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ORIGINAL ARTICLE

First Haploid Genetic Map Based on Microsatellite Markers in Senegalese Sole (*Solea senegalensis*, Kaup 1858)

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Abstract The Senegalese sole (*Solea senegalensis*, Kaup 1858) is a flatfish species of great value for aquaculture. In this study, we develop the first linkage map in this species based on microsatellite markers characterized from genomic DNA libraries and EST databases of Senegalese sole and from other flatfish species. Three reference gynogenetic families were obtained by chromosome-manipulation techniques: two haploid gynogenetics, used to assign and order microsatellites to linkage groups and another diploid gynogenetic family, used for estimating marker–centromere distances. The consensus map consists of 129 microsatellites distributed in 27 linkage groups (LG), with an average density of 4.7 markers per LG and comprising 1,004 centimorgans (cM). Additionally, 15 markers remained unlinked. Through half-tetrad analysis, we were able to estimate the centromere distance for 81 markers belonging to 24 LG, representing an average of 3 markers per LG. Comparative mapping was performed between flatfish species LG and model fish species chromosomes (stickleback, Tetraodon, medaka, fugu and zebrafish). The usefulness of microsatellite markers and the genetic map as tools for comparative mapping and evolution studies is discussed.

Keywords Linkage map · Senegalese sole · Microsatellite markers · Flatfish · Comparative mapping

Introduction

Senegalese sole (*Solea senegalensis*; Soleidae; Pleuronectiformes) is a flatfish species with $2n=42$ chromosomes and a small genome size as compared with teleost (Hinegardner 1968; Hinegardner and Rosen 1972). This species is distributed from Atlantic African and European coasts to western Mediterranean coasts and represents a very promising species for marine aquaculture. In fact, its production is being increased significantly every year, mainly in Southern Europe (Diaz-Ferguson et al. 2012), mostly due to the rapid larval development and its high growth rate (Imsland et al. 2003). However, Senegalese sole farming still presents some difficulties such as the low production, poor-quality sperm in F1 males (Cabrita et al. 2006) and morphological abnormalities (spinal malformations during metamorphosis, irregular pigmentation, lacking eye migration) (Gavaia et al. 2002; Soares et al. 2002; Aritaki and Seikai 2004; Villalta et al. 2005) and recurrent bacterial and viral infections (Zorrilla et al. 2003; Prat 2004; Arijo et al. 2005; Díaz-Rosales et al. 2009). A notable amount of genetic information has been reported to date in this species which can aid to solve these problems, including the development of molecular markers (Funes et al. 2004; Porta and Alvarez 2004; Castro et al. 2006; Chen et al. 2008; Molina-Luzón et al. 2012) for estimating genetic diversity and supporting breeding programs (Porta et al. 2006a, b; De la Herrán et al. 2008); gene expression evaluation (Fernandez-Trujillo et al. 2007; Guzmán et al. 2011; Infante et al. 2011; Ponce et al. 2011); cytogenetical

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studies (Vega et al. 2002); and the development of a bioinformatics platform based on EST sequences and microarrays (Soleamold; Cerdà et al. 2008). Notably, a preliminary physical map has been drawn for this species based on bacterial artificial chromosomes (García-Cegarra et al. 2013), and chromosome-manipulation techniques have been applied to obtain haploid and diploid gynogenetics, and triploid progenies (Molina-Luzón et al. 2014).

However, contrary to the situation of other cultivated flatfish with consolidated breeding programs, such as turbot (*Scophthalmus maximus*; Bouza et al. 2007, 2008; Hermida et al. 2013), brill (*Scophthalmus rhombus*; Hermida et al. 2014), Atlantic halibut (*Hippoglossus hippoglossus*; Reid et al. 2007), Japanese flounder (*Paralichthys olivaceus*; Coimbra et al. 2003; Kang et al. 2008; Castaño-Sánchez et al. 2010) and tongue sole (*Cynoglossus semilaevis*; Ji et al. 2009), Senegalese sole still lacks a marker-based genetic map useful for QTL screening and marker-assisted selection applications. Microsatellite loci or simple sequence repeats (SSR) are widely used, among other purposes for linkage map construction, because they are highly polymorphic, codominant and easily assayed. Within microsatellites, those derived from expressed sequence tag (EST) have proven to be useful for comparative analyses because they are included in gene regions that are expected to be more conserved than anonymous microsatellites (Bouza et al. 2008, 2012; Molina-Luzón et al. 2012; Navajas-Pérez et al. 2012).

Linkage maps are useful tools in studies for identification and tracking of quantitative trait loci (QTLs) related to productive traits, such as resistance and survival to infections (Massault et al. 2010a, b; Ødegård et al. 2011; Rodríguez-Ramilo et al. 2011, 2013). Also, genetic maps provide information on genome sizes and their evolution by comparative mapping with related species (Williams 1998). Development of linkage maps and genome sequencing in fish such as stickleback (*Gasterosteus aculeatus*; Jones et al. 2012), Tetraodon (*Tetraodon nigroviridis*; Jaillon et al. 2004), medaka (*Oryzias latipes*; Kasahara et al. 2007; Ahsan et al. 2008), fugu (*Takifugu rubripes*; Aparicio et al. 2002) and zebrafish (*Danio rerio*; Barbazuk et al. 2000) have enriched our knowledge on chromosomal rearrangements occurring throughout evolution in teleosts by using a comparative genomic approach (Schoen 2000; Danzmann and Gharbi 2001). In fact, many comparative studies have shown a close relationship between linkage groups of farmed fish species such as tilapia (*Oreochromis niloticus*, Lee et al. 2005), rainbow trout (*Oncorhynchus mykiss*, Rexroad et al. 2005), gilthead seabream (*Sparus aurata*, Franch et al. 2006) and European seabass (*Dicentrarchus labrax*, Chistiakov et al. 2008), Sarropoulou et al. (2008) to chromosomes of one or several model species tilapia. In flatfish, similar studies have been conducted in turbot (Bouza et al. 2012) and extended to brill (Hermida et al. 2014); flounder (Castaño-Sánchez et al. 2010), halibut (Reid et al. 2007) and tongue sole (Chen et al. 2014). Additionally, the relationships between LGs of non-model species can be established by using chromosomes of a model species as a bridge, in the so-called stepping-stone approach, as performed by Cerdà et al. (2010) in turbot and halibut using Tetraodon chromosomes as a link.

Here, we describe the first genetic microsatellite-based map in Senegalese sole. For this, we developed new microsatellite markers (both anonymous and EST-derived) and found three gynogenetic reference families (two haploid and one diploid). The haploid gynogenetic families were useful to construct the female map. These haploid families have been prevalent in the developing of linkage map in various fish species (Slettan et al. 1997; Postlethwait et al. 1994; Johnson et al. 1996; Kocher et al. 1998; Kelly et al. 2000; Sun and Liang 2004; Poompuang and Na-Nakorn 2004; Bouza et al. 2007) because they show several advantages over F1 crosses. They save time and cost (obviating the construction and maintenance of pedigrees), are easy to obtain (by eggs fertilization with UV radiate sperm) and simplify the genotyping of material (allelic segregation in heterozygous mother can be directly determined in the haploid offspring). On the other hand, the haploid families have some limitations such as the recombination-rate estimation in only female and the impossibility of mapping phenotypic characters (nonviable embryos).

On the other hand, the diploid gynogenetic family was used to estimate the marker–centromere distance by half-tetrad analysis.

Finally, comparative mapping against the chromosomes of model fish species was performed to gain information on flatfish and teleost synteny.

Materials and Methods

Biological Samples and DNA Analysis

A sample of 46 wild individuals coming from a natural population of the coast of Huelva (SW Spain) and from the broodstock of the IFAPA Centre *Agua del Pino* of Junta de Andalucía (Spain) were used to develop new microsatellite markers in Senegalese sole.

Two haploid gynogenetic families, HGF1 (112 embryos) and HGF2 (98 embryos), were used to analyse marker segregation and mapping. This number of offspring is in accordance with the statistical power to detect a minimum and a maximum intermarker distance of 5 and 35 cM, respectively ($P<0.05$; Lie et al. 1994).

In addition, a diploid gynogenetic family, DGF (70 larvae), was used for positioning the centromeres within each LG. The parents and 20 embryos or larvae of each gynogenetic family were genotyped with three microsatellites to confirm their

haploid and diploid constitution, respectively, and their exclusive maternal inheritance.

Genomic DNA of adults was extracted from blood samples and muscle tissue following the protocol recommended by Macherey-Nagel kit for NucleoSpin® Blood and the phenol–chloroform–isoamyl alcohol procedure, as described by Sambrook and Russell (2001), respectively. Genomic DNA was extracted from progenies by the same method using the full embryo or larvae followed, in both cases, by amplification of genomic DNA with GenomiPhi (GE Healthcare, Chalfont St. Giles, UK).

Microsatellite Marker Development

Anonymous microsatellites were obtained from three enriched genomic libraries following the method by De la Herrán et al. (2008). Sequencing of clones containing microsatellite repeats was performed following the method of Sanger et al. (1977) using the ABI Prism commercial kit BigDie Terminator v.3.1 in an ABI Prism 3100 automated sequencer (Applied Biosystems).

The new microsatellites linked to EST (EST-SSRs) were isolated from an EST database of Senegalese sole (http://www.pleurogene.ca), and the selection criteria were as described by Molina-Luzón et al. (2012). The EST-SSRs sequences were submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Searches for homology and annotation were conducted first at protein level (BLASTx) and then at nucleotide level (BLASTn) when no information was available. *E* values<10–5 over a minimum of 80 bp alignments were used for blast searches.

Additionally, we cross-amplify 16 microsatellites designed for other flatfish, such as turbot (Coughlan et al. 1996; Navajas-Pérez et al. 2012), flounder (Coimbra et al. 2003; Castaño-Sánchez et al. 2010) and halibut (Reid et al. 2007). The selection of these markers was based on the reported variability, their location in the genetic map and their conservation degree, choosing those amplified in more than one species.

Polymerase chain reaction (PCR) conditions were optimised by using DNA from eight adult fish, varying the annealing temperature, and the primer and $MgCl_2$ concentrations. Genetic diversity was evaluated in 20–38 adults from a natural population, and the polymorphism at each locus was checked using an ABI 3100 Avant sequencer (Applied Biosystems). Alleles were designated according to the PCR product size, which was determined using Gene Scan™ 500 LIZ™ Size Standard (Applied Biosystems) as the reference marker and analyzed using the GENMAPPER 3.7 software (Applied Biosystems).

For each microsatellite, the number of alleles (*A*), the observed and expected heterozygosity (*Ho* and *He*, respectively) and the estimated frequency of null alleles was determined using the program CERVUS 3.0 (Kalinowski et al. 2007). Deviations from Hardy–Weinberg equilibrium (HWE) for each locus were estimated by exact tests using GENEPOP 4.0 (Raymond and Rousset 1995).

Polymorphic microsatellites were genotyped in the mother of both families (HGF1 and HGF2) to look for informativeness. Finally, heterozygote microsatellites were used to construct the linkage map by genotyping the progeny.

Linkage Analysis

The JOINMAP 3.0 software was used to construct the genetic map (Ooijen and Voorrips 2002). We first checked for Mendelian segregation (1:1 haploid gynogenetic families) for further mapping by applying a chi-square test with a significance level of $P<0.05$ and also considering Bonferroni correction for multiple tests. Then, independent segregation between all pairs of markers was evaluated using contingency chi-square tests against the null hypothesis (1:1:1:1). Distances (two-point analysis) between marker pairs showing linkage were calculated to establish linkage groups in the map. Only recombination frequencies with a LOD score ≥3 and recombination frequencies below <0.4 were considered for establish linkage between markers. Finally, markers were ordered within each LG under the same criteria (recombination frequencies <0.4; LOD score ≥3), although, in some cases, a LOD score of 2 was accepted.

For a more accurate estimation of the distance between markers, the Kosambi map function, which considers a decreasing interference effect as the distance increases (1994), was applied. The different linkage groups with microsatellites arranged in map distances, centimorgans, were graphically represented using MAPCHART 2.0 (Voorrips 2002).

Microsatellite–Centromere Distance Estimation

All mapped microsatellites were amplified in the DGF mother, and the heterozygous loci were amplified in its progeny.

The marker–centromere distance (*d*) was estimated from the frequency of heterozygotes in the offspring (*y*) using the formula, $d=y/2$ and expressed in centimorgans. For this analysis, we considered complete interference (Thorgaard et al. 1983).

Comparative Mapping Analyses

Initially, comparative mapping was performed by comparing the Senegalese sole linkage groups with regard to the chromosomes of model fish species: stickleback, Tetraodon, medaka, fugu and zebrafish available in the platform Ensembl (www.ensembl.org / index.html). Through BLAST searches, homologies were found between the flanking microsatellite sequences of mapped loci and the genome sequences of model

species. The selection criteria of the hits were E value$<10^{-5}$ and minimum alignment >80 pb.

Additionally, we carried out a comparative mapping analysis within flatfish using the genetic maps of turbot (Bouza et al. 2012), brill (Hermida et al. 2014), flounder (Castaño-Sánchez et al. 2010), halibut (Reid et al. 2007) and tongue sole (Chen et al. 2014). The presence of common markers among different maps enabled us to establish the relationship between LGs of these species (chromosomes in the case of tongue sole). Due to the small number of shared markers, we used the information gained from model species comparisons as a reference to associate LG/chromosome within flatfish. Thus, we selected the chromosomes of model species with highest number of significant matches using Senegalese sole mapped microsatellite sequences as an anchor between the different LGs/chromosomes of flatfish.

Results

Development and Genetic Diversity of New Microsatellite Markers

A total of 127 SSR markers, 85 anonymous (from three enriched libraries constructed in this study), 26 EST-derived (from Soleamold bioinformatics platform; Cerdà et al. 2008) and 16 from other flatfish were validated and checked for polymorphism in a sample of 20–38 individuals of Senegalese sole (Electronic Supplementary material Table S1). Eighty-two markers were polymorphic (64.56 %; 60 anonymous, 15 ESTs and 7 from other flatfish) and were used for map construction. The mean number of alleles per locus was 5.719±2.681 (range, 2–12), average observed heterozygosity 0.562±0.188 (range, 0.105–1.000) and average expected heterozygosity 0.585±0.181 (range, 0.102–0.876). Hardy–Weinberg equilibrium test (HWE; $P<0.05$) and the estimated null allele frequency (NAF>0.100) revealed heterozygote deficit in six loci: Mss043 ($P=0.002$ and NAF=0.244), Mss056 ($P=0.081$ and NAF=0.266), Mss074 ($P=0.016$ and NAF=0.330), Mss106 ($P=0.005$ and NAF=0.533), Mss126 ($P=0.055$ and NAF=0.255) and Est-57 ($P=0.012$ and NAF=0.343), representing putative candidate loci for null allele presence (Electronic Supplementary material Table S1). The EST-SSRs sequences were submitted to GenBank, and five revealed homologies with annotated genes (Electronic Supplementary material Table S3).

Map Construction

A total of 171 polymorphic microsatellites, 82 characterized in this study (60 anonymous, 15 EST-SRRs, 7 from other flatfish) and 89 previously reported in Senegalese sole (10 by Funes et al. 2004; 10 by Porta and Álvarez 2004; 8 by De la Herrán et al. 2008 and 61 by Molina-Luzón et al. 2012) were amplified in the HGF1-mother. Among these, 105 were heterozygous. In this first linkage-map analysis based on the HGF1 family (Fig. 1; Table 1), 24 LGs were identified including 96 (91.42 %) microsatellites (LOD values≥3), 9 (9.37 %) markers remaining unlinked (*UL*). Six microsatellites (6.25 %; Mss157, Mss014, CSse27G19, Mss100, Mss013 and Mss130) showed significant deviation from Mendelian segregation at $P<0.05$, but none after Bonferroni correction. The order of the 96 linked microsatellites was established at LOD≥3, except for two loci (Mss024 in LG13 and CSse27G19 in LG14) ordered at LOD=2 (Fig. 1). The position of one microsatellite (Mss096) could not be determined with confidence, so it was considered as *accessory* and located next to the microsatellite linked with the highest LOD score (Smax-E273 at LG5) (Fig. 1; Electronic Supplementary material Table S4).

A second linkage analysis was carried out in the HGF2-mother with the 66 remaining non-informative markers in HGF1-mother, 39 of them being heterozygous. Thirty-seven microsatellites distributed across all linkage groups identified in the HGF1 map were used to anchor maps of both families. In this second map, based on HGF2 family, 22 LGs were identified (LOD≥3) with a total of 64 linked (84.21 %) and 12 unlinked (15.78 %) microsatellites (Table 1). Six microsatellites (7.89 %; Est-48, CSse6A20, Mss042, Smax-02, Est-22 y Est-60) showed deviation ($P<0.05$) from Mendelian segregation (1:1) but none after Bonferroni correction (Fig. 1; Electronic Supplementary material Table S4).

A consensus linkage map was finally constructed using information of both families and contained 27 LGs with a total of 129 linked microsatellites (89.6 %), 15 unlinked (10.4 %) and two *accessories* (Fig. 1; Table 1). Marker density ranged from 2 (LG18 to LG27) to 14 markers per group (LG1 and LG3), with a mean of 4.7 markers per group (Table 2). LG length ranged from 3.2 cM (LG19) to 109.9 cM (LG1), with an average size of 36.9 cM (Table 2). The total length of the map was 1,004 cM, as estimated from the sum of distances of LGs, excluding accessory markers. Eighty-three microsatellites came from enriched libraries (64.34 %), 41 from ESTs (31.78 %) and 5 from cross-species amplification (3.87 %; 2 from turbot [Sma-E273 and Sma-E50] and 3 from flounder [Poli200TUF, Poli129TUF, and Poli16-76TUF]).

Among the 27 LGs (Fig. 1; Electronic Supplementary material Table S4), eight were a consensus between both haploid families (LG2 to LG8 and LG12), whereas, in six LGs, it was not possible because common marker pairs were not available (LG1, LG9, LG10, LG11, LG13 and LG15). Another four LGs were constructed with one LG of one haploid family together with unlinked microsatellites of the other haploid family (LG14, LG16, LG17 and LG18), and

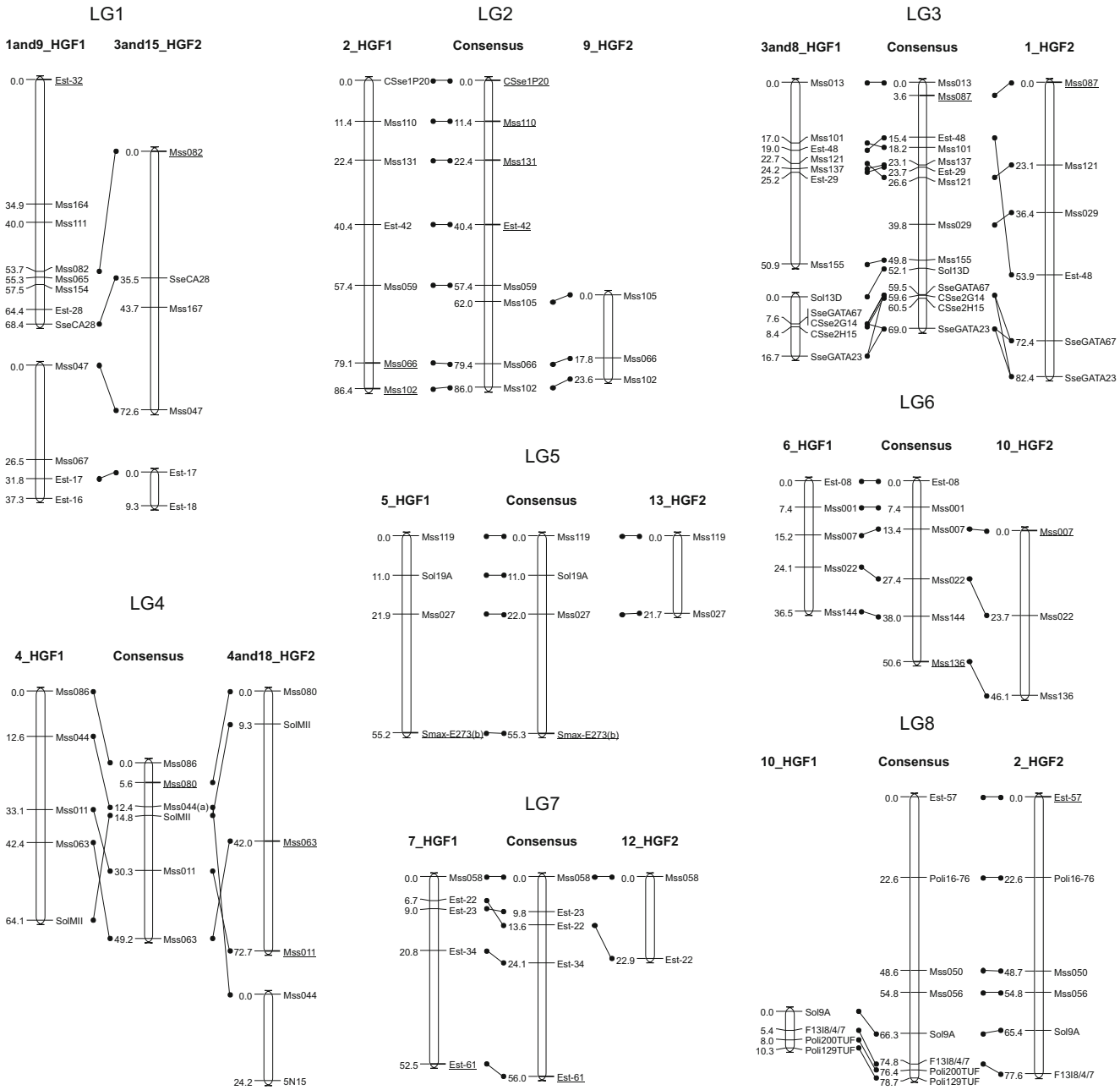

Fig. 1 Genetic map of Senegalese sole (*S. senegalensis*). Consensus LGs, when available, are represented between both haploid LGs (HGF1 on the *left* and HGF2 on the *right*). Markers positioned with a LOD=2 are *underlined*. *Accessory* markers: **a** 5N15, 24.2 cm; **b** Mss096, 36.0 cm, indicating the distance to the closest marker in the map

finally, nine LGs were formed by doublets of markers from only one family (LG19 of the LG27).

Marker–Centromere Distance Estimation

Using the DGF, we estimated the marker–centromere distances in 81 mapped microsatellites which were heterozygous in the mother of this family. We could estimate the distance to the centromere from eight markers in LG1 to a single marker in LG20, LG24, LG25 and LG26, with an average of three markers per LG (Electronic Supplementary material Table S5).

The frequency of heterozygotes (y) ranged from 0 (Est-15/LG20) to 0.957 (F13l8/4/7 and Mss061) with an average of 0.635±0.235 (Electronic Supplementary material Table S5). A large number of microsatellites (about 50 %) showed a heterozygote frequency >0.667, a value corresponding to independent segregation with respect to the centromere under the assumption of no interference.

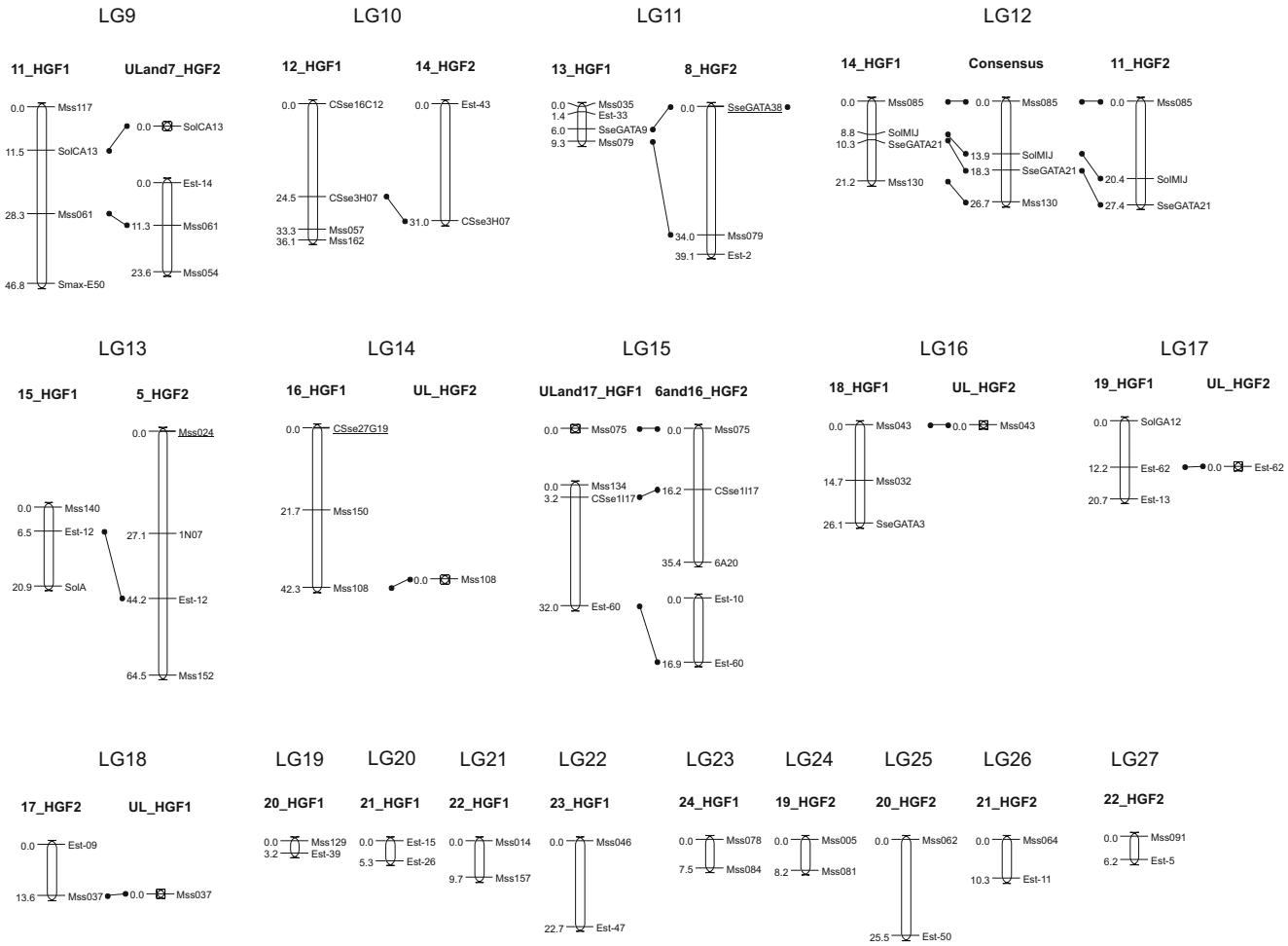

Fig. 1 (continued)

Additionally, we performed an analysis of the microsatellite segregation distortion in offspring of DGF to look for deleterious alleles. Significant deviation ($P<0.05$) was detected for 14 markers (17 %), two of them (Poli200TUF at LG8 and SseGATA9 at LG11) after Bonferroni correction. However, this analysis can be carried out only when at least a total of six homozygous offspring are observed (Lindner et al. 2000). This is the case for all microsatellites except Mss046 at LG22 and Est-8 at LG6, and therefore, they were excluded for this analysis. These results suggest the existence of deleterious alleles associated with these markers (Electronic Supplementary material Table S5) determining a decreased viability of the homozygous class. Nevertheless, we did not observe a total loss of viability for any marker. Only Est-8 and Mss046 showed a complete absence of one homozygous class, but they had been discarded because they did not reach the minimum number of homozygous genotypes required for the analysis.

Table 1 Comparison of linkage maps between haploid families and the consensus one

	Consensus	HGF1	HGF2
No. markers	144	105	76
Anonymous	83	65	46
EST	41	28	20
Flatfish	5	3	2
LGs	27	24	22
Unlinked	15	9	12
Total length (cM)	1,004	782.1	754.6

Comparative Mapping

The 129 and 15 sequences containing mapped or unlinked microsatellites, respectively, were compared with the genomes of stickleback, Tetraodon, medaka, fugu and zebrafish by BLASTn in the Ensembl database. A total of 41 microsatellite sequences (30 %; 13 anonymous and 28 EST-linked) showed significant hit with any model species (Table 3), 25.7 % (9 anonymous and 28 EST-linked) with stickleback; 19.4 % (9 anonymous and 19 EST-linked) with Tetraodon;

Table 2 Number of markers and length per LG in Senegalese sole maps

Consensus map			HGF1 map			HGF2 map		
LG	No. markers	Length (cM)	LG	No. markers	Length (cM)	LG	No. markers	Length (cM)
LG1	14	109.9	LG1	8	68.4	LG3	4	72.6
			LG9	4	37.3	LG15	2	9.3
LG2	8	86	LG2	7	86.4	LG9	3	23.6
LG3	14	69	LG3	7	50.9	LG1	6	82.4
			LG8	5	16.7			
LG4	7	49.2	LG4	5	64.1	LG4	4	72.7
						LG18	2	24.2
LG5	5	55.3	LG5	5	55.2	LG13	2	21.5
LG6	6	50.6	LG6	5	36.5	LG10	3	46.1
LG7	5	56	LG7	5	52.5	LG12	2	22.9
LG8	8	78.7	LG10	4	10.3	LG2	6	77.6
LG9	6	46.8	LG11	4	46.8	LG7	3	23.6
LG10	5	37.1	LG12	4	36.1	LG14	2	31
LG11	6	48.4	LG13	4	9.3	LG8	3	39.1
LG12	4	26.7	LG14	4	21.2	LG11	3	27.4
LG13	6	85.4	LG15	3	20.9	LG2	4	64.5
LG14	3	42.3	LG16	3	42.3			
LG15	6		LG17	3	32	LG6	3	35.4
						LG16	2	16.9
LG16	3	26.1	LG18	3	26.1			
LG17	3	20.7	LG19	3	20.7			
LG18	2	13.6				LG17	2	13.6
LG19	2	3.2	LG20	2	3.2			
LG20	2	5.3	LG21	2	5.3			
LG21	2	9.7	LG22	2	9.7			
LG22	2	22.7	LG23	2	22.7			
LG23	2	7.5	LG24	2	7.5			
LG24	2	8.2				LG19	2	8.2
LG25	2	25.5				LG20	2	25.5
LG26	2	10.3				LG21	2	10.3
LG27	2	6.2				LG22	2	6.2

22.2 % (7 anonymous and 25 EST-linked) with medaka; 27.1 % (11 anonymous and 28 EST-linked) with fugu and 14.6 % (5 anonymous and 16 EST-linked) with zebrafish: four significant hits in LG1 and LG2; three in LG3, LG15, and LG17; two in LG9, LG10, LG11, LG13, LG25 and LG26 and one in LG4, LG6, LG8, LG14, LG16, LG19, LG20, LG22, LG23 and LG27 considering all species. Table 3 shows the relationships of Senegalese sole LGs with the chromosomes of these model species. Note that the unlinked microsatellite CSse3A04 showed homology with the same chromosomes in model species that the microsatellites Est-13, Est-62 (LG17) and Est50, Mss062 (LG25), and therefore, it likely pertains to one of these LGs, or LG17 and LG25 can be considered as only one LG.

Only one marker showed homology with all model species (Est-33/LG11; a ribosomal protein), always in a single chromosome (see Molina-Luzón et al. 2012). When zebrafish (belonging to *Ostariophysi* and phylogenetically more distant to Pleuronectiformes) was excluded, the number of microsatellite sequences conserved in a single chromosome in the remaining four model species belonging to *Acanthopterygii* increased to 6 (Mss047 and Est-28 at LG1, Est-57 at LG8, CSse3H07 at LG10, Est-33 at LG11 and CSse6A20 at LG15). In this case, only one sequence was annotated, CSse3H07, corresponding to the interferon regulatory factor (see Molina-Luzón et al. 2012).

To establish the relationship between Senegalese sole LGs to other flatfish species, we used both shared mapped markers among flatfish and significant homologies with model fish genomes. Only 3 out of the 16 cross-amplified microsatellites (Poli16-76TUF, Poli 129TUF and Poli 200TUF from flounder) could be integrated in the Senegalese sole map

Table 3 Marker sequence homology between the Senegalese sole linkage groups and the chromosomes of the model fish species

Senegalese sole	Stickleback	Tetraodon	Medaka	Fugu	Zebrafish
Est-16 (LG1)	10–20	8	11–16	7–9–12	16–19–25
Est-32 (LG1)	9	17	1	4–17	1
Est-28 (LG1)	9	18	1	17	–
Mss47 (LG1)	9	18	1	17	–
Est-42 (LG2)	17	–	5	19	–
Mss102 (LG2)	9–12–17–19	11	5–6–23	3–9–18–19	4–6–8–11–25
Mss105 (LG2)	–	–	–	19	–
Mss131 (LG2)	–	11	5	11–19	–
CSse2H15 (LG3)	17–19	5	3–6	7–9–13	7–25
Est-29 (LG3)	–	–	3	13	–
Mss29 (LG3)	–	8	–	7	–
Mss44 (LG4)	1–13	12	9–13	21	–
Est-8 (LG 6)	2–19	13	6	9–13	–
Est-22 (LG7)	–	–	23	18	4–25
Est-34 (LG7)	7	2	17–18	1–8	5
Est-57 (LG8)	15	1	4	20	–
Est-14 (LG9)	13–14	4–12	12	6–21	5
Mss54 (LG9)	14	4	–	6	–
CSse3H07 (LG10)	12	–	7	3	–
Mss57 (LG10)	12	9	–	3	23
Est-2 (LG11)	1–7–15–18	10–13–14–16	13–14–22	2–9–11–15–16	5–15–20
Est-33 (LG11)	18	14	24	16	20
CSse1N07 (LG13)	–	1–3	–	1–5–8–14–19	–
Mss24 (LG13)	4	–	–	–	14
Est-27G19 (LG14)	11	2–13	8	5	–
CSse6A20 (LG15)	15	10	22	2	N0
Est-10 (LG15)	15–18	10–14–16	13–22	2–16	5–13
Est-60 (LG15)	15	10	–	2	–
Mss32 (LG16)	10–20	–	16	7–12	16
Est-13 (LG17)	16	2–3	2–21	1–8	1–9
Est-62 (LG17)	16	2–3	21	1–8	–
CSse3A04 UL	–	2	21	1	9
Est-39 (LG19)	5	–	19	–	–
Est-15 (LG20)	20	–	16	7–15	16
Est-47 (LG22)	1–7–13	–	9–13–14	6–11–15–21	5–15
Mss84 (LG23)	4–12	9	7	3–14	–
Est-50 (LG25)	21	–	21	1	9
Mss62 (LG25)	–	–	–	1	–
Est-11 (LG26)	2–19	5–13	6–13	9–13	7
Mss64 (LG26)	2–9	5–18	3	13–17	7
Est-5 (LG27)	3–7	–	–	2–20	20

(Fig. 1, Electronic Supplementary material Table S4). These three microsatellites linked to LG9 in flounder (Castaño-Sánchez et al. 2010) also proved to be linked within a single linkage group of Senegalese sole (LG8).

Due the low number of shared mapped markers, we used homology data from model species to establish the relationship of flatfish linkage groups to Senegalese sole map. Zebrafish was discarded due to the low homology shown. Significant hits were compared with those found in turbot (Bouza et al. 2012), brill (Hermida et al. 2014), flounder (Castaño-Sánchez et al. 2010), halibut (Reid et al. 2007) and tongue sole (Chen et al. 2014). Following this stepping-stone strategy, we could established the correspondence of 15 LGs of Senegalese sole with 13 of turbot, 13 of brill, 10–12 of

flounder, 10 of halibut and 15–16 chromosomes of tongue sole (Table 4).

Discussion

Microsatellite Development

Construction of enriched libraries has been widely used to obtain microsatellite markers in other flatfish such as turbot (Pardo et al. 2007; Ruan et al. 2011), flounder (Coimbra et al. 2001, 2003) and halibut (Reid et al. 2004). Advances in new-generation sequencing (NGS) technologies have enabled to largely increase genomic resources by developing whole genome and transcriptome sequencing projects, which represent a valuable source of molecular markers, e.g. in turbot (Chen et al. 2007; Bouza et al. 2008, 2012; Cerdà et al. 2008; Pardo et al. 2008; Ruan et al. 2011; Vera et al. 2011; Navajas-Pérez et al. 2012), brill (Hermida et al. 2014), tongue sole (Liu et al. 2007; Sha et al. 2010) and Senegalese sole (Molina-Luzón et al. 2012).

EST-SSRs are usually more conserved than anonymous ones and are associated to coding sequences providing functional information. They proved to be useful in studies for developing linkage maps, QTL screening and comparative evolutionary studies in several flatfish species like turbot (Chen et al. 2007; Bouza et al. 2007, 2008, 2012; Ruan et al. 2010; Rodríguez-Ramilo et al. 2011; Navajas-Pérez et al. 2012), brill (Hermida et al. 2014), flounder (Coimbra et al. 2003; Kang et al. 2008; Castaño-Sánchez et al. 2010), halibut (Douglas et al. 2007; Reid et al. 2007) and tongue sole (Liu et al. 2007; Sha et al. 2010; Chen et al. 2014).

In this work, we used both methods to identify microsatellites in Senegalese sole. Thus, we found 70.6 % (60/85) and 57.7 % (15/26) polymorphic microsatellites from the enriched genomic libraries and the EST database, respectively. This difference have been previously reported in Senegalese sole (32/33 and 37/50, respectively, Molina-Luzón et al. 2012). Because EST-derived microsatellites are in gene regions, they are more conserved than anonymous microsatellite loci and usually show less variability.

The average number of alleles per locus was 5.72 (range, 2 to 12) in accordance with previous data in Senegalese sole and other fish (Funes et al. 2004; Porta and Alvarez 2004; Pardo et al. 2007; Wang et al. 2007; Sha et al. 2010; Molina-Luzón et al. 2012; Navajas-Pérez et al. 2012;). Hardy–Weinberg equilibrium tests and null allele frequency estimations revealed heterozygote deficit at some microsatellites likely caused by the presence of null alleles. Map construction did not present any problem in our study with these markers since only one heterozygote parental was used for segregation analysis.

Cross-species amplification was successful in 16 out of 31 checked microsatellites (51 %), results in other flatfish being highly variable according to species. For instance, these were cross-amplified between flounder and halibut (22 %, Iyengar et al. 2000; 63.9 %, Kang et al. 2008), or 11 % between turbot, flounder and halibut (Fortes 2008). It has been suggested that species belonging to the order Pleuronectiformes display a higher evolutionary rate or lower conservation in the flanking regions of microsatellites than other fish (Bouza et al. 2002; Arijo et al. 2005; Castro et al. 2006). In this sense, our data,

Table 4 Correspondence of the Senegalese sole linkage groups with other flatfish through homologies found with model species chromosomes

Senegalese sole	Stickleback	Tetraodon	Medaka	Fugu	Turbot*-brill*	Flounder*	Halibut*	Tongue sole*
LG1	Cr 9	Cr18	Cr1	Cr17	LG9	LG7	LG11	Cr9
LG2	Cr17	Cr11	Cr5	Cr19	LG11	LG16	LG15	Cr11
LG3	–	Cr5	Cr3	Cr13	LG4	LG4	LG6	Cr5
LG4	–	Cr12	–	Cr21	LG12	–	LG14	Z
LG6	–	Cr13	Cr6	–	LG6	LG11	LG3	Cr6
LG9	–	Cr4	Cr12	Cr6	LG14	LG6–LG9	LG13	Cr14
LG10	Cr12	Cr9	Cr7	Cr3	LG10	LG1	LG2	Cr10
LG11	Cr18	Cr14	Cr24	Cr16	LG23	LG8	LG1	Cr7
LG14	Cr11	–	Cr8	Cr5	LG13	–	–	Cr17
LG15	Cr 15	Cr10	Cr22	Cr2	LG3	LG2	LG7	Cr1
LG17	Cr 16	Cr2	Cr21	Cr1	LG20	LG3-LG20	LG12	Cr8-Cr16
LG19	Cr5	–	Cr19	–	LG21	–	–	Cr8
LG20	Cr20	–	Cr16	–	LG2	–	–	Cr14
LG23	–	Cr9	Cr 7	–	LG10	LG1	LG2	Cr10
LG25	Cr21	–	Cr21	Cr1	LG20	–	–	Cr16

*Turbot (Bouza et al. 2012), brill (Hermida et al. 2014), flounder (Castaño-Sánchez et al. 2010), halibut (Reid et al. 2007), tongue sole (Chen et al. 2014)

with limited success in cross amplification, indicate the inefficiency of using primers across species for comparative mapping in flatfishes.

Linkage Map

This first consensus map of Senegalese sole is composed of 27 LGs (six more than the haploid complement; Vega et al. 2002), including 129 linked microsatellites (90 % of the 144 available for mapping) and 15 unlinked microsatellites (10 %). The presence of a higher number of LGs than the haploid chromosome number of the species (21) is a common feature when constructing linkage maps in fish, including those with a high number of markers (e.g. tilapia, 525 markers; Lee et al. 2005). When marker density is low, linkage groups can be split into several fragments, the most distant markers identified as small independent LGs (Pérez et al. 2005; Cerdà et al. 2010). The 14 doublets in the Senegalese sole map (from LG18 to LG27) described in this study is a reflection of this situation.

Map lengths, either with low or medium marker density, represent an approximation to the actual size (Postlethwait et al. 1994). In Senegalese sole, map length was 1,004 cM, a figure in the range observed in other flatfish (741 cM in flounder, Coimbra et al. 2003; 1,402 cM in turbot, Bouza et al. 2012; 1,500 cM in halibut, Reid et al. 2007). These values are lower than those of other fish such as 2,750 cM in rainbow trout (Guyomard et al. 2006), in part related to the small genome of Pleuronectiforms, with average 0.6 pg of *C* value (www.genomesize.com). Moreover, recombination rate of females and males usually differ and correspondingly their map length. This difference does not alter the order of markers, but change estimates of distances, rendering different map sizes (Gilbey et al. 2004). In teleost, the recombination rate is usually lower in males than in females (Sakamoto et al. 2000; Waldbieser et al. 2001; Wang et al. 2007). In Senegalese sole, the heterogametic sex is unknown, but in flatfish such as turbot (Bouza et al. 2012) and flounder (Castaño-Sánchez et al. 2010), in which the heterogametic sex is the female, the recombination values were higher in females than in males (1.6:1 and 1.4:1, respectively).

Centromere–Marker Distances

The analysis of centromere–marker distance enables to study phenomena such as crossing-over or interference, although these distances are also influenced by chromosome structure (Kauffman et al. 1995). Centromere position provides additional information to linkage maps since its position influences the distribution of crossovers during meiosis and recombination rate is usually lower close to centromeres (Choo 1998; Sakamoto et al. 2000; Danzmann and Gharbi 2001).

Several studies have examined the localisation of centromeres in species such as zebrafish (Kauffman et al. 1995), medaka (Sato et al. 2001), common carp (*Cyprinus carpio*, Aliah and Taniguchi 2000) and Atlantic salmon (*Salmo salar*, Lindner et al. 2000), as well as within flatfish species such as turbot (Martínez et al. 2008), halibut (Reid et al. 2007) and tongue sole (Ji et al. 2009). In this study, we founded a diploid gynogenetic family to localise centromeres through half-tetrad analysis, a usual method for this purpose in fish (Martínez et al. 2008). We were able to estimate the centromere distance for 81 markers belonging to 24 LG, representing an average of three markers per LG. Additionally, we calculated the distance to the centromere of several markers (13) which remained unlinked, but which may be useful when, in subsequent analyses, they are consistently mapped.

In these analyses, values of $y=1.00$ occur when there is a single crossover between the marker and the centromere in all analyzed meiosis, indicating strong interference. None of the analysed microsatellites showed that value in our study; however, y values were higher than expected for independent segregation under the assumption of no interference (0.667), 50 % for markers showing values above this threshold and 14.8 % higher than 0.9. These values are very similar to those found by Martínez et al. (2008) in turbot (48.1 % and 10.1 %, respectively), indicating strong meiosis interference in Senegalese sole. This situation has been reported in other flatfish such as turbot (Martínez et al. 2008) and in most fish species studied (Kauffman et al. 1995; Lindner et al. 2000; Matsuoka et al. 2004; Nomura et al. 2006).

In a half-tetrad analysis in diploid gynogenetic families, an unequal number of homozygous classes should be expected when one of them has reduced their viability due to the presence of a deleterious allele (Lindner et al. 2000). We found that 14 markers showed significant deviation ($P=0.05$), two of them after Bonferroni correction (Poli200TUF at LG8 and SseGATA9 and LG11). Although several studies in fish have not detected deviations (Lindner et al. 2000; Matsuoka et al. 2004; Li and Kijima 2005, 2006), similar results were observed in other flatfish, such as turbot (Martínez et al. 2008).

Comparative Mapping

Some authors have suggested that chromosomal rearrangements through evolution would have determined a lack of synteny between teleost species as compared with other lineages of tetrapods (Sémon and Wolfe 2007; Hufton et al. 2008). However, several studies (Kasahara et al. 2007; Kucuktas et al. 2009; Kai et al. 2011; Bouza et al. 2012) demonstrated high macrosyntenic conservation between linkage groups of teleost. Thus, genetic maps represent valuable tools for comparative genomics (Kai et al. 2005), since it enables to transfer information from deeply sequenced genomes to poorly studied species,

providing knowledge on the organisation of their genomes and their evolution (Cristescu et al. 2006; Wang et al. 2007).

In this study, we included six microsatellites belonging to other flatfish for comparative mapping in pleuronectiforms, but microsatellites from turbot were not useful. The three linked microsatellites from flounder LG9 (Coimbra et al. 2003; Kang et al. 2008) and located in the halibut LG20 (Reid et al. 2007), appeared to be linked to Senegalese sole LG8, allowing to establish the relationship between these three LGs.

However, frequently, as in our case, cross-amplification is difficult, and genome sequencing and bioinformatics tools are necessary for a syntenic study. During the last decade, high-coverage genomes have been assembled into chromosomes in several fish, such as stickleback, Tetraodon, medaka, fugu and zebrafish. Also, the genomes of several commercial fish species have been sequenced or are in progress, including rainbow trout, Atlantic salmon, Atlantic cod, gilthead seabream, European seabass and tilapia (Sarropoulou and Fernandes 2011), and within flatfish turbot and tongue sole recently published (Chen et al. 2014). With the use of this genomic information, markers in a linkage map can be detected in a model-species genome and thus analyse their conservation (Guyon et al. 2012). We analysed the homology of microsatellite sequences of the Senegalese sole linkage map with respect to genomes of model species. This approach revealed that only 41 of the 144 sequences analysed showed homology with sequences of model species. The highest homology was found with fugu (27.1 %), stickleback (25.7 %), medaka (22.2 %), Tetraodon (19.4 %) and zebrafish (14.6 %). These results are similar to those reported in turbot by Bouza et al. (2012), tilapia by Lee et al. (2005), goby (*Cottus gobio*) by Stemshorn et al. (2005), gilthead seabream by Franch et al. (2006) and barramundi (*Lates calcarifer*) by Wang et al. (2011), appearing in all of them higher homology with *Acanthopterygii* (fugu, stickleback, Tetraodon and medaka) than *Ostariophysi* (zebrafish). This fact supports phylogenetic data reported by Miya et al. (2003) in teleosts.

The teleost genome is interesting from an evolutionary perspective because several copies of the same gene exist as a result of recent genome duplication (Steinke et al. 2006). We found homologous sequences in several chromosomes for a single marker, even under stringent conditions, indicating that they might belong to paralogous regions. Also, several linked microsatellites in Senegalese sole were located in the same chromosome and with the same order in several model species (Table 3), suggesting the existence of syntenic blocks between these species and a low rate of chromosomal rearrangement in these regions during the evolution of *Acanthopterygii* (Kasahara et al. 2007; Kai et al. 2011). On the other hand, the comparison with model species has added utility for mapping unlinked markers.

Relationships between LGs and the chromosomes of model species have been established for several flatfish species (Reid et al. 2007; Castaño-Sánchez et al. 2010; Cerdà et al. 2010; Bouza et al. 2012). In Senegalese sole, these correspondence found in previous studies was useful to establish the relationships between LGs of Senegalese sole with other flatfish (Table 4). Thus, for example, LG1 of Senegalese sole and LGs 6, 7, 9 and 11 of turbot, flounder, tongue sole and halibut, respectively, are syntenic through their homologies with the same chromosomes of the model-species. Moreover, LG10 and LG23 of Senegalese sole should be grouped in a single LG because they showed homology with only one chromosome or one LG of the model and flatfish species, respectively. This situation is similar for LG17 and LG25. Both grouped occur also in turbot and brill (LG10 and LG20, respectively) (Table 4). These cases could be due to fusions of one or more chromosome pairs, because the karyotype of Senegalese sole presents a lower chromosome number than in turbot or flounder (Kim et al. 1988; Bouza et al. 1994). In addition, the homologies found between single Senegalese sole LGs with pair flounder LGs may also indicate the possible chromosomal rearrangements that occurred during Senegalese sole evolution. These results constitute a preliminary step for the establishing a future synteny between these species.

Furthermore, these synteny analyses could be used for the detection of regions of interest in Senegalese sole. Thus, for example, in studies of survival and resistance against infections with *Aeromonas salmocida* in turbot (Rodríguez-Ramilo et al. 2011), QTLs have been located in LG6 and LG9, which show homology with LG1 to LG6 of Senegalese sole, indicating its possible location. Recently, *dmrt1* has been reported as the putative sex determinant gene in tongue sole with a ZW system (Chen et al. 2014), the Z chromosome showing synteny with Sengalese sole LG4 in this study.

In conclusion, we have developed the first female linkage map in Senegalese sole using microsatellite markers. Relationships between LGs of flatfish species and chromosomes of model species are suggested through comparative mapping analyses. Although our data are limited, they could provide an effective approach for evolutionary genome fish studies and could be useful in future breeding programmes in Senegalese sole.

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