**ARTICLE: DISCOVERIES**

# Integrated gene mapping and synteny studies give insights into the evolution of a sexual proto-chromosome in *Solea senegalensis*

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## Abstract

The evolution of genes related to sex and reproduction in fish shows high plasticity; and, to date, the sex determination system has not been identified in more than a few species. *Solea senegalensis* has 42 chromosomes in pairs, and an XX/XY chromosome system for sex determination, while related species show the ZZ/ZW system. Candidate genes involved in these processes (*dmtr1*, *dmtr2*, *dmtr3*, *sox3*, *sox6*, *sox*, *sox9*, *lh*, *cya19a1a*, *amh*, *vasa*, *aqp3*, and *nanos3)* have been studied. Sequencing produced 56 contigs, from which 93 genes could be annotated. By including 9 previous mapped bacterial artificial chromosomes (BACs) and one containing the *fshβ* gene an integrated map has been produced, in which 15 pairs out of 21 have at least one BAC, important for distinguishing those chromosomes of *S. senegalensis* being similar in shape and size. The sequenced size of the 21 BACs represents about 10 % of the total size of the genome of *Senegalensis*. Multicolor fluorescence in situ hybridization mFISH showed the following co-locations in the same chromosomes: *dmrt1-dmrt2-dmrt3*, *dmtr4-sox9-thrβ*, *aqp3-sox8, cyp19a1a-fshβ, igsf9b-sox3* and *lysg-sox6.* Synteny studies showed that the co-location of *dmrt1-dmrt2-drmt3* in the largest metacentric chromosome of *S. senegalensis* is coincident with the Z chromosome of *Cynoglossus semilaevis* – a finding that would make this potentially a proto-sexual chromosome. Phylogenetic studies of 10 concatenated candidate genes show the close proximity of *S. senegalensis* to *Danio rerio*, in which temperature during early life determines sex. Comparative mapping with other species provides evidence of the preferential association of these candidate genes in particular chromosomes, instead of random distribution.



## Key words

Integrated genetic maps, sex determination, sexual differentiation, reproduction, *Solea senegalensis*, Senegalese sole, multicolor in situ hybridization (mFISH), gene mapping, bacterial artificial chromosomes (BAC) library

## Introduction

Integrated genetic maps are a powerful tool both genetic and evolutionary studies of species because they bring together data from gene sequencing and the location of genes on chromosomes. Physical maps represent the real position of a gene within the chromosomes and this is an essential first stage for structural and functional genome characterization. Synteny analysis in non-model species with few resources provides information of existing genes in that region and allows identification of candidate genes associated with traits of interest (Diopere et al. 2014). Previous studies have demonstrated that physical linkage maps in model fish species can be used to search for regions of interest in commercial species. Furthermore, the relationships between the linkage groups of non-model species can be established, firstly using common markers, and secondly using the chromosome of a model species as a bridge (Sarropoulou et al. 2008).

At present, BACs (bacterial artificial chromosomes) are the main tool for constructing physical maps and for studying structural and functional genes, since the clones are quite stable and easy to handle (Cação et al. 2013). The physical mapping based on BACs is considered to be an important intermediate stage in the sequencing strategy with the next generation sequencing (NGS) platform, to carry out a precise genome assembly. Together they represent an efficient approach for anchoring genomic and linkage data onto physical chromosomes (García-Cegarra et al. 2013). After improvements in resolution and accuracy, the FISH (fluorescence in situ hybridization) technique has become an indispensable tool for filling the gaps that exist in a genome sequencing (Gan et al. 2012), and has established itself as a method for assembling high-resolution physical maps (Greulich-Bode et al. 2008).

Various genetic maps have been produced and their data have been integrated in order to use them as tools for genetic and genomic studies in species of commercial interest, to promote breeding programs, for the mapping of QTL (Quantitative Trait Loci), and in evolutionary studies. Examples of these are the cases of the catfish (*Ictalurus punctatus)* (Xu et al. 2007), seabass (*Lates calcarifer*) (Xia et al. 2010), Nile Tilapia (*Oreochromis niloticus*) (Katagiri et al. 2005; Guyon et al. 2012), Asian seabass (*Lates calcarifer)* (Xia et al. 2010), the *Gossypium* family (Gan et al. 2012), common carp (*Cyprinus carpio)* (Zhao et al. 2012) and half-smooth tongue sole (*Cynoglossus semilaevis)* (Zhang et al. 2014) between others; in all these species, BAC-based physical maps havebeen produced and the physical and genetic maps have been integrated, with the aim of locating and studying QTLs. This knowledge has then been used to improve various important traits of these species, which are of interest for production and commercial exploitation.

Thus, genetic maps are becoming increasingly important for studies of commercial or potentially commercial species because, using the information provided to them, fish-farmers are then able to improve the system of cultivation using the latest scientific knowledge of the species (García-Cegarra et al. 2013; Hermida et al. 2014; Zhang et al. 2014).

*Solea senegalensis* is a member of Pleuronectiformes order, which is composed of about 570 species. The adult Senegalese sole has both eyes on the right side of the head, whereas in the larva stage the fish has bilateral symmetry; the left eye migrates during metamorphosis (Padrós et al. 2011). The Senegalese sole is a target species of great interest for aquaculture because of its relatively fast growth rate, good commercial prospects and high market demand; it is therefore an interesting species for diversification in marine aquaculture (Imsland et al. 2004). Although significant advances have been achieved in the last years in larval rearing and on growing stages, the sexual dysfunction of those males produced in captivity still represent a major bottleneck to reproduction in captivity, and the establishment of breeding programs (Guzmán et al. 2009).

Teleost fishes are the most abundant vertebrates on Earth and show all known vertebrate systems for sex determination. Sex determination is a fundamental developmental process, affecting the sexual differentiation of gonads, and leads to sex-specific differences in behavior, physiology and morphology. There are two sex-determining systems: genotypic sex determining (GSD), attributed to the genetic segregation of genes (Toyota et al. 2014) and environmental sex determining (ESD), by which environmental factors regulate sex-determination (Mankiewicz et al. 2013). It has been proposed that sex determination and sexual differentiation are closely related (Uller and Helanterä 2011) because genetic and environmental factors work together in a hierarchical network to produce either male or female phenotypes. Depending on the species the equilibrium may be biased towards one or other mechanism (Heule et al. 2014).

Until now, in fish, sex master genes have been described only in medaka (in which the determining male gene is *DMY* or *Dmrt1bY*) and in rainbow trout (in which the sex determining gene is *sdy*): they are still unknown in flatfish. The *Dmrt* family of transcription factors involved in gonad development shares a common DNA-banding domain (the DM domain) but otherwise these factors show little sequence conservation, and are responsible for sexual dimorphism in diverse organisms (Kopp 2012). *Dmrt* transcription factors seem to work on tissue-specific developmental regulators directing populations of cells towards either male or female traits. One of the members of the family, *Dmrt1* (Double sex and MAB-3 (MAB) related to transcription factor 1, is considered to be the first preserved gene in the sex-determination/sexual differentiation cascade between phyla (Marchand et al. 2000). *Dmrt1bY* is known to play an important role in early testicular differentiation in metazoans, although it can

work as an upstream or downstream regulator in the sex cascade depending on the species (Hodgkin 2002). *Dmrt1* genes (or *Dmrt1* paralogs) have risen to the top of the sex determination hierarchy in at least three distantly-related clades: the *DMRT1* paralog named *DMY* in some fish species of the genus *Oryzias* (Matsuda et al. 2003); the paralog *DM-W* gene in some amphibian species of the genus *Xenopus* (Yoshimoto et al. 2008); and the Z-chromosome-linked gene *Dmrt1* in avian species (Chue and Smith 2011; Smith et al. 2009). However *Dmrt1*-related sex-determining systems reported in *Oryzias latipes* and *Oryzias curnivotus* (Matsuda et al. 2002, 2003; Nanda et al. 2002) and in *Xenopus laevis* (Yoshimoto et al. 2008) are an exception and appear to be a recent evolution event, considering that closely-related species do not have these systems in common (Bewick et al. 2011).

Interestingly *Dmrt1* may play a key role as a downstream regulator in testis differentiation, development and spermatogenesis, as reflected by the male-biased expression of *Dmrt1* in all vertebrate groups, including fish species (Amberg et al. 2010).

The sex-determining gene *sdy* (sexually dimorphic on the Y chromosomes) in rainbow trout and conserved in many salmonid species, has been produced through the evolution of an immune-related gene. This finding provides additional proof of evolutionary plasticity in vertebrates, and shows that master sex determining genes can arise from the *de novo* evolution of genes that have not previously been implicated in sex determination (Yano et al. 2014).

The determination of genetic sex is often accompanied by the formation of sex chromosomes, which have a degenerated origin and high content of repetitive DNA; as a result it is very difficult to decipher their genetic content and organization (Cioffi et al. 2010). As a consequence very few sex chromosomes have been sequenced although the development of NGS techniques is providing new insights (Hallast et al. 2015).

A male heterogametic system is called an XX/XY system, and a female heterogametic system is ZZ/ZW. Both types exist in teleosts and have been found in closely-related species (Mank et al. 2006). Notably, sex chromosomes in fish are mostly homomorphic without differentiation, a finding that could explain the existence of different sex chromosomes systems among teleosts and even among related species (Mank and Avise 2009).

The line between GSD and TSD is quite narrow (5º C or less for Southern flounder, *Paralichthys lethostigma*) and TSD could occur naturally (Luckenbach et al. 2009). The high proportion of neo-males is a general phenomenon in many species (with unknown GSD) produced by aquaculture; it causes commercial losses and unwanted bias in breeders' stocks (Kopp 2012). In species important for aquaculture there are several commercially-desirable

traits associated with sexual dimorphism, particularly growth rate, time and age of maturation, color patterns and fin shape (Viñas et al. 2012).

In addition, the possibility of the XX natural male developing in the Japanese flounder (*Paralichthys olivaceus*) and its relationship with the decline of natural populations has recently been revealed (Lukenbach et al. 2009), and this makes it more urgent to decipher the mechanism by which the sex is controlled in important species

The half-smooth tongue sole (*Cynoglossus semilaevis*) is an important marine fish whose cultivation has recently been exploited commercially in China. It presents considerable similarities with *S. senegalensis* and has been found to have heteromorphic sex chromosomes and a ZZ/ZW sex determination system (Chen et al. 2007). However in *S. senegalenesis* a XX/XY system without heteromorphic sex chromosomes has been described (Molina-Luzón et al. 2014). *S. maximus*, a species of flat fish close to *C. semilaevis* and *S. senegalensis*, shows the same system as the former rather than that of the latter (Haffray et al. 2009) Although a major sex-determining region has been described in *S. maximus* (Martinez et al. 2009), and several candidate genes related to sex determination and gonad differentiation have been mapped related to that region (Viñas et al. 2012), no sex chromosomes have been found in that species.

Temperature-dependent sex determination (TSD) is the most common form of ESD in fishes, although different patterns have been reported and attributed an adaptive significance related to growth and fitness. Hence, in flat fishes, where females reach adult sizes larger than males, faster growth is observed at temperatures that produce the greatest proportion of females, that in turn favor the maintenance of ESD (Lukenbach et al. 2009).

A key enzyme in sexual differentiation and ESD is the Cytochrome P450 aromatase (P450 arom). This enzyme acts on the biosynthesis of 17-b-estradiol and its expression is influenced by environmental factors. Analysis of regulator regions in the promoter of *cyp19a1a* have revealed binding sites for a number of transcription factors (Piferrer et al. 2005). Some of these elements are fork head box (Fox) and SRY/Sox, among others (Yamaguchi et al. 2007).

In the Japanese flounder and other fishes, high temperatures suppress the expression of *foxl2*, *cyp19a1a* and *fshr* (the receptor for the follicle-stimulating hormone) in the XX gonad (Yamaguchi et al. 2008). It has been reported (Piferrer et al. 2008) that the relationship between high temperatures and increased methylation of the *cyp19a1a* gene leads to decreased expression and male development

In the study described in this paper, an integrated genetic mapping has been carried out with sex-determination, sexual differentiation and reproduction-related genes (*dmtr* families, *sox* families, *lhb*, *cya19a1a*, *amh*, *vasa*, *aqp3*, and *nanos3*, all of them named candidate genes) and

completed with some previously published genes (Ponce et al. 2011; García-Cegarra et al. 2013). Information obtained from these maps is fundamental not only for understanding development mechanisms in vertebrates and evolution phenomena but also, by the determination of synteny relationships, for contributing to aquaculture research and improving the production of target species. For this reason, this study will contribute new information about genes/chromosomes which are important in sex-determination and sexual differentiation processes and in reproduction in the Senegalese sole. These genes may be the keys to improving the cultivation of this specie. Synteny studies also provide information that is important for related species.

## Results and Discussion

### Sequencing of BAC clones

The NGS technology has revolutionized genetic research, due to the rapidity, volume of information generated and the low cost of this technique compared with Sanger sequencing (Jiménez-Escrig et al. 2012). Whole genome sequences can thus be obtained in a brief time. Two general steps are followed in the assembly of the genome sequence: first, it is necessary to generate sequences of contigs and scaffolds; and second, these need to be anchored on genome-wide maps (Hastie et al. 2013).

In this study, 13 candidate genes associated with sexual differentiation and reproduction were isolated from a BAC library using specific primers (Table 1). Eleven of them were then sequenced using Roche 454 technology (Table 2) and a total of 93 genes were annotated from the 13 BAC clones (Table 3). .

Sequences organized in contigs obtained in each BAC can be found as Supplementary Material (Supplementary Material 1) and the complete name of the genes annotated can be found in the Supplementary Material (Supplementary Material 2). The BAC containing *fshβ* from García-Cegarra et al. (2013) was also included in Table 1 because is a candidate gene.

BACs containing the *dmtr1* gene (BAC11O20 and *sox 8* gene (BAC10K23) were partially sequenced using Sanger, and sequence homologues to these genes were obtained (Supplementary Material 1).

Table 2 shows the quality characteristics of the sequenced BACs. Most of the BACs have less than 4% contamination, which means sequencing has been satisfactory in terms of quality. Only one BAC showed 16% contamination (BAC6P22), and all contaminated sequences of this BAC were discarded. Hence from the 63 initial contigs only 11 were considered in annotation. Genes found inside useful contigs were verified as genes from eukaryotic species. In general the N50 value is high, which suggests that the average size of contigs is also high.

**Table 2.** Characteristics and quality data of NGS applied in BAC clones.

| **BAC Name *(candidate gene)*** | **Reads[a]** | **Average size of reads (bp)[b]** | **Mb[c]** | **Total contigs[d]** | **Useful contigs[e]** | **N50 (bp)[f]** | ***E. coli* contamination** |
|---|---|---|---|---|---|---|---|
| BAC2K18 (*lhb*) | 6835 | 493 | 3.37 | 12 | 3 | 40197 | [g]nd |
| BAC6P22 (*nos3*) | 7669 | 429 | 3.29 | 63 | 11 | 23281 | 16.38% |
| BAC8O7 (*sox6*) | 5535 | 470 | 2.6 | 11 | 4 | 27172 | 3.27% |
| BAC9J21 (*sox9*) | 6486 | 476 | 3.08 | 160 | 13 | 2505 | 14% |
| BAC12N15 (*vasa*) | 3263 | 471 | 1.54 | 17 | 9 | 17616 | 3.20% |
| BAC16E16 (*dmrt2*) | 12425 | 562 | 6.98 | 3 | 2 | 12264 | 0.28% |
| BAC19H9 (*cyp19a1a*) | 10334 | 443 | 4.58 | 15 | 1 | 47966 | 0.60% |
| BAC20D8 (*aqp3*) | 13298 | 453 | 6.0 | 36 | 4 | 19681 | 1.98% |
| BAC21O23 (*dmrt4/dmrta1*) | 5188 | 469 | 2.43 | 14 | 1 | 7284 | 3% |
| BAC30H22 (*amh*) | 7552 | 447 | 3.37 | 6 | 2 | 35655 | 5.92% |
| BAC32B8 (*sox3*) | 6524 | 475 | 3.1 | 14 | 6 | 21596 | 1.90% |

[a] Reads: Total number of sequences obtained per BAC.

[b]Average size of each sequence obtained.

[c]Sequenced Mb: total DNA sequenced, in Mb, resulting after multiplication of number of reads by average size of read.

[d]Contigs: Total number of contigs assembled after the sequencing of each BAC.

[e]Useful contigs: Contigs with useful information for annotation genes and additional studies; contigs shorter than 500 bp were discarded.

[f]N50: Contigs were numbered by their total bp; N50 measures the length of the central contig.

[g]nd: not determined.

From the genetic perspective, the mechanisms of sex determination may differ between closely related species. Fish provide a paradigmatic example because their sex determination mechanisms range from the environmental to various different modes of genetic determination. There is no known evolutionary significance of this remarkable plasticity (Heule et al. 2014).

Knowledge of the sex-determining mechanism can improve female development and production in some species because of sexual growth dimorphism. In related species such as the half-smooth tongue sole (*C. semilaevis*), as in other fish species, this difference in growth rate between male and females has been detected; for aquaculture there is commercial interest in being able to achieve monosex stocks, because females grow 2-3 times faster than males (Shao et al. 2010). These differences have been also observed in *S. senegalesis*, especially in high-density cultivation (Sánchez et al. 2010). The study of genes related to sex-determining should help aquaculture producers to obtain the predominantly female stocks desirable for maximum returns.

The NGS results showed that *dmrt 2* and *3* were annotated in the same BAC but in different contigs. The gene *dmrt1* was found by Sanger sequencing within the BAC11O20, and it cannot be completely discounted that *dmrt1* is linked with the other members of the *DMRT* gene family. FISH mapping of these BACs confirmed their location on the same chromosome arm. These three genes of the *Dmrt* family have a conserved DM domain and an arrangement widely conserved in vertebrates, this being *dmrt1-dmrt3-dmrt2* (El-Mogharbel et al. 2007). This gene cluster organization has also been studied in *O. latipes*, *T. rubripes* and *D. rerio* (Brunner et al. 2001), and it could be argued that this is an ancestral gene arrangement in vertebrates. The evolution of these genes is still not clear because of lack of data in basal metazoans. However, using their available whole-genome sequences, it can be deduced that the DM domain probably arose during early metazoan evolution, after the divergence of the choanoflagellates, and subsequently the domain expanded in the metazoan lineage (Bellefroid et al. 2013) and, more accurately, during the interval between *Trichoplax* and eumetazoans (Wexler et al. 2014).

With respect to the *dmrt4* gene (also named *dmrta1* because of its DMA domain), this gene was isolated in a different BAC. Two genes were annotated in the only useful contig obtained (Table 3).

**Table 3.** BACs studied with the candidate gene used for PCR-4D, and the genes annotated within each BAC (full names of the genes in Supplementary Material).

| Name of BAC | Candidate gene | Genes annotated |
|---|---|---|
| BAC2K18 | *lhb* | *rtn4, lhb, ube2g1, nmcp2, tm9sf2, cox20* |
| BAC6P22 | *nanos3* | *rfx1, dcaf15, khsrp, slc25a23, rgs5, c19orf53, gng10, ssx2ip, nanos 3, cc2da1a, umod, scl26a11, sgsh, sirt7, cpp110, gnao, mri1, pcyt2, mafg, npb, map2k5, skor1b* |
| BAC8O7 | *sox6* | *calc, psma1, ric3, lmo1, rergl, insc, sox 6, etnk1* |
| BAC9J21 | *sox9* | *dhx15, ccdc149b, wnk1, sh3rf1, sstr2, trim16, fbxl5, rasd1, cbr4, usp3l, med9, palld, glnd* |
| BAC12N15 | *vasa* | *dpp10, ddx4(vasa),cnga, gpd2, ube3a, atp1b, nr4a2, hsf2bp, ankrd10, tuba1c, taar5* |
| BAC16E16 | *dmrt2* | *dmrt2, dmrt3* |
| BAC19H9 | *cyp19a1a* | *gldn, dmxl2, cyp19a1a* |
| BAC20D8 | *aqp3* | *aqp3, nol6, wdr54, hdac11, rhobtb2, dbln-a* |
| BAC21O23 | *dmrt4(dmrta1)* | *dmrt4, fabp2* |
| BAC30H22 | *amh* | *ssbp3, ell, fkbp8, peak1, oaz1, amh, dot1* |
| BAC32B8 | *sox3* | *pdzd11, stard10, rab6a, arr3, atp11c, inppl1b, p2ry4, sox 3, gdpd2, mcf2l, zbed1* |

*Sox3, Sox6, Sox8* and *Sox9* are transcriptional factors that belong to the SRY-related family. They are characterized by a conserved DNA-binding domain, termed the high mobility group box, and by their ability to bind to the minor groove of DNA. *Sox3* is the SRY (sex-related Y chromosome), responsible for sex determination in mammals. *Sox6* could be involved in the maturation of sperm in vertebrates. *Sox8* is involved in Sertoli cell development and in spermatogenesis (Hagiwara 2011).

It has long been thought that *Sox9* is the direct target gene of SRY during mammalian sex determination. *Sox9* is initially expressed on the lateral side of the bi-potential genital ridge and

upregulated in the Sertoli cell precursors in the XY male gonad immediately after the onset of SRY gene expression (Chaboissier et al. 2004).

The anti-Müllerian hormone, also known as AMH, is a protein member of the transforming growth factor-beta gene family which mediates male sexual differentiation. Anti-Müllerian hormone causes the regression of Müllerian ducts which would otherwise differentiate into the uterus and fallopian tubes. It is activated by *Sox9* and causes the irreversible regression of the Müllerian ducts. Because AMH expression is critical to sex differentiation at a specific time during fetal development, it appears to be tightly regulated by several transcription factors (Sox, GATA, etc.) and by FSH (Follicle stimulating hormone) (Kopp 2012).

Gonadotropins (LH (Luteinizing hormone) and FSH) are pituitary hormones regulated by the corresponding hypothalamic releasing factors; they act as the main regulators of male and female gonadal functions and sexual hormone synthesis. Comparative expression of these in the pituitary of wild and cultivated broodstocks has been studied and significant differences have been found (Guzmán et al. 2012). Moreover, cytochrome P450 aromatase (*cyp19a1a* gene) is associated with gonadal differentiation, since it guides the conversion from androgens to estrogens, and its activation or inactivation determines the ovarian or testicular differentiation respectively in lower vertebrates (Guiguen et al. 2010). Fish possess two forms of the *cyp19* gene encoding P450 aromatase, named *cyp19a1a* and *cyp19a1b*. The isoform *cyp19a1a* encodes the gonadal form of aromatase predominantly expressed in the ovary, while the second form is expressed in the brain. In Japanese and Southern flounder *cyp19a1a* levels increase during early female differentiation, and gonad histology in larger juveniles confirmed that high expression of that isoform coincided with ovarian differentiation (Luckenbach et al. 2005). Similarly, in a number of other fishes, increases in *cyp19a1a* are related to female differentiation, and suppression of *cyp19a1*a is necessary for both normal and temperature-induced male differentiation (Baroiller et al. 1999; Piferrer et al. 2005; Rashid et al. 2007). Blast analysis confirms that the gene contained in BAC 19H19 is that corresponding to *cyp19a1a.*

Both the *vasa* and *nanos* families are involved in the specification of primordial germ cells (PGCs) in sexual reproduction (Cho et al. 2014). The *vasa* gene is associated with sexual dimorphism in early development stages in fish species, including the pleuronectiform half-smooth tongue sole (*C. semilaevis*) (Huang et al. 2014). In Senegalese sole four *vasa* transcripts have been described, two of them being ovary-specific in their expression (Pacchiarini et al. 2013). Meanwhile, the *nanos* family has an important role in the development of embryonic germline cells, germline stem cell maintenance and neuronal homeostasis and, specifically, *nanos3* is detected in primordial germ cells in both sexes (Bhandari et al. 2014).

Aquaporins (AQP) are water channels, and a subclass of them, termed aquaglyceroporins, are also able to transport glycerol and perhaps urea and other small solutes (Hara-Chikuma and Verkman 2006). One of them, *aqp3*, is involved in the hydration of oocytes and hence is important for their cryopreservation (Chauvigné et al. 2012).

To determine the relationships existing among BAC contigs a micro-synteny study was performed with other totally or partially sequenced fish species (Supplementary Material 4). These analyses were carried out using the Genomicus (Muffato et al. 2010) website which takes the information from the *Ensembl* database.

To create these figures, the species stickleback (*G. aculeatus)* was used as reference genome, with the exception of BAC2K18 where the reference species selected was the zebrafish (*D. rerio)*. Reference genes are the candidate genes discussed above. Most but not all of these genes were found in the same locus as in the Senegalese sole. The differences between species are reported in the corresponding figures. For BAC2K18 the species *D. rerio* was used because the *lhb* gene could not be found in *G. aculeatus*.

The conservation of the sequenced regions has been compared by micro synteny with the species *G. aculeatus*. The order of the contigs provided for the species *S. senegalensis* was therefore according to this species; and, in parallel, the results obtained in other fish species depending on these two species were observed. Overall, the regions analyzed corresponding to the Senegalese sole are quite well conserved, with the biggest differences between the two species being found outside these areas. For some genes such as *nanos3* the results show that the more recently diverged species are more conserved than those that have been separated for longer. In other cases like *sox6*, isoforms of this gene are found within the analysis, indicating the different evolution of the region studied. Genes such as *sox3* that show inversion in several species, such as the zebrafish, cave fish and medaka, is also observed. Blocks that appear colorless correspond to genes that are not represented in the reference species in that particular region. For DMRT the preserved organization, as *Dmrt1-dmrt3-Dmrt2* is confirmed.

In addition, studies on the structure of genes was carried out in *dmrt2* and *amh* (Supplementary Material 3) confirming our previous results regarding the structure and size of the genes in *S. senegalensis*, which are shorter than in other species (García –Cegarra et al. 2013).

## m-FISH analysis of BACs

Multicolor Ffluorescence *in situ* Hybridization (m-FISH) allows the simultaneous location of various genes on chromosomes to be visualized using specific probes.

Results of the location of BACs listen in Table 1 in individual chromosomes are shown oin Table 4. The presence of candidate genes in these BACs was confirmed by Sanger sequencing. In addition BACs with the *fshβ*-containing gene (BAC7H22), which has been studied previously in our laboratory (García-Cegarra et al. 2013) was also included, as well as the rest of BACs studied there and in a previous work (Ponce et al. 2011). All metaphases analyzed showed 21 pairs of chromosomes as described by Vega et al. (2002).

**Table 4.** Characteristics of the location of BACs in chromosomes by FISH.

| **Name of BAC ( *candidate gene*)** | **Number of signals[a]** | **Location of signals[c]** | |
|---|---|---|---|
| | | **Chromosome type** | **Position within chromosome** |
| BAC2K18 (*lh*) | Multiple | Dispersed | |
| BAC6P22 (*nos3*) | 2 | MT | pTL |
| BAC7H22[b] (*fsh*) | 2 | STC | qSC |
| BAC8O7 (*sox6*) | 4 | STC | qSTL |
| | | A | I |
| BAC9J21 (*sox9*) | 4 | MT | SC |
| | | A | qI |
| BAC10K23 (*sox8)* | 2 | STC | qSTL |
| BAC11O20 (*dmrt1*) | 2 | MT | SC |
| BAC12N15 (*vasa*) | 2 | SMT | pSTL |
| BAC16E16 (*dmrt2*) | 6 | MT | STL |
| | | STC | qSC |
| | | A | I |

| | | | |
|---|---|---|---|
| BAC19H9 (*cyp19a1a*) | 2 | STC | qI |
| BAC20D8 (*aqp3*) | 2 | STC | qTL |
| BAC21O23 (*dmrt4*) | 4 | M | STL |
| | | A | TL |
| BAC30H22 (*amh*) | 2 | A | STL |
| BAC32B8 (*sox3*) | 2 | A | SC |

[a]Number of signals per metaphasic plate.

[b] From García-Cegarra et al. (2013).

[c]MT: metacentric chromosome; SMT: submetacentric; STC: subtelocentric; A: acrocentric; SC: subcentromeric position; I: interstitial; STL: subtelomeric; TL: telomeric. q: long arm; p: short arm.

The FISH technique allowed us to locate the BAC clones on 10 different chromosome pairs (Figure 1). Some BACs presented four or six signals, instead of two, as would be expected (Table 4). In these cases a main signal was observed, which would have the complete BAC sequence, and one or two secondary signals. These secondary signals would be BAC regions which do not possess all the genes contained in the BAC, and show less intensity of signals. If just principal signals are considered, the total number of different chromosomes detected decreases to nine chromosomes.

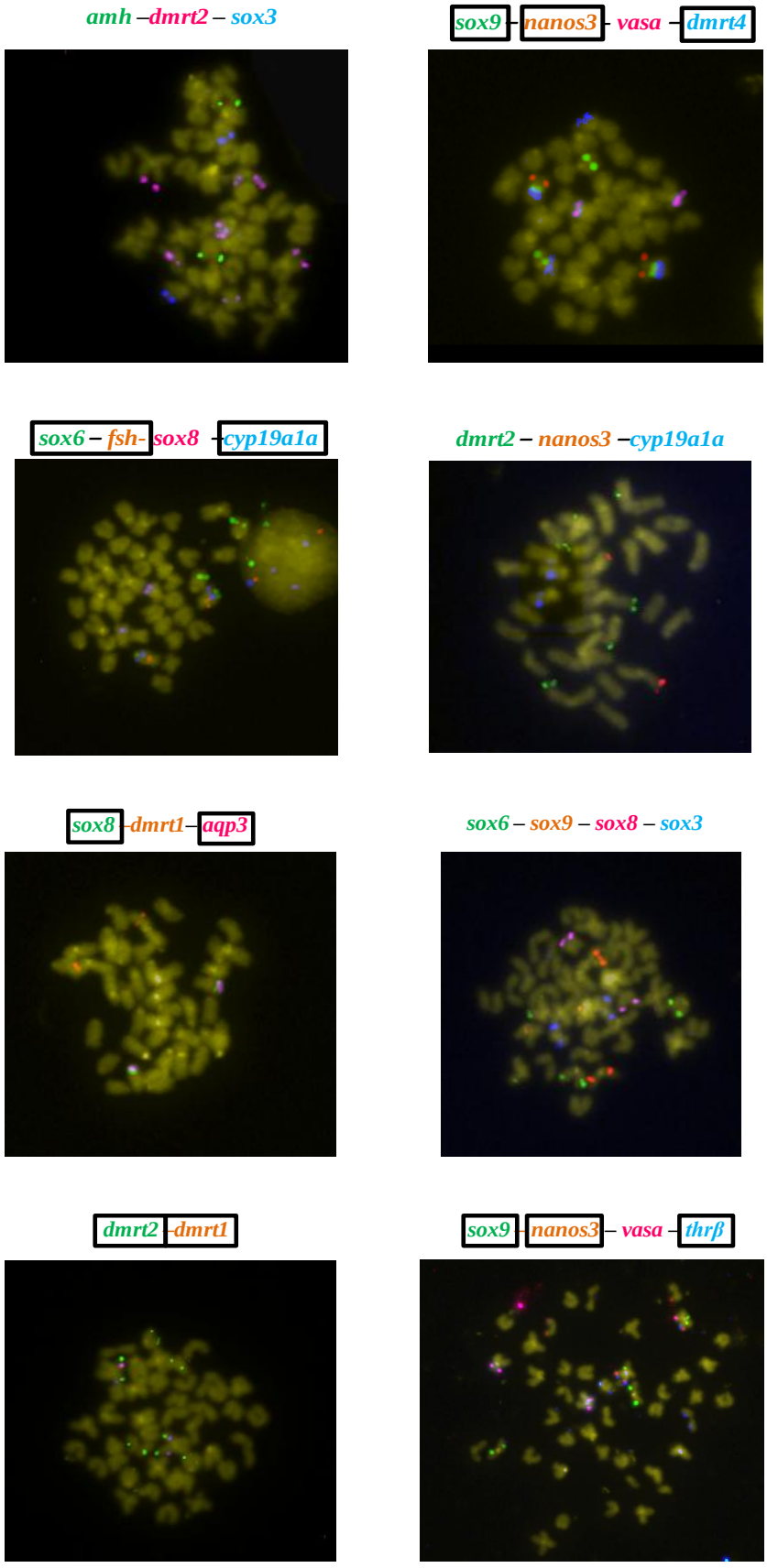


**FIG. 1.** Hybridization after mFISH of the BACs isolated in the library which have as candidate gene the names indicated above the image in colored type. The colors correspond to the

fluorochrome used, namely: fluoresceinsothiocyanate = FITC (green), Spectrum Orange (red), TexasRed (pink) and diethylaminocoumarine = DEAC (blue). Genes co-located are included in boxes.

From the FISH results it could be proved that 9 BAC clones produced single signals, 3 BACs were localized in two pairs, and one in three pairs. The gene *lhb* produced multiple signals but none of them was clearly a main signal so it could not be assigned to a specific chromosome.

The results of mFISH allowed us to study the relationships between several BACs and candidate genes (Fig 1). Table 5 summarizes all the BACs containing candidate genes that were used in mFISH including the BAC containing the *fshβ* (García-Cegarra et al. 2013).

Results showed that BACs containing *dmrt2* and *dmrt3* genes produced signals on 3 chromosome pairs: one of them was the pair of largest metacentric chromosomes (the main signal) co-hybridized with *dmrt1*; the second was a pair of subtelocentric chromosomes (co-hybridized with both *aqp3* and *sox8*); and the third was a pair of acrocentric chromosomes. The gene s*ox9* also hybridized on two pairs: one signal was on a metacentric pair (co-hybridized with *dmrt4*, *nanos3* and *thrβ*) and the other signal was on a pair of acrocentric chromosomes (co-hybridized with *thrαa*). Moreover, the gene *fshβ* also co-hybridized with *cyp19a1a* and *sox6* (Table 5).

**Table 5.** BAC-mFISH combinations with candidate genes. The crosses show the hybridizations carried out, and the co-locations are represented by the colored boxes.

| | ***fshβ*** | ***sox9*** | ***dmrt2*** | ***dmrt4*** | ***sox 8*** | ***dmrt 1*** | ***sox3*** | ***sox6*** | ***amh*** | ***nanos3*** | ***vasa*** | ***cyp 19a1a*** | ***aqp3*** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***fshβ*** [b] | | x | x | x | x | x | x | x | a | x | x | x | |
| ***sox9*** | | | x | x | x | x | x | | x | x | x | x | |
| ***dmrt2*** | | | | x | x | x | x | x | x | x | x | | x |
| ***dmrt4*** | | | | | x | x | x | x | x | x | x | | x |
| ***sox8*** | | | | | | x | x | | x | x | x | x | x |
| ***dmrt1*** | | | | | | | | | x | | | x | x |
| ***sox3*** | | | | | | | | x | x | x | x | x | |
| ***sox6*** | | | | | | | | | x | x | x | x | |
| ***amh*** | | | | | | | | | | x | x | x | x |
| ***nanos 3*** | | | | | | | | | | | x | x | x |
| ***vasa*** | | | | | | | | | | | | x | x |
| ***cyp 19a1a*** | | | | | | | | | | | | | x |
| ***aqp3*** | | | | | | | | | | | | | |

[a] Empty boxes represent mFISH that were not carried out because they were not necessary, since relationships were already determined.

[b] *fshβ*, from García-Cegarra et al. (2013)

As a result, a cytogenetic map was produced (Figure 2), in which up to 9 chromosome markers were obtained. This is an important point because the karyotype of Senegalese sole contains twelve pairs of acrocentric chromosomes of which the size and shape are difficult to distinguish. In the last decade there has been increasing research in the field of *S. senegalensis* aquaculture; this species has been widely studied, from hatching eggs through to the adult age (Barroso et al. 2013). Cytogenetic studies have also been undertaken to complete our knowledge at the genetic level with the object of characterizing its karyotype. This characterization will be useful for both taxonomic and evolutionary research and to obtain cytogenetic and molecular markers which enable the species to be reliably identified, to combat commercial fraud and to help in making decisions on methods of production. Vega et al. (2002) characterized for the first time the karyotype of *S. senegalensis*; subsequently, the need to find markers for each pair of chromosomes has been addressed. Manchado et al. (2006) located the 5S rDNA, and determined by FISH that two pairs of chromosomes hybridized within the karyotype using that probe. Meanwhile, Cross et al. (2006) confirmed this location together with the major ribosomal family (45S rDNA) and (GATA)n and (TTAGGG)n signals, and observed a co-localization between 5S and 45S rDNA in one chromosome pair. Ponce et al. (2011) localized the BAC containing the lysozyme gene; this was the first time that BAC clones had been used as probes in the FISH technique for *S. senegalensis*. On the basis of these first studies and obtaining new BACs useful for chromosome markers, García-Cegarra et al. (2013) produced a preliminary BAC-based cytogenetic map of the Senegalese sole, in which 11 markers were presented. Hence, the present work has followed on from this previous research, to complete the karyotype characterization of this species, focusing on BACs containing genes associated with sex-determining and sex-differentiation, using the M-FISH technique. The combination of all the cytogenetic results provides the characterization of 15 pairs of chromosomes out the total of 21 in the karyotype of *S. senegalensis* (Figure 2). The FISH technique has been also used in the cytogenetic characterization of other species of flatfish such as the turbot (*S. maximus)*; this technique has also been used to consolidate the linkage map produced in this species, thus helping to bring together several previously established linkage groups (Taboada et al. 2014).

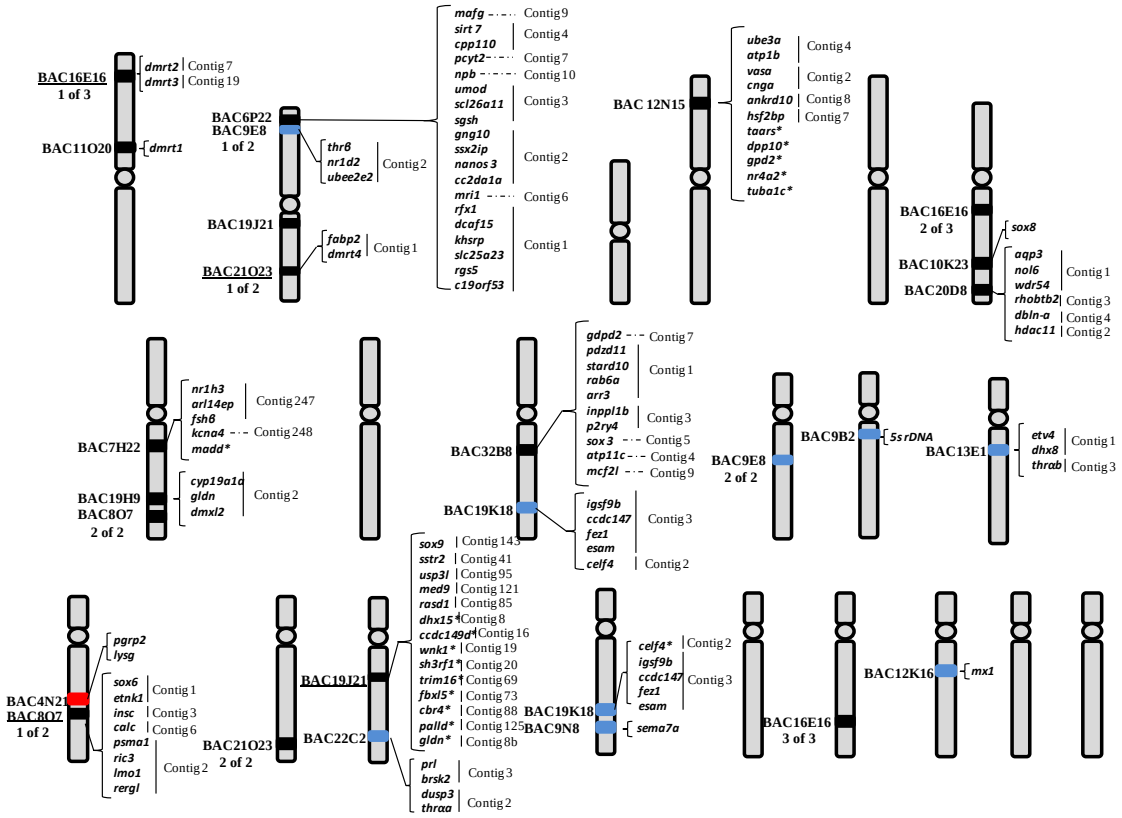


**FIG. 2**. Characterization of the karyotype of *S. senegalensis:* integration of the results obtained in Ponce et al. (2011) (red box), García-Cegarra et al. (2013) (blue box) and the BACs provided in this study (black box). BAC names underlined indicate main signals of those BACs with more than one signal. * Indicates that contigs are not ordered by synteny.

## Integrated genetic map

The BAC16E16 which contains the *dmrt2* and *dmrt3* genes is co-located with the BAC clone BAC11O20, which contains the *dmrt1* gene. These three genes are linked in other species. If we compare our species with *C. semilaevis*, the location of this family can be found on the Z chromosome (Figure 3), which is the sexual chromosome (Supplementary Material 4). These two species belong to the same order, and they diverged just 50 million years ago (Campbell et al. 2013). Since this *DMRT* family is located on a sexual chromosome of the half-smooth tongue sole, the closeness of the two species suggests that something similar could occur in the Senegalese sole, and that this metacentric chromosome could be considered a sexual proto-chromosome. It is remarkable that *sox3*, *sox6*, *sox 8* and *sox9* did not co-hybridize in *S. senegalensis* in contrast to *C. semilaevis (*Figure 2 and Figure 3).

The sex chromosomes of the half-smooth tongue sole are derived from the same ancestral proto-chromosome in vertebrates as the X and Y in birds (Chen et al. 2014). In addition, the gene

(*DMRT1*) located on the Z chromosome, which determines the male sex in birds, shows characteristics of convergent evolution with a similar function in this species of flatfish (Chen et al. 2014). Sexual chromosomes in flatfish are heterogeneous, so in the turbot, no heteromorphic sex chromosomes have been described (Pardo et al. 2001), whereas they do appear in the half-smooth tongue sole (Zhuang et al., 2006). In *S. senegalensis* metaphasic preparations are commonly obtained from larvae because of the difficulty in obtaining preparations from males and females with sufficiently high resolution to distinguish this possibility. In both the turbot and the half-smooth tongue sole, a ZZ/WZ system for sex determination has been described (Hu et al., 2014). However, in *S. senegalensis*, there is evidence of a XX/XY system (Molina-Luzon et al. 2014).

In *C. semilaevis* the *dmrt1* gene has two copies in the male and just one in the female (Chen et al. 2014), but it is still unknown whether *S. senegalensis* has a different number of copies of this gene. It is known that the sex-determining mechanisms in the two species are different (Molina-Luzón et al. 2014), so the master genes for sex-determining will not necessarily be the same. For example, the *dmrt1bY* gene responsible for sex determination in *O. latipes* (medaka) is not found in the closely-related species *O. celebensis* (Kondo et al. 2003). In some species it has been reported that the sex-chromosomes are linked to multigene families (Salvadori et al. 2009; Utsunomia et al. 2014); in the Senegalese sole, this chromosome possesses BACs which contain histone families (L. Rebordinos et al. unpublished work). Nevertheless, it has not been demonstrated that there is a direct relationship between multigene families and the sex chromosomes.

Although the evolution of sex chromosomes involves suppression of recombination, probably in order to maintain sex-related co-adapted gene blocks, we have no evidence of this in males and females of *S. senegalensis*; in addition, the DNA for the library was from a pool of larvae, and the same for the chromosome preparations (Tripathi et al. 2009).

It is known that the differentiation process of sex chromosomes involves structural changes that result in the partial or complete suppression of crossing-over between the 2 primitive homomorphic chromosomes (Charlesworth 1978). The evolution of sex chromosomes generally involves the accumulation of repetitive elements by different strategies that lead in all cases to heteromorphism. Hence, in general, it is the Y chromosome that is the first to be involved in differentiation by the accumulation of repetitive elements around the sex-determining region. However, in some groups, the 18 S rDNA has been accumulated mainly in the X chromosome, as in the fish species *Hoplias malabaricus* (Cioffi et al. 2010), and even between karyomorphs of the same species (Bertollo et al. 1997).

Kejnovsky et al. (2009) proposed repetitive DNA as the initial mechanism involved in the evolution of sex chromosomes. GATA repetitions, the main component of the so-called *Bkm* (banded krait minor) satellite DNA isolated from the W chromosome of the female snake *Elaphe radiate* (Jones and Singh 1985), have been associated with sex chromosomes and their evolution, and have allowed the detection of differences between sex chromosomes in the guppy fish (Nanda et al. 1990). We have found a concentration of (GATA)n at a specific location in the toad fish *Halobatrachus didactylus*, the youngest member of that family but whithout differences between males and females (Merlo et al. 2007). Although previous studies did not find accumulation of that sequence in any of the chromosomes of *S. senegalensis* (Cross et al. 2006), this is not a surprise considering the small size of the species' genome (Cerdá and Manchado 2013): a different strategy could have been used. Hence, if we look at the chromosome number of related species, we find that the turbot has 2n= 44, and that the difference in chromosome morphology compared with *S. senegalensis* is one more acrocentric pair than in *S. senegalensis*. It is possible that a Robertsonian fusion has been responsible for the largest metacentric chromosome, the one that contains the cluster *dmrt1-dmrt2-dmrt3* and, interestingly, also contains the histone gene families. This chromosome is homologous to the sexual chromosome in *C. semilaevis* (Figure 2). Analysis of possible differences between males and females is currently in progress. Although interstitial telomeric (TTAGGG)n sequences were not found on this chromosomes (Cross et al. 2006), its origin in a robertsonian fusion cannot be excluded because the loss of telomeric sequences can occur after such rearrangements (Almeida-Toledo et al. 2000).

Differences in sex determination between related species have also been reported at the chromosome level. Hence progenies from the turbot (*S. maximus*) and brill (*S. rombus*) are monosex depending on the direction of the crossing (Purdom and Thacker 1980).

A recent theoretical model raises the possibility of transitions between the XY/XX and ZZ/ZW systems and environmental sex determination, with some known species like *Xiphophorus maculatus* still at this intermediate stage; that it to say, in the same population some fishes have ZW and others XY systems (Gamble and Zarkower 2012).

The *dmrt4* gene is associated with the development of germ cells in *Monopterus albus* (Shen et al. 2014), and during embryonic development (early gastrula stage) in *Oreochromis aureus* (Cao et al. 2010); however, it also intervenes in other processes, such as the development of the olfactory system in *Orizyas latipes* and *Xenopus laevis* (Veith et al. 2006). In *S. senegalensis* the reproduction phase has some peculiarities: the reproductive behavior involves a courtship in which male and female swim in synchrony with the genital ducts kept closely together while the gametes are liberated and fertilized. In F1 progeny anomalies of this behavior have been shown,

consisting of not swimming in synchrony, and swimming to the surface to liberate and fertilize the gametes, coincident with the absence of some potent odorants important in chemical communication (Norambuena et al. 2012).

It is well-known that cytochrome P450 aromatase is an important enzyme for sexual differentiation, due to its role in conversion from androgens into estrogens (He et al. 2009). In the case of the gene *sox6*, its implication in sex-determining is not well known. It has been found that this gene of the SRY-related high-mobility-group box could be involved in sperm maturation in vertebrates. Nevertheless in rainbow trout it has been discounted as the primary sex-determining gene (Alfaqih et al. 2009). In turbot *S. maximus*) it has been reported that *cyp19* and *sox6* are linked (Viñas et al., 2012). This co-location between *cyp19* and *sox6* has also been observed in *S. senegalensis* by means of FISH analysis. Moreover the *fshβ* gene, which belongs to the gonadotropin family, has also been located in the same locus. In the species *C. semilaevis* this arrangement can also be observed when the two cytogenetic maps are compared (Figures 2 and 3). Therefore, the linkage of *cyp191a* and *sox6* genes appears to be conserved within the Pleuronectiformes order, although more species have to be analyzed to conclude definitively that this assumption is correct.

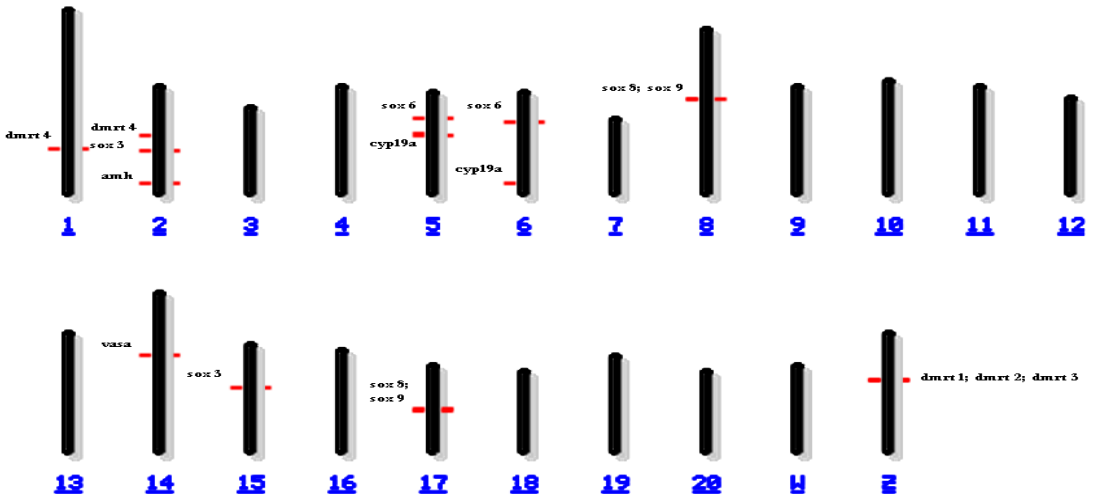


**FIG. 3.** Cytogenetic map of half-smooth tongue sole (*Cynoglossus semilaevis)*. Protein sequences of candidate genes were aligned to make sure that the same proteins were being compared (Supplementary material 4). Obtained using the Map Viewer (NCBI).

*fsh* and *lhb* : are produced in multiple locations in *C. semilaevis.*

*nanos3* and *aqp3*: were not located in *C. semilaevis*

## Comparative mapping

A comparative mapping was carried out with the following species: *G. aculeatus* (Figure 4), *D. rerio* (Figure 5), *T. nigroviridis* (Figure 6) and *O. latipes* (Figure 7).

In this kind of diagram it is possible to visualize, very rapidly, the degree of similarity in the genome between the two species compared. Thus, the more thin lines that the diagram presents, the more chromosome rearrangements that have occurred between the species and, consequently, the greater the genetic distance between the species. On this basis, the comparative mapping shows that, between the zebrafish and Senegalese sole, more rearrangements occurred than between any of the other species and the Senegalese sole. Conversely, the mapping shows that the fewest chromosome rearrangements have occurred between the stickleback and the Senegalese sole. Some authors have reported that Pleuronectiformes have developed from Perciformes fishes (Ivankov et al. 2008; Flores and Martínez 2013), so this could reflect the closer relationship between *S. senegalensis* and *G. aculeatus* with respect to the other species. In zebrafish the situation is different from that of the stickleback, because the time of divergence between *D. rerio* and *S. senegalensis* (260 Ma) is much longer than that between *G. aculeatus* and *S. senegalensis* (110 Ma) (Betancur et al. 2013).

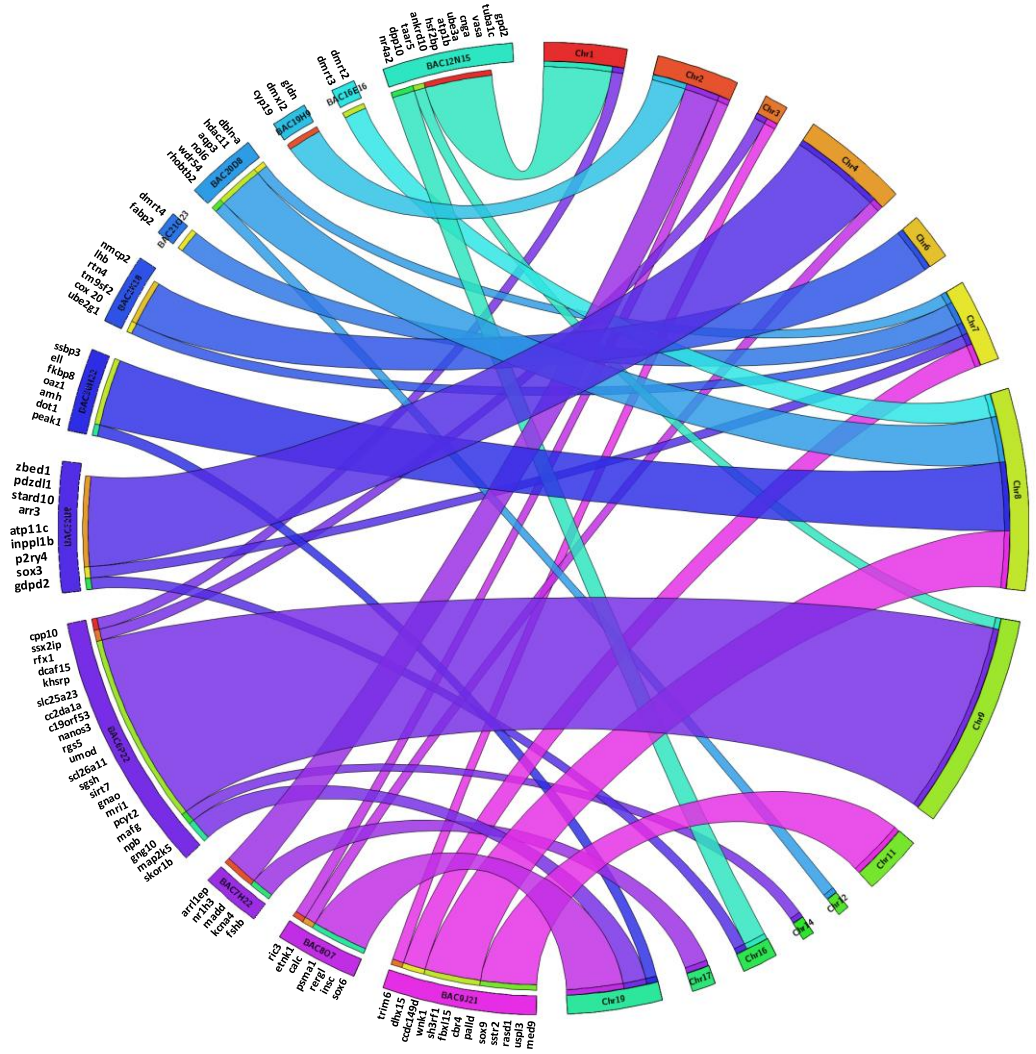


**FIG. 4.** Circos analysis in the species *Gasterosteus aculeatus* (stickleback). On the left side the distribution of the BACs of *S. senegalensis* can be observed. Indicated within each BAC are the genes found by annotation, and their corresponding localizations in the *G. aculeatus* genome, whose chromosomes are represented on the right side of the figure. BACs analyzed are those given in Table 3, plus the BAC containing the *fshβ* from García-Cegarra et al. (2013).

Shown in Figure 4 are the results of comparison between *S. senegalensis* and *G. aculeatus*, where BAC20D8, which contains *aqp3*, co-locates with BAC16E16, which contains the *dmrt2* gene, similar to our results in *S. senegalensis.* Both of these clones also co-locate with BAC10H22 (which contain the *amh* gene) and BAC9J21. These last two BACs do not co-locate in *S. senegalensis.*

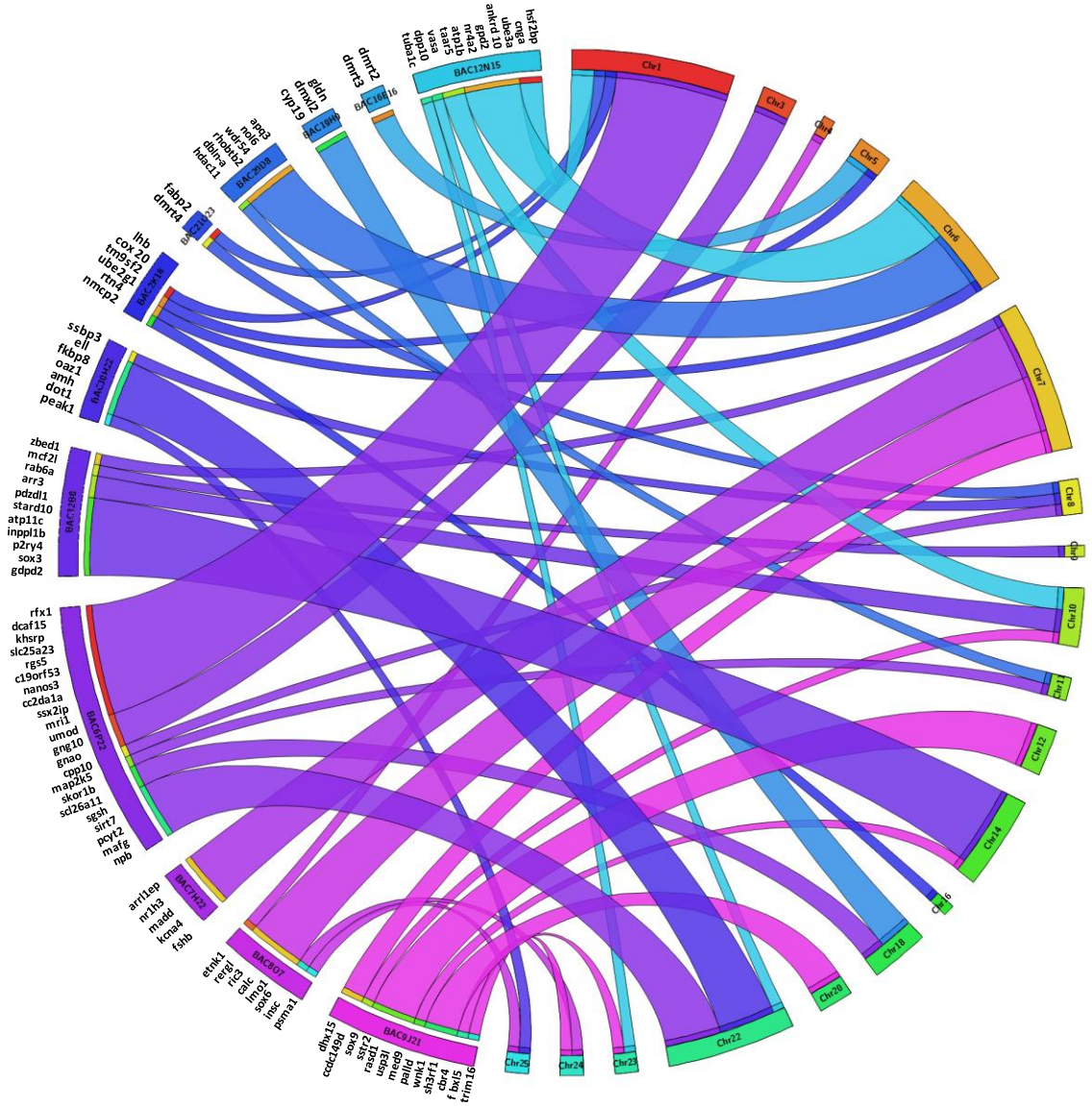


**FIG. 5.** Circos analysis in the species *Danio rerio* (zebrafish). On the left side the distribution of the BACs of *S. senegalensis* can observed. Indicated within each BAC are the genes found by annotation, together with their corresponding localizations in the *D. rerio* genome, whose chromosomes are represented on the right side of the figure. BACs - analyzed are those given in Table 3, plus and the BAC containing the *fshβ* from García-Cegarra et al. (2013).

In Figure 5 comparison between *S. senegalensis* and *D. rerio* indicates a partial co-location of BACs 20D8 and BAC12N15 in chromosome 6, although a candidate gene appears in only one of them. BAC9J21 which contains the *sox9* gene is located in seven different chromosomes in *D. rerio*. The s*ox9* gene co-locates with *vasa* in chromosome 10; however this co-location is not found in *S. senegalensis*.

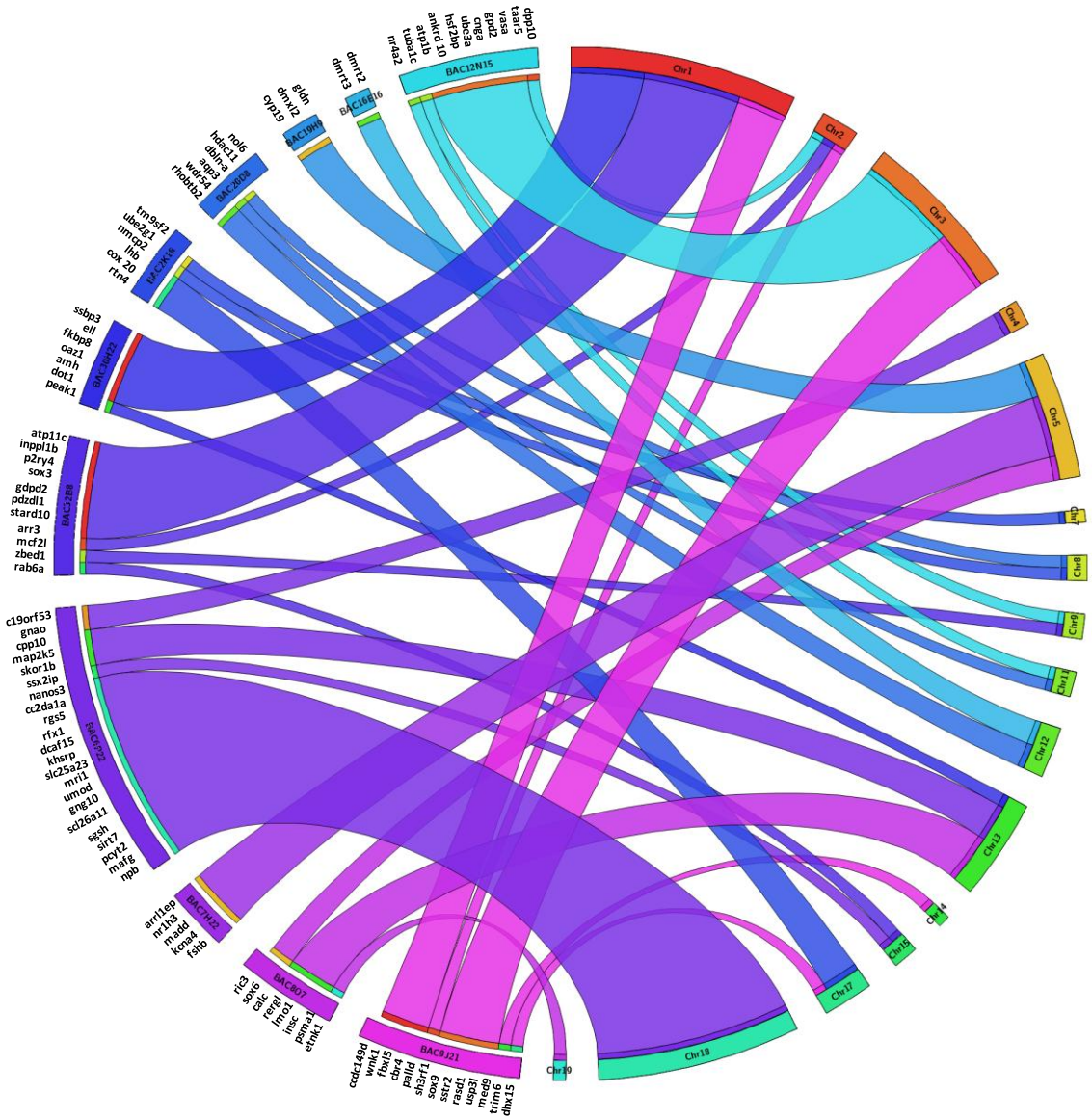


**FIG. 6.** Circos analysis in the species *Tetraodon nigroviridis* (pufferfish). On the left side the distribution of the BACs of *S. senegalensis* can be observed. Indicated within each BAC are the genes found by annotation, and their corresponding localizations in the *T. nigroviridis* genome, whose chromosomes are represented on the right side of the

figure. BACs analyzed are those given in Table 3, plus the BAC containing the *fshβ* from García-Cegarra et al. (2013).

In Figure 6 comparison between *S. senegalensis* and *T. nigroviridis* shows that BAC 9J21 and BAC 12

N15 co-localize only partially, although the part involved in co-location contains the candidate genes *sox9* and *vasa*, respectively.

In the comparison between *O. latipes* and S.senegalensis (Figure 7) the most relevant result is the partial location of BAC9J21 and BAC10H22, containing candidate genes *sox9* and *amh*, respectively. The *dmrt2* contained in BAC16E16 has the same behavior.

All the genes found by annotation in each BAC are distributed in 9 chromosomes in the Senegalese sole, but the above comparative analysis demonstrates that the same genes are distributed in a total of 14, 19, 16 and 17 chromosomes in *G. aculeatus*, *D. rerio*, *T. nigroviridis* and *O. latipes* respectively (Figures 4 to 7, and Table 6). It would be expected that the 12 target genes associated with sex-determination, sexual-differentiation and reproduction would be distributed in fewer chromosomes in *G. aculeatus* than in the other species, but that does not happen. When only the candidate genes are investigated, the species *T. nigroviridis* and *O. latipes* show a similar number of chromosomes bearing sex genes as *S. senegalensis*. It is well-known that *T. nigroviridis* has a very compact genome (Zaucker et al. 2014), and that property has also been described for flatfishes (Cerdà and Manchado, 2013). From an evolutionary point of view, such different genomes as those of *S. senegalensis* and *T. nigroviridis* could have evolved in a similar way to optimize the expression of genes with similar functions. Evidence of that kind of evolution could be the co-localization of the three genes, *fshβ*, *sox6* and *cyp19a1a*, in both *S. senegalensis* and *T. nigroviridis.* This co-localization can, in fact, be observed in the Circos comparison of these two species (Figure 6). *D. rerio* also possesses two of these three target genes, *sox6* and *fshβ*, on the same chromosome, which is consistent with the genome of *D. rerio* being somewhat less compact than that of *T. nigroviridis* but more compact than that of the other two species. It would thus support the theory about compact genome optimization in *T. nigroviridis* and *S. senegalensis*, and would highlight the greater rearrangement among these two species as mentioned above because of their evolutionary distance and genome size.

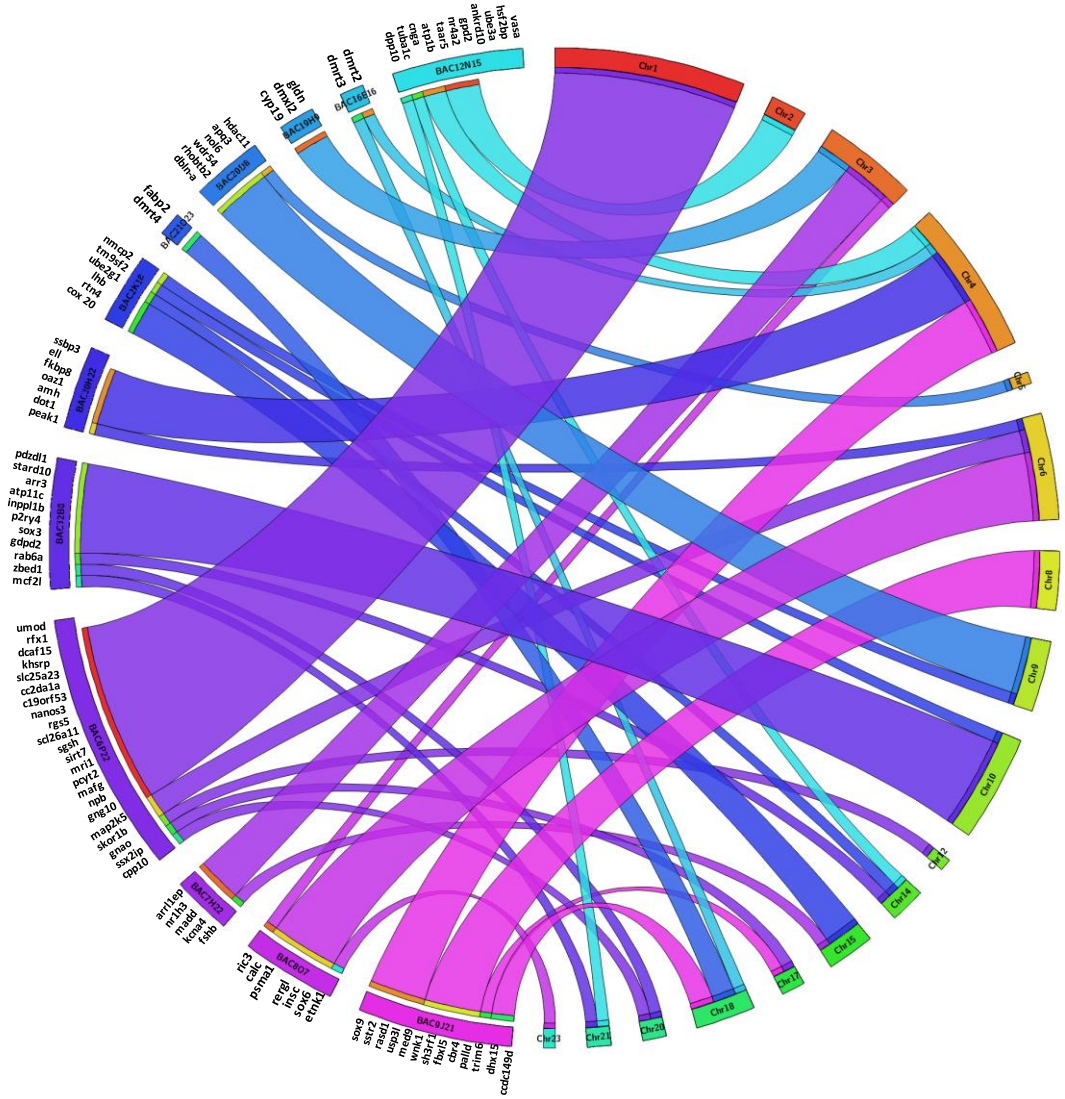


**FIG. 7.** Circos analysis in the species *Oryzias latipes* (Medaka). On the left side the distribution of the BACs of *S. senegalensis*, can be observed. Indicated within each BAC are the genes found by annotation, and their corresponding localizations in the *O. latipes* genome, whose chromosomes are represented on the right side of the figure. BACs analyzed are those given in Table 3, plus the BAC containing the *fshβ* from García-Cegarra et al. (2013)

**Table 6.** Distribution among the chromosomes of the five different species analyzed, of: [a] all genes, and [b] the genes implicated in sex-determination and reproduction, annotated as present within the BACs of *S. senegalensis*.

| Species | Location of BACs in chromosomes[a] | Location of candidate genes in chromosomes[b] | N° of chromosomes (>1 candidate gene in BACs)[c] | N° of candidate genes missing[d] |
|---|---|---|---|---|
| ***S. senegalensis*** **2n= 42** | 9 | 5 | 2 | 0 |
| ***G. aculeatus*** **2n= 42** | 12 | 9 | 1 | 0 |
| ***T. nigroviridis*** **2n= 42** | 15 | 6 | 3 | 1 (*dmrt4)* |
| ***O. latipes*** **2n= 48** | 15 | 7 | 3 | 1 (*vasa)* |
| ***D. rerio*** **2n= 50** | 17 | 8 | 2 | 1 (*lhb*) |

[a] Number of chromosomes in which BACs were located

[b] Number of chromosomes in which candidate genes were located

[c] Co-location of candidate genes in chromosomes

[d] Number and names of candidate genes missing in the chromosomes of the studied species related to second column

In *G. aculeatus* genes *dmrt2* and *dmrt3* are located on the same chromosome, similar to *D. rerio* and *T. nigroviridis*. This result is the same for *S. senegalensis*. However, in *O. latipes* gene *dmrt2* is not co-located on the same chromosome as *dmrt3* but gene *dmrt4* is co-located with *dmrt3*. This kind of deviation has also been observed in a micro-synteny study, in which *dmrt1* did not appear with the normal arrangement described in the bibliography. In zebrafish, the genes *fshβ* and *sox6* are on the same chromosome, as are some of the genes corresponding to the

whole of the BAC. In *T. nigroviridis* the gene *cyp19a1a* is also located with *fshβ* and *sox6*, similar to the Senegalese sole. In the case of *G. aculeatus* and *O. latipes* these candidate genes are not co-located, but other genes belonging to their BACs are on the same chromosome, in spite of these species having the same number of chromosomes, pointing to different evolutionary strategies.

Table 6 shows that the number of chromosomes with BACs located is increased with the number of chromosomes of the species (column 1), but the candidate genes do not follow this distribution, instead they appear grouped in particular chromosomes (column 2). Column 3 confirms the synteny of candidate genes.

## Phylogenetic analysis

The JTT phylogenetic tree obtained using sequences of 10 concatenated Genes (Supplementary material 5) showed a good resolution and a robust branch support (Figure 8). Using a concatenated protein sequence provided more robustness to the result, since this approach gives a more accurate tree (Gadagkar et al. 2005).

The phylogeny clearly separates the two Classes included, i.e. Sarcoptherygii and Actinoptherygii. Mammals are together in the same clade, and *Latimeria chalumnae* is found between marine vertebrates and land vertebrates. The coelacanth is considered the most ancient of the living osteichthyes and its evolutionary proximity to our own fish ancestor affords a view about the first fish to have crawled from the sea onto land (Amemiya et al. 2013). The ray-finned fish species are grouped together and among the clades it can be observed that modern fishes are in the same clade, and older fishes are in a different clade. These results also agree with that of the comparative mapping, where *D. rerio* was the species most distant from *S. senegalensis*. The analysis with concatenated sex-related genes supports the phylogenetic relationships previously established between Sarcoptherygii and Actinoptherygii, and among fish species also (Betancur et al. 2013).

It has been proposed that sex determination signals and mechanisms evolve so rapidly that the master gene rarely stays at the top of the hierarchy for very long, although the rest of the genes acting down on the network are more highly conserved (Heule et al., 2014). The location of *S. senegalensis* and *O. latipes* on the tree could be due to this, but the proximity of these two species to each other gives important clues regarding the genes involved in processes of sex-determination, sexual differentiation and reproduction studied in this paper.

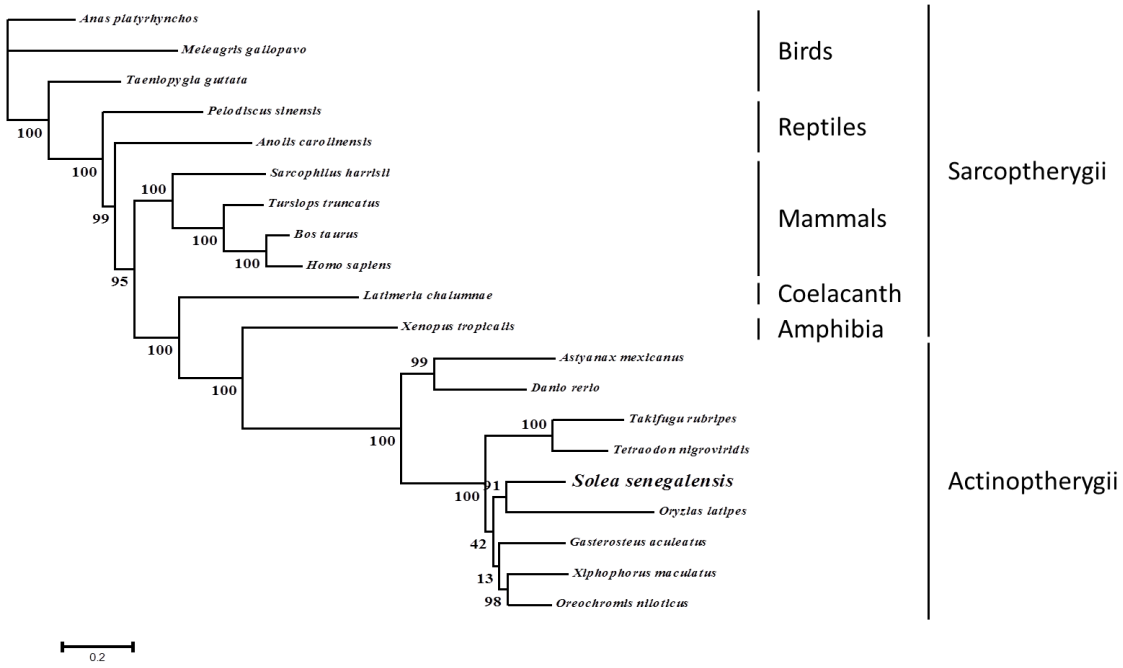


**FIG. 8.** This phylogenetic tree was created using the JTT model from concatenated candidate genes (10 genes available in all species: *amh*, *cyp19a1a*, *dmrt2*, *dmrt3*, *dmrt4*, *lhb*, *nanos3*, *sox3*, *sox6*, *vasa*) (Supplementary Material 5).

## Materials and Methods

A BAC library of Senegalese sole was used, taking as starting material a pool of larvae before mouth opening (3 days after hatching). Larvae were washed with DEPC water, then frozen directly in liquid nitrogen. High molecular weight genomic DNA was isolated from the samples, digested with *Bam* HI, cloned into the CopyControl TM pCC1 BAC tm (Epicentre Biotechnologies, USA) and transformed into the host cell DH10B tm (Invitrogen, Life Technologies, USA). The library comprised 29,184 positive clones distributed in 384-well plates (76 plates in total). Approximately, 99.99% of the clones contained nuclear DNA inserts of Senegalese sole (average size, 285 kb) with 0.01% of empty clones found.

### PCR screening of *S. senegalensis* BAC library

The strategy used to find and isolate the BAC clones was the 4D-PCR method modified by Asakawa et al. (1997). In this method, the library is pooled in four dimensions that are used as template DNA. The first dimension contains ten pools and corresponds to [1, 11 or 21] plates, [2, 12 or 22] plates… [10, 20 or 30] plates respectively. The second dimension includes three pools which (together with the first dimension) reveal the correct plate in which the BAC clone could be: [1-10] plate, [11-20] plate or [21-30] plate. The third and fourth dimensions give the

information about the well in which the BAC clone is located. In this case the third dimension consists of twenty four pools (columns) and the fourth dimension has sixteen pools (rows). The crosses between these dimensions will provide the positive well.

Genes relevant (candidate genes) for the sex-determining process were chosen, including members of the *SOX* (Sry-related high mobility group box) family such as *sox3*, *sox6*, *sox8* and *sox9*; members of the *DMRT* (The Doublesex and Mab-3 Related Transcription factors) family, such as *dmrt1*, *dmrt2* and *dmrt4*; Cytochrome P450 aromatase 19a (*cyp 19a1a*); Anti-Mullerian Hormone (*amh*); Follicle Stimulating Hormone (*fshβ*); Luteinizing Hormone (*lh*); Nanos 3 (na*nos3*); and ATP-dependent RNA helicase DEAD box protein 4 (*vasa*). Primer pairs of these genes were used to carry out the 4D-PCR (Table 7), and were designed from Senegalese sole sequences available in the Solea DataBase (SoleaDB, http://www.scbi.uma.es/soleadb), and using orthologous sequences from different fish species available in the ENSEMBL database. PCR was performed in a total volume of 25 µL using MyTaq$^{TM}$ HS red DNA polymerase (Bioline), and containing 5x My Taq Red Reaction Buffer (which comprises 5mM dNTPs, 15mM $MgCl_2$, stabilizers and enhancers); 50pg-10ng of Template; forward and reverse primers 10 µM each; My Taq HS DNA Polymerase 5u/µl and water. The PCR conditions were: denaturing at 95 °C for 2 min, followed by 30 cycles of 15 s at 95 °C, 15 s at 65 °C and 30 s at 72 °C.

**Table 1.** Primer sequences used for screening by 4D-PCR in the *S. senegalensis* BAC library.

| Gene | Primer sequences (5'→3') | Tm |
|---|---|---|
| *aqp3* | Fwaqp3:GCAGCGCTGATCGTTTGTAT<br>Rvaqp3:ATACGTGGTCCGAGGTCTCTT | 57ºC |
| *amh* | FwAMHSse:CTCTTTACAGTCCTTGCCTCCTCT<br>RvAMHSse: GGACCCACCTCCTCTTCTCTTAT | 59ºC |
| *cyp 19a1a* | FwCyp19a1aSse:CTCTTTACAGTCCTTGCCTCCTCT<br>RvCyp19a1aSse: GGACCCACCTCCTCTTCTCTTAT | 57ºC |
| *dmrt1* | Fwdmrt1: GCTGCAGGAACCACGGCTACGTGTC<br>Rvdmrt1: GGACTGCAAATACCAAGCTCCTCCTC | 63ºC |

| | | |
|---|---|---|
| *dmrt 2* | FwDmrt2Sse:GGAGCGCGAGTACAAAGAGCGRGAG<br>RvDmrt2Sse: CATGTTCTGCACSGCCGTGGCGTTG | 60ºC |
| *dmrt 4* | FwDmrt4Sse: CGAAGTGCGCCMGMTGYAGRAACC<br>RvDmrt4Sse: GYTCGATGGATYTSACAABGTCMCC | 60ºC |
| *fshβ*[a] | FwFSHSe:GGCGTCTGTACTGCAACCAGC<br>RvFSHSe:CACTTCATAGGTCCAGTCCCCG | 59ºC |
| *lhb* | FwLHSse: GGAGACGACCATCTGCAGCGGCC<br>RvLHSse: CAGCTCACAGCCACCGGGTACGTG | 60ºC |
| *nanos3* | FwNos3Sse:CAAGCACAACGGAGAATCTG<br>RvNos3Sse:GTCCACCTTTGGACAGAAGC | 56ºC |
| *sox3* | FwSox3Sse:CATAACTCTGGGGTCAGCAAG<br>RvSox3Sse:AGGACATCATCGGGTACTGG | 57ºC |
| *sox6* | FwSox6Sse: CAGGAGAAACAGCCATACTACG<br>RvSox6Sse:ACCGCATCAACTGTTTGTACTC | 57ºC |
| *sox8* | FwSox8: GAGCCCCAGCCACTACAGCGAGCAC<br>RvSox8: GTGGTGTAGACGGGCTGGTCCCAGC | 63ºC |
| *sox9* | FwSox9:GACTGGWCYYTKGTRCCSATGCC<br>RvSox9:CTGCTCAGCTCDCCRATGTCCAC | 60ºC |
| *vasa* | FwVasaSse:CGAGAGCAGTATCAGCGAGAG<br>RvVasaSse:GAAACCAAGGAGCGAGTCAA | 56ºC |

[a] García-Cegarra et al. (2013)

## Chromosome preparations

Chromosome preparations were made from *S. senegalensis* larvae 1 to 3 days-old. The specimens were pre-treated with 0.02% colchicine for 3 h to accumulate a larger number of metaphasic cells. Then they were subjected to hypotonic shock with KCl (0.4%), and finally fixed in a freshly-prepared solution of absolute ethanol-acetic acid (3:1). Larvae were homogenized in Carnoy, and the preparations were dropped onto wet slides by splashing on a hot plate with damp paper to create the necessary moisture for good spread of the chromosomes.

## mFISH analysis

## FISH probes

To isolate the BAC prior to labeling, a single colony containing the clone was grown on LB containing chloramphenicol at 37ºC overnight. BAC-DNA was extracted using the BACMAX$^{TM}$ DNA purification kit (EPICENTRE), following the manufacturer's instructions. The insert was extracted by digestion with *Eco*RI and analyzed by agarose gel electrophoresis (0.8%). The probes were amplified by DOP-PCR and then labeled by normal PCR using four different fluorochromes, i.e. Texas Red (Life Technologies), Spectrum Orange dUTP, Fluorescein-Isothiocyanate (FITC) (Abbott Molecular/ENZO) and diethylaminocoumarin (DEAC) (Vysis, Downers Grove, USA) using the protocol described in Liehr et al. (2009). Finally the probes were precipitated using a protocol with NaAc and ethanol.

## Hybridization and post-hybridization washes

For hybridization, chromosome preparations were pre-treated with pepsin solution at 37ºC and fixed with paraformaldehyde solution. Finally, the preparations were dehydrated with ethanol series of 70%, 90% and 100%, and air-dried before hybridization. Hybridization was carried out by denaturation of the probes and chromosome preparations in parallel, following the protocol described by Yang et al. (2009) with some modifications.

The post-hybridization treatment consisted of serial washes of SSC, Tween20 (Panreac) and PBS. The preparations were then dehydrated with ethanol and counterstained with Antifade-DAPI solution (VectorLabs). Hybridization images were obtained with a digital CCD camera (Olympus DP70) coupled to a fluorescence microscope (Olympus BX51 and/or Zeiss Axioplan using software of MetaSystems, Altlussheim, Germany).

## BAC clone sequencing and Bioinformatic analysis

Each BAC isolated from the library was checked by PCR, using primers indicated in Table 1, to ascertain if it contains the candidate gene. Partial sequencing by Sanger was also carried out. Clones identified as positive according to both criteria were sent for NGS sequencing by Roche Technology. DNA was isolated using the Large-Construct Kit (Qiagen), then digested and separated with the restriction endonucleases *Hae*II and *Rsa*I. The fragments generated were ligated to AP11/12 adapters using T4 DNA ligase, and were pre-amplified using the single primer AP11 and the Elongase Enzyme Mix (Invitrogen), according to supplier's recommendations. Pre-amplified products were purified, cloned and sequenced by the same procedure.

The functional and structural annotations of the gene sequences present in each BAC were performed in a semi-automated process. Protein and EST from *S. senegalensis* and related species were compared. The homologous sequences obtained were used to get the best predictions. Finally, all available information was used to create plausible models and, when it was possible, functional information was added. Using the Apollo genome editor (Lewis et al. 2002), Signal map software (Roche Applied Science) and Geneious basic 5.6.5 (http://www.geneious.com), the results were individually tested and adjusted in the final annotation process. Each clone was analyzed by BLAST analysis using the protein sequence to verify the annotation performed by the Annotation Company, because automatic annotation can make mistakes that must be rectified by hand.

The genome comparison with other species was carried out using the CIRCOS software (Krzywinski et al. 2009), which works with a linear data array where the data are included. The principle of this program is used to identify similarities among different versions of genomic arrangement. These results can also help to determine the relative closeness of these different species. The Circos program provides an efficient and scalable way to illustrate relationships between genomic positions, and the elements of the image allow the rearrangement to be easily understood (Krzywinski et al. 2009). The species compared were: the green spotted pufferfish (*Tetraodon nigroviridis*); zebrafish (*Danio reriro*); medaka (*Oryzias latipes*) and stickleback (*Gasterosteus aculeatus*). The ENSEMBL database (www.ensembl.org) was used to learn on which chromosomes the genes under study are localized in each species. With the object of comparing our species with other related species, such as the half-smooth tongue sole, a cytogenetic map of this species was produced, using data obtained from the NCBI Map Viewer (Figure 4).

### Phylogenetic analysis

To carry out the phylogenetic analysis a protein sequence of 10 candidate genes obtained by the NGS, arranged in concatenated form, was used; and ten protein sequences (*amh, cyp19a1a, dmrt2, dmrt3, dmrt4, lhb, nanos3, sox3, sox6, vasa*) from twenty species were analyzed (Figure 8). The sequence alignment was performed with the MAFFT tool (Katoh and Toh 2008). The ProtTest 3 program (Darriba et al. 2011) was used to determine the best-fit phylogenetic model. The resulting best-fit model predicted by the ProtTest program was the JTT model with the +I +G +F improvements, and with a corrected Akaike Information Criterion (AICc) of 224.511,629 and -LnL of -112.197.305. This model was running on the PhyML 3.0 program (Guidon et al. 2010); the branch support was tested by the fast likelihood-based method using aLRT SH-like (Anisimova et al. 2011). Finally, the tree editing was carried out in the MEGA6 program (Tamura et al. 2013).

## Conclusions

The species S*olea senegalensis* is currently the subject of many ongoing studies in respect of diverse areas of knowledge. The purpose of present work is to study c BACs, i.e. clones, which possess genes with functions in sex-determining, sexual differentiation and reproduction. The purpose is to pinpoint the genomic locations that control these aspects which are of major interest to aquaculture, both in this species and in all species susceptible to commercial production by aquaculture. This is because, in general, it is more desirable to obtain female stock than male, because females have a significantly faster rate of growth.

This work presents the first mFISH performed in this species. This technique has provided interesting results, particularly the co-location of several candidate genes. These data suggest the possibility of a specialization of chromosomes due to the small and compact size of the genome presented by this order. Support for this result has been gained by comparative analyses with another species, *T. nigroviridis*, whose compact genome also has this organization, as well as with *S. maximus*, another flatfish which shows co-location between the relevant genes *sox6* and *cyp19a1a*.

Synteny studies showed that the co-location of *dmrt1-dmrt2-drmt3* in the largest metacentric chromosome of *S. senegalensis* is coincident with the Z chromosome of *C. semilaevis* – a finding that would make this potentially a proto-sexual chromosome.

This study brings us one step closer to the possibility of integrating our data (and other unpublished data) with information about linkage groups obtained in this species, thereby achieving a complete and robust characterization of the karyotype of *S. senegalensis.*

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