157A>G	F: TTCTGGAGCAGATTTTAATCA R: CATCCGACAGAATAGATCTG	54	2.0	40	NheI	A: 128+22 G: 148	A/G = 0.952/0.048 (21)
IL1B	c.76+215A>G	F: CTATGATATGGCAGCTGTACGC R: CATTTCCTTTCCTTGAATATAAA	54	2.0	40	PauI	A: 143 G: 122+21	A/G = 0.975/0.025 (20)
IL1B	c.76+310A>G	F: CTATGATATGGCAGCTGTACGC R: CATTTCCTTTCCTTGAATATAAA	54	2.0	40	SspI	A: 119+24 G: 143	A/G = 0.868/0.132 (19)
IL1B	c.300+263C>T	F: CATGCTGGAGAGCATAGTGG R: CAGACAACAGGACAATAATAGACAT	58	1.0	35	XceI	C: 270+24 T: 294	C/T = 0.929/0.061 (21)
IL1B	c.300+329C>T	F: GGTGTTTAGGGTTTGTTAAAAGGC R: TGTGTCTCTTCATGCGCAATCGA	58	2.0	35	Eco147I	C: 127+23 T: 150	C/T = 0.976/0.024 (21)
IL1B	c.300+431A>C	F: GGTGTTTAGGGTTTGTTAAAAGGC R: TGTGTCTCTTCATGCGCAATCGA	58	2.0	35	Bsu15I	A: 128+22 C: 150	A/C = 0.905/0.095 (21)
TCRB	c.*241C>T	F: GTTCAAAGGGAAAGCAGAACTAC R: CTCTGAAAAGTTTTTATTTAGATGAAT	58	1.0	35	Eco47III	C: 258+49 T: 307	C/T = 0.818/0.182 (22)
SOX10	c.86+148C>A	F: AATTATAAGCTCAAATTGTTGATTT R: AAATAAAGTTTGAGTAAAAATTATAT	55	2.0	40	DraI	C: 129 A: 104+25	A/C = 0.773/0.237 (22)

Comparative genomics

The genomes of teleost fishes, the spotted green pufferfish *Tetraodon nigroviridis* (v. 7.40), the tiger pufferfish or fugu *Takifugu rubripes* (v. 4.40), medaka *Oryzias latipes* (v. 1.41), the three-spined stickleback *Gasterosteus aculeatus* (v. 1.41) and zebrafish *Danio rerio* (v. 6.40), were downloaded from ftp://ftp.ensembl.org/pub/. Local BLAST searches were performed under default settings using individual sequences of 186 sea bass genomic clones as queries and the different genomes' repeat-masked sequences as subjects. Hits with $e < 10^{-5}$ were considered significant. The aligned region and the flanking sequence of the subject (±1 kb) were then extracted from the genomes. Searches for non-repeat-masked sequences and a Smith–Waterman alignment were performed with the FASTA v.2.0 package (Pearson & Lipman 1988) with sea bass microsatellite clones as queries. Microsatellites were identified and characterized with a PERL script based on the MISA script (http://pgrc.ipk-gatersleben.de/misa). The parameters were set for the detection of di- to hexanucleotide motifs with a minimum of 6, 4, 3, 3 and 3 repeats respectively. For the analysis of the structure of the transcripts, local SQL expressions were developed on the Ensembl (Hubbard *et al.* 2007) databases (v. 40 and 41 for both Ensembl and MART ftp://ftp.ensembl.org/pub). The MAPCHART software v2.2 (Voorrips 2002) was used for the graphical visualization of the maps. The above procedure was automated in a pipeline; Perl scripts are available upon request to JL.

Results

Development of new microsatellite markers

Fourteen new type II (non-coding) polymorphic dinucleotide microsatellites (*DLA0038–DLA0051*) were isolated from two $(AC)_{12}$ and $(AG)_{12}$ microsatellite-enriched DNA libraries of *D. labrax* (Table S1). The screening of 510 sea bass liver ESTs and 179 brain ESTs resulted in the selection of 111 sequences containing putative microsatellites. Thirty-one type I markers (13 extracted from the liver and 18 from brain cDNA libraries respectively) showed polymorphism in the population sample of 21 unrelated sea bass individuals. The polymorphic microsatellites were designated *DLA0250E–DLA269E* and *DLA271E–DLA281E* (Table S1). Locus *DLA0254PXN1* showed strong homology with *pentraxin-1*, a member of the family of pentraxins representing cytokine-inducible acute phase proteins, which are implicated in a variety of functions, such as innate immunity, sperm-egg fusion and neuronal synapses.

Analysis of the nucleotide sequence of several *D. labrax* genes and cDNAs revealed two sequences containing polymorphic type I microsatellites. *DLA0261ERA* contains a dinucleotide motif located at intron 3 of the *ERA* gene encoding estrogen receptor-α, and *DLA0281NROB1* is situated within the *NROB1* gene. DAX-1, a product of the *NROB1* gene, has a DNA-binding domain and functions as an anti-testis gene by acting antagonistically to the *Sry* locus (Zhang *et al.* 2000). Thus, the addition of 45 new microsatellites (31 type I) reported here increases the total number of available microsatellite loci of *D. labrax* to 246, including 59 type I markers. Of the 59 coding (type I) markers, eight microsatellites reside in known genes.

Development of AFLPs

The analysis of 64 combinations of EcoRI/MseI primers in the Venezia Fbis family revealed a total of 221 AFLPs, which, on average, corresponded to 3.45 polymorphic bands per primer combination (Table S2). Various numbers of markers were produced depending on the primer combinations. The combinations of MseI-CAG/EcoRI-ACC and MseI-ACC/EcoRI-ACC were the most informative primers, resulting in 14 and 10 polymorphic bands respectively. Among the 221 AFLPs detected, 44 were heterozygous in both parents, while the remaining 177 were sex-specific [i.e. heterozygous in the male (96 bands) or female (81 bands) parent].

Identification of SNPs

For SNPs, we screened the following sequences: 3′ untranslated regions (UTRs) of mRNA encoding estrogen receptor β_1 (*ERB1*; AJ489523) and β_2 (AJ489524), cyclooxygenase 2 (*COX2*; AY336080), gonadotropin-releasing hormone receptor (*GRHR*; AJ419594), actin β (*ACTB*; AJ537421) and T-cell receptor β (*TCRB*; AJ493441); intronic sequences of genes encoding sex-determining region Y-box 10 (*COX10*; AY247003) and fructose 1,6 diphosphate aldolase (*FDA*; AJ493441) and the complete genomic sequence of the *interleukin-1*β gene (*IL1B*; AJ311925). A total of eleven SNPs were developed (Table 1). These SNPs include *ACTB* c.*575T>C, *ERB1* c.*147C>T and *TCRB* c.*241C>T and are located at the 3′ UTRs of the corresponding mRNAs. The *SOX10* c.86+148C>A was found in the intronic sequence of the *sex-determining region Y-box 10* gene. Within the *IL1B* gene, a total of seven polymorphic nucleotide substitutions were identified. Four of them, c.76+52C>T, c.76+157A>G, c.76+215A>G and c.76+310A>G, are located in intron 1. Three other SNPs, c.300+263C>T, c.300+329C>T and c.300+431A>C, reside in intron 2 of the *IL1B* gene (Fig. S1). All polymorphisms found in the *IL1B* gene were in strong linkage disequilibrium.

Linkage mapping

As mentioned above, the first-generation microsatellite-based linkage map (Chistiakov *et al.* 2005) was used as a framework for incorporating new markers, mainly AFLPs. Of the 45 newly developed microsatellites, 28 were informative. Of the 246 polymorphic microsatellites in total, 201 were informative (i.e. heterozygous at least in one of the parents) in the Venezia Fbis family. Before linkage analysis, AFLPs were checked to determine whether their frequency distribution in the F_1 progeny followed Mendelian inheritance, either 3:1 or 1:1 (for dominant markers heterozygous in both parents or in one of the parents respectively), using a chi-squared test. The screening resulted in the elimination of 15 AFLPs the observed genotype distribution of which significantly deviated from Mendelian inheritance. The remaining 221 AFLP markers were used in linkage analysis.

Linkage mapping resulted in a new version of the linkage map of *D. labrax* consisting of 368 genetic markers: 190 microsatellites, 176 AFLPs and two SNPs grouped in 35 linkage groups. Sixty-seven markers (11 microsatellites, 47 AFLPs and nine SNPs) remained unlinked. The current linkage map includes 24 anonymous type I microsatellites derived from ESTs and six genes including *peptide Y* (*DLA0237*; LG1), *CYP19* (*DLA0238*; LG6), *SOX10* (LG7), *PXN1* (*DLA0254*, LG16), *ERA* (*DLA0261*; LG17) and *TCRB* (LG27).

The total length of the sex-averaged map is 1373.1 cM (Fig. 1). The number of markers ranged from 2 to 39 per linkage group. Among linkage groups, LG1 is the longest having a size of 162.4 cM; LG18 is a fusion of the first-generation LG18 and LG21. In the map, the intermarker distance varies from 0 to 27.3 cM, with an average of 3.67 cM; 71.5% of the intermarker intervals are in the range from 0 to 5 cM, 16.7% range from 5 to 10 cM, 10.3% range from 10 to 20 cM and only 1.5% are larger than 20 cM.

We also built sex-specific linkage maps. The male map has a length of 1046.9 cM, an average distance between markers of 3.03 cM and a maximum marker interval of 24.3 cM (Fig. S2). The length of the female map is 1380.0 cM, with an average intermarker distance of 4.05 cM and a maximum marker interval of 37.8 cM (Fig. S3). The female map is thus 1.32-fold that of the male map. Most linkage groups are smaller in males than in females.

Comparative mapping between the sea bass linkage map and the genomes of five model fish species

Similarity searches of the microsatellite sequences of *D. labrax* against the genome sequences of five teleosts (green spotted pufferfish, fugu, medaka, three-spined stickleback and zebrafish) revealed a high percentage of evolutionarily conserved regions (ECRs) between pairs of species.

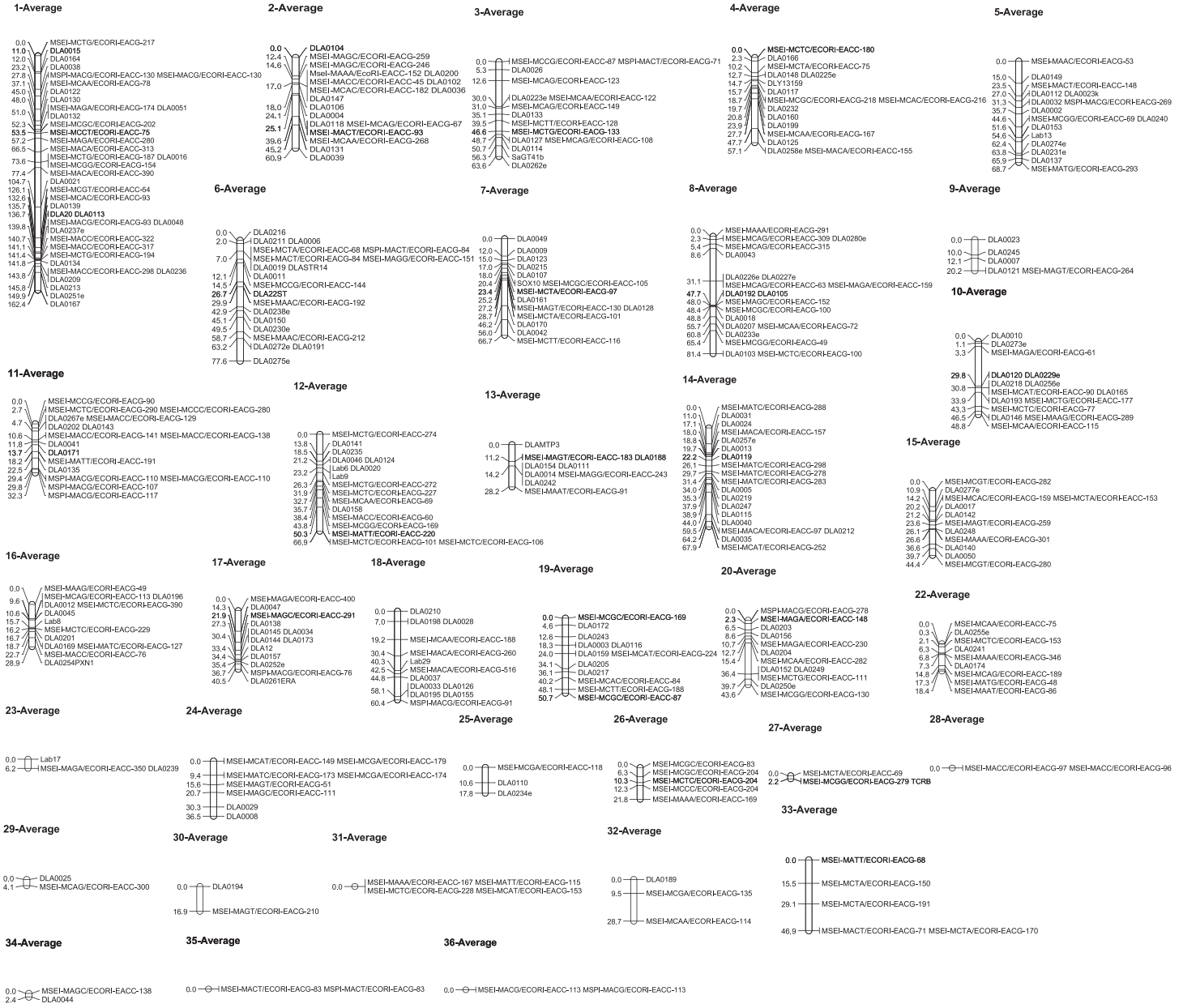

Figure 1 Sex-averaged second-generation linkage map of European sea bass. Distances in Kosambi centimorgans (cM) are given to the left of each ideogram. Microsatellites are indicated as *DLAxxx*, AFLP markers as *MSExxx/ECORIxxx* and type I markers as *DLAxxxE*.

Species	Stickleback	Fugu	Pufferfish	Medaka	Zebrafish
Total hits ($e < 10^{-5}$)	66	40	39	35	6
No. hits with $e < 10^{-10}$	53	29	24	24	2
Total hits in chromosomes	58	0	29	24	6
Total hits in genes	31	17	18	21	4
Total hits in exons	8	10	10	5	4
Total hits in introns	21	7	8	14	0
Total hits in UTRs	2	0	0	2	0
Mean size alignment (bp)	284.89	278.98	289.54	234.46	135.33
Mean alignment in *Dicentrarchus labrax* (%)	71.51	60.74	64.54	56.92	31.36
Minimum alignment (bp)	90	47	90	84	94
Maximum alignment (bp)	835	829	815	605	157
Mean identity (%)	75.46	74.07	73.07	75.37	77.95
Minimum identity (%)	60.3	60.5	62.2	61.2	73.7
Maximum identity (%)	98.9	89.7	89.9	88	85.4

Table 2 Comparison of 186 microsatellite sequences of European sea bass with the three-spined stickleback (*Gasterosteus aculeatus,* v. 1.41), fugu (*Takifugu rubripes*, v. 4.40), spotted green pufferfish (*Tetraodon nigroviridis*, v. 7.40), Japanese medaka (*Oryzias latipes*, v. 1.41) and zebrafish (*Danio rerio*, v. 6.40).

The percentage of ECRs decreased from the stickleback, where we observed the highest similarity (66/196 = 33.6%), to pufferfish, fugu and medaka (approximately 20%) and finally to the zebrafish genome (only 6/196 = 3.1%) (Table 2). A similar trend was also observed with a higher threshold of $e < 10^{-10}$; there was still great similarity with the stickleback genome (53 out of 196 sequences) and less so with the fugu, medaka, pufferfish and zebrafish genomes. The mean size of the aligned region against the species mentioned above ranges from 135 bp against the zebrafish to 285 bp against the stickleback, with mean sequence identity ranging from 73% to 78% (Table 2).

ECRs, which flank microsatellites associated with ESTs or genes, seem to be more informative for comparative mapping than those within non-coding genomic regions. More specifically, in the sea bass – stickleback comparison, while 58 out of 66 ECRs refer to stickleback chromosome positions (Tables 2 and 3), 13 of them are type I markers (46% of the 28 included) and only 45 are non-coding microsatellites (27% of the 165 used in the analysis). These data are compatible with the sequence conservation of *cis*-regulatory elements and their distribution is close to (i.e. proximal) and/or inside structural genes (Davidson 2006). A majority of ECRs (47.0%) are located in genes, mainly in introns.

A comparison between the stickleback genome and the sea bass linkage map reveals several regions that display evidence for conservation of synteny as shared putative homologues (i.e. two or more putative homologues located in the same region in both species; see Table 3 and Fig. 2). The large LG1 of sea bass showed homology with LGV of stickleback, while the sex-determining LGXIX of stickleback had the highest number of shared sequence fragments with sea bass at LG6 (Peichel *et al.* 2001). Overall, for 66 sequences shared between the sea bass and stickleback, there were 101 pairs of sequences that map to the same sea bass linkage group. Of these pairs, 74 (73%) were located on the same chromosome or assembly in stickleback. Similarly, for the comparison to the spotted green pufferfish, there were 36 pairs of sequences mapped to the same sea bass linkage group and 18 of these pairs (50%) were located on the same chromosome or assembly in pufferfish. For the comparison to medaka, there were 34 pairs of sequences mapped to the same sea bass linkage group and 13 of these pairs (38%) were located on the same chromosome or assembly in medaka. This indicates substantially greater conservation of synteny between sea bass and stickleback than between sea bass and pufferfish or between sea bass and medaka. The comparison cannot be made for fugu where no assembly is available.

Likewise, comparative studies may help in resolving the linkage of non-chromosomal markers and/or regions of well-studied species, tentatively assigning them to already described chromosomes (Table 3). For instance, similarity hits between three markers of sea bass LG1 to corresponding stickleback non-chromosomal markers could be linked to *G. aculeatus* chromosome V, which appears orthologous to sea bass LG1 on the basis of four markers.

Discussion

The genomic resources of European sea bass now include a medium-density linkage map, a large number of ESTs based on 14 tissue libraries (A. Canário, personal communication), a BAC library (Whitaker *et al.* 2006), a radiation hybrid panel (F. Galibert, personal communication) and a growing inventory of candidate genes. In comparison with the first version of the linkage map of European sea bass (Chistiakov *et al.* 2005), the updated map includes new types of markers (AFLPs and a few SNPs) in addition to a larger number of microsatellites.

Compared to microsatellites, AFLPs present several problems because of the low information content of these markers, which are generally informative only from one parent or

Table 3 Oxford plot comparing the sea bass linkage groups (LG) to the chromosomes of the three-spined stickleback (S), spotted green pufferfish (T), medaka (M) and zebrafish (Z); Fugu (F) sequence data are assembled into scaffolds, so no chromosomes are available.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	22	24	25	34
S non-chr	3						1	1													1		1	1
S-I													1											
S-II					2				1															
S-III										2						1								
S-IV		5																						
S-V	4																							
S-VI											3													
S-VII			2							1				4										
S-VIII				3																				
S-IX							3																	
S-X									1															
S-XI							1	5																
S-XIII																				2				
S-XIV																			1					
S-XV													2											
S-XVI															1									
S-XVII																								
S-XVIII																	5							
S-XIX						6																		
S-XX																1		1						
F non-chr	2	2	2	2	1	6	2	5		2	1	2	1	1	2	2	2	1		2	1	1		
T non-chr	1						2	2		1				1				1	1					1
T-1		1		2																				
T-2	3														1									
T-3								3																
T-5						1																		
T-7														1										
T-9																					1			
T-10												1												
T-12																				2				
T-13						4																		
T-14																	2							
T-15										1														
T-16													1											
T-17											2													
T-18							1																	
T-20		1	1																					
M non chr	1					2	2	1	1		1			1				1						1
M-1							1																	
M-2																								
M-3					1				1															
M-4				1																				
M-5	1																							
M-6						3																		
M-7																								
M-8								3																
M-9																				2				
M-10		1																						
M-14														1										
M-17										1														
M-18			2							1														
M-19	3																							
M-22												1												
M-24																	1							
Z-3						1																		

Table 3 Continued.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	22	24	25	34
Z-5		1																1						
Z-6							1																	
Z-8																				1				
Z-18					1																			

Only the sea bass LGs (24 out of 35) and the five species chromosomes (or scaffolds) that show a hit ($e < 10^{-5}$) are presented. Sea bass sequence hits against unique chromosomes (excluding non-chromosomal sequences) are shaded.

one sex in our case and exhibit a potentially higher error rate in genotyping. As a consequence, AFLP markers largely developed the existing map and in a few cases, we may expect AFLP markers to link existing maps. Here, this happened when LG18 and LG21 of the first-generation map fused into a single linkage group designated LG18 + 21 through the link between two AFLPs, *MSEI-MCAA/ECORI-EACC-188* and *MSEI-MACA/ECORI-EACG-260.*

The total sex-averaged length was increased 1.7-fold, from 815 to 1373.1 cM. The number of linkage groups has also increased from 25 to 35, including six new linkage groups that contain only AFLPs. In the current map, 29 linkage groups have three or more markers. The recombination length is close to the estimated map size of *D. labrax* (1769.5 cM; calculations are based on the method of Postlethwait *et al.* 1994). We expect that the addition of new markers will make the direct correspondence of the 24 linkage groups to the 24 chromosomes (Sola *et al.* 1993) a biological reality for the European sea bass. This may also be independently achieved by the development of physical maps, such as BAC clone paired-end sequencing (H. Kuhl, personal communication), assembly of a partially or fully sequenced genome or/and the recombination-independent radiation hybrid map (F. Galibert, personal communication).

With the addition of markers, the new map became denser, as is reflected by a decrease in the intermarker spacing from 5.03 to 3.67 cM. Despite the increase in length of the current sex-specific maps compared to the first generation maps, their average intermarker interval has decreased from 3.5 to 3.03 cM (male, M) and from 5.59 to 4.05 cM (female, F). It is consistent with the results from the first-generation map (Chistiakov *et al.* 2005). The F:M recombination ratio in sea bass is typical for most vertebrate species, whose sex recombination rate was measured. In higher vertebrates, this ratio varies between 1.0 and 2.0 (Dib *et al.* 1996; Dietrich *et al.* 1996; Mellersh *et al.* 1997). The F:M ratio in sea bass is the lowest among those reported in fishes, for which F:M recombination usually exceeds 2.0, such as in rainbow trout (Sakamoto *et al.* 2000), zebrafish (Singer *et al.* 2002), Arctic char (Woram *et al.* 2003) and fugu (Kai *et al.* 2005). However, in fish such as Japanese flounder, a higher recombination rate was shown in males compared to females (Coimbra *et al.* 2003). The association of sea bass LG6 with stickleback LGXIX, which hosts the sex-determining locus at the distal end and the somewhat reduced recombination rates in the male, make this LG the target for further analysis. The mechanism of sex determination in sea bass remains unknown. The *DAX1* (*NROB1*) gene was shown to be involved in sex differentiation in several vertebrates, but not in sea bass (Martins *et al.* 2007). Sex differentiation is dependent on strain and parents and is environmentally (temperature) induced (Pavlidis *et al.* 2000; Piferrer *et al.* 2005). As the male-specific suppression in recombination rate (Haldane's rule) seems common in a range of fishes and vertebrates, sexual heterogeneity is likely to be an ancestral feature.

In the updated map, microsatellites represent half of the mapped markers. Sequenced markers such as microsatellites and ESTs can be easily related to physical maps and genome sequences. In addition, the current map is sufficiently dense to be used for the medium-resolution mapping of economically and physiologically important QTL. Mapped genes such as *SOX10*, *ERA* and *CYP19* (with known polymorphic markers inside) are functional candidates for searching quantitative traits related to sex differentiation and maturation, whereas *TCRB* seems significant in mapping traits associated with the resistance to pathogens and immunity. The collection of microsatellites would be useful for marker-assisted selective breeding to establish commercially improved strains of farmed sea bass (Chatziplis *et al.* 2007). The collection of markers is also critical for characterizing the genetic background of wild and cultured sea bass to maintain heterozygosity of cultured stock and for assigning parentage (García de León *et al.* 1995).

A growing number of the sea bass microsatellites originate from coding regions, which may improve the chances for matches between genomes. A total of 33.6% of the sea bass microsatellite sequences match with three-spined stickleback and a lower, but still considerable number with other Acanthopterygii. Only forty hits (20.4%) were found between the sea bass and pufferfish. Using the same stringency ($e < 10^{-5}$), Stemshorn *et al.* (2005) and Franch *et al.* (2006) reported higher rate of similarity for microsatellite sequences between the bullhead sea bream and pufferfish (45.0%) and between the gilthead sea bream and pufferfish (30.4%). Using a more stringent threshold for BLASTN searches ($e < 10^{-8}$),

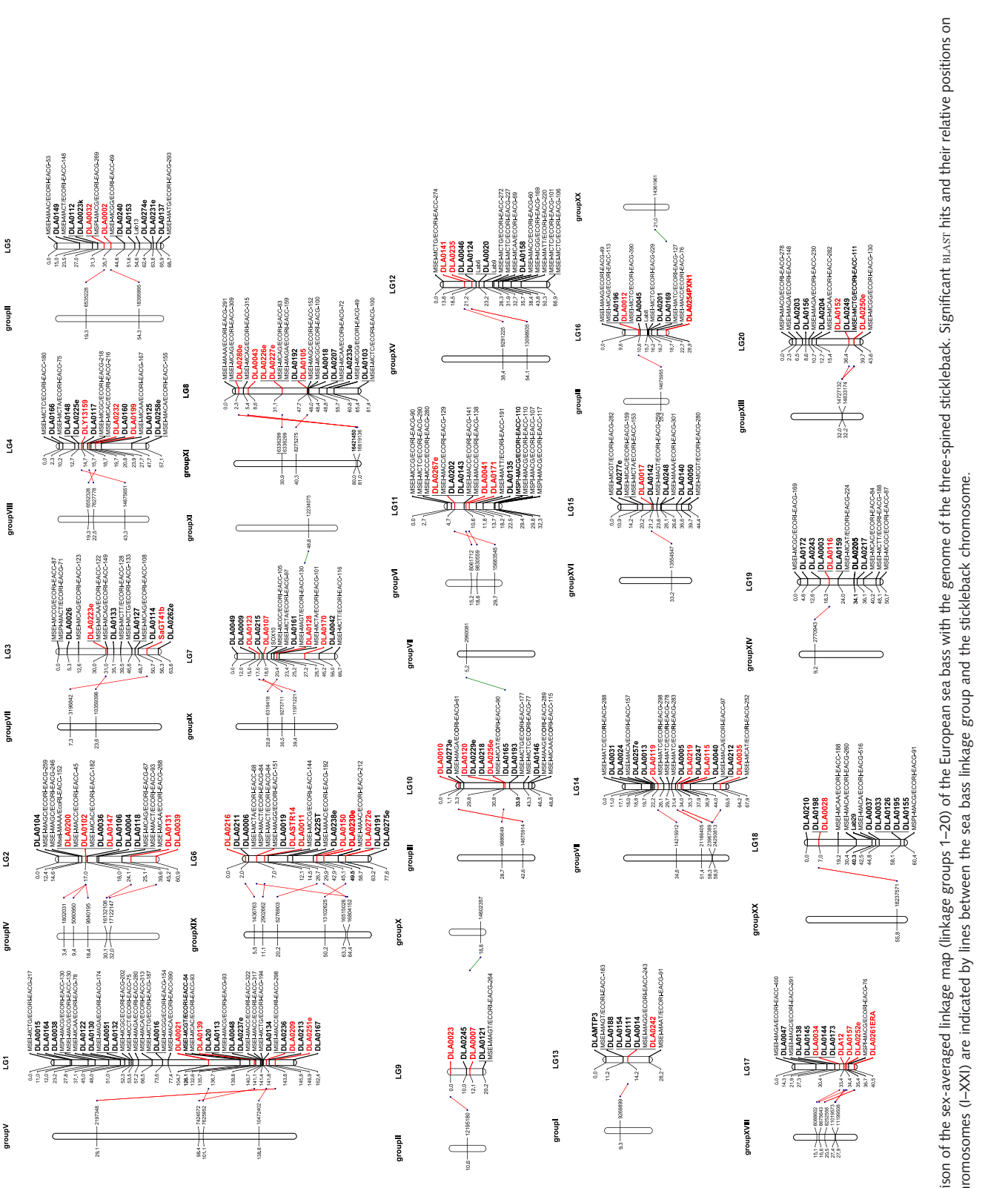

Figure 2 Comparison of the sex-averaged linkage map (linkage groups 1–20) of the European sea bass with the genome of the three-spined stickleback. Significant BLAST hits and their relative positions on the stickleback chromosomes (I–XXI) are indicated by lines between the sea bass linkage group and the stickleback chromosome.

Wang *et al.* (2007) found high homology in 11% and 22.9% of the loci for tilapia and barramundi respectively. In sea bream, a majority of ECRs (72%) were found either in transcribed regions or in introns of the green spotted pufferfish genome, but a substantial proportion could not be associated with any known gene (Franch *et al.* 2006).

Nearly half of the ECRs are found in stickleback genes (31 out of 66), and most of them are in introns (Table 2). The average size of highly similar ECRs observed between the sea bass and the three-spined stickleback (285 bp) is among the highest values obtained in a synteny analysis between sea bream and fugu (182 bp; Franch *et al.* 2006) and between fugu and human (199 bp; Walter *et al.* 2005).

As expected, non-Acanthopterygii such as zebrafish showed the lowest number of matches. Phylogenetic distance is a key aspect here. The high level of conservation of synteny between sea bass and stickleback fits with the paraphyly of the Perciformes and Gasterosteiformes. It opens very good perspectives for a detailed comparison between the genome of a model species, such as three-spined stickleback or fugu and a phylogenetically related non-model species, such as European sea bass. However, to have better insight into the evolution of fish chromosome number, more type I markers (i.e. SNPs and microsatellites in ESTs and/or genes) are needed for comparative mapping.

In the future, the linkage map of *D. labrax* will be further enriched with type I markers, such as genes, ESTs and SNPs. The high frequency of ECRs or homologous synteny blocks facilitates the identification of comparative anchor points, some of them covering a broad phylogenetic range. The linkage map of sea bass will become even more suitable and robust for fine-mapping QTL, functional genomics and comparative analysis with other teleosts. A promising development is that partial and even full genome sequencing has become affordable.

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Supporting information

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

Figure S1 Locations of SNPs within the nucleotide sequences of *Dicentrarchus labrax* genes.

Figure S2 Male sex-specific linkage maps of European sea bass. Distances in Kosambi centimorgans (cM) are given to the left of each ideogram. Microsatellites are indicated as *DLAxxx*, AFLP markers as *MSEIxxx/ECORIxxx* and type I markers as *DLAxxxE*.

Figure S3 Female-specific linkage map of European sea bass. Distances in Kosambi centimorgans (cM) are given to the left of each ideogram. Microsatellites are indicated as *DLAxxx*, AFLP markers as *MSEIxxx/ECORIxxx* and type I markers as *DLAxxxE*.

Table S1 Characteristics of newly developed type I and II polymorphic microsatellites of the European sea bass.

Table S2 AFLP primer combinations used and number of markers generated with each primer combination specific for the male parent, female parent and both parents of European sea bass.

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