Chromosoma (2017) 126:261–277
DOI 10.1007/s00412-016-0589-2

RESEARCH ARTICLE

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

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Received: 10 September 2015 / Revised: 31 March 2016 / Accepted: 4 April 2016 / Published online: 14 April 2016

Abstract The evolution of genes related to sex and reproduction in fish shows high plasticity and, to date, the sex determination system has only been identified in a few species. *Solea senegalensis* has 42 chromosomes and an XX/XY chromosome system for sex determination, while related species show the ZZ/ZW system. Next-generation sequencing (NGS), multi-color fluorescence in situ hybridization (mFISH) techniques, and bioinformatics analysis have been carried out, with the objective of revealing new information about sex determination and reproduction in *S. senegalensis*. To that end, several bacterial artificial chromosome (BAC) clones that contain candidate genes involved in such processes (*dmrt1*, *dmrt2*, *dmrt3*, *dmrt4*, *sox3*, *sox6*, *sox8*, *sox9*, *lh*, *cyp19a1a*, *amh*, *vasa*, *aqp3*, and *nanos3*) were analyzed and compared with the same region in other related species. Synteny studies showed that the co-localization of *dmrt1-dmrt2-drmt3* in the largest metacentric chromosome of *S. senegalensis* is coincident with that found in the Z chromosome of *Cynoglossus semilaevis*, which would potentially make this a sex proto-chromosome. Phylogenetic studies show the close proximity of *S. senegalensis* to *Oryzias latipes*, a species with an XX/XY system and a sex master gene. Comparative mapping provides evidence of the preferential association of these candidate genes in particular chromosome pairs. By using the NGS and mFISH techniques, it has been possible to obtain an integrated genetic map, which shows that 15 out of 21 chromosome pairs of *S. senegalensis* have at least one BAC clone. This result is important for distinguishing those chromosome pairs of *S. senegalensis* that are similar in shape and size. The mFISH analysis shows the following co-localizations in the same chromosomes: *dmrt1-dmrt2-dmrt3*, *dmrt4-sox9-thrb*, *aqp3-sox8*, *cyp19a1a-fshb*, *igsf9b-sox3*, and *lysg-sox6*.

Keywords Integrated genetic maps · Sex phenotype *Solea senegalensis* · Multicolor fluorescence in situ hybridization (mFISH) · Bacterial artificial chromosome (BAC)

Introduction

Solea senegalensis is a flatfish species belonging to the Pleuronectiformes order, which comprises about 570 species. This species has been identified as a target species for diversification in marine aquaculture due to its growth rates and flesh quality (Imsland et al. 2004). Although significant advances have been achieved in recent years concerning the procedures for larval rearing and ongrowing, sexual dysfunction of males reared in captivity still remains as a major bottleneck for the expansion of the aquaculture industry, thus limiting the establishment of commercial breeding programs (Guzmán et al. 2009).

Until now, among all fish species, sex master genes have been described only in *Oryzias latipes* (in which the determining male gene is *DMY* or *Dmrt1bY*) and in *Oncorhynchus mykiss* (in which the sex determining gene is *sdy*): in flatfish they are still unknown. The transcription factors that are

Electronic supplementary material The online version of this article (doi:10.1007/s00412-016-0589-2) contains supplementary material, which is available to authorized users.

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members of the double-sex and mab-3-related (*dmrt*) family are involved in gonad development and share a common DNA-binding domain (the DM domain). These factors show a little DNA sequence conservation and are responsible for sexual dimorphism in diverse organisms (Kopp 2012). In particular, the *dmrt1* gene is considered the first conserved gene in the sex determination/sexual differentiation cascade among several phyla (Marchand et al. 2000).

The genetic determination of sex is often associated with the formation of sex chromosomes. These chromosomes are usually genetically degenerate and have a high content of repetitive DNA, making it difficult to analyze both genetic content and gene organization (Cioffi et al. 2010). In teleosts, both heterogametic systems, the XX/XY and ZZ/ZW types, have been reported. Notably, these sex chromosomes are mostly homomorphic without morphological differentiation that could explain the existence of different systems of sex chromosomes even among closely related species (Mank et al. 2006; Mank and Avise 2009). From the genetic perspective, fish provide a paradigmatic example because their sex determination mechanisms range from the environmental to various different modes of genetic determination. The evolutionary significance of this remarkable plasticity is unknown (Heule et al. 2014).

Cynoglossus semilaevis is a flatfish species closely related to Senegalese sole whose genome has been recently described (Chen et al. 2014). This species possesses heteromorphic sex chromosomes with a ZZ/ZW sex determination system (Chen et al. 2007). In contrast, *S. senegalensis* lacks heteromorphic sex chromosomes and a putative XX/XY determination system has been proposed (Molina-Luzón et al. 2014). Interestingly, in *Scophthalmus rhombus*, an ancient XX/XY system that changed to a ZZ/ZW mechanism in *S. maximus* has been reported (Haffray et al. 2009; Taboada et al., 2014a). Although a major sex-determining region has been described in *S. maximus*, and several candidate genes related to sex determination and gonad differentiation have been mapped close to that region (Viñas et al. 2012), no heteromorphisms of sex chromosomes have been found in that species. In all these flatfish species, genetic and environmental influences on sex determination have been determined.

In aquaculture, it is essential to unravel the sex-determining mechanisms particularly for those species with sexual dimorphism in several traits such as growth. This is the case of some flatfish species such as *Hipoglossus hipoglossus*, *C. semilaevis*, and *S. senegalensis*, in which females grow faster than males do (Tvedt et al., 2006; Shao et al. 2010; Sánchez et al., 2010). Strategies to produce all-female stocks are based on a clear and independent identification of genetic and phenotypic sexes (Chen et al. 2009). Therefore, the identification of genetic markers linked to sex determination has become a major issue for producing monosex stocks.

Integrated genetic maps are a powerful tool for both genetic and evolutionary studies. Maps bring together data from gene sequencing and physical mapping on chromosomes. At present, bacterial artificial chromosomes (BACs) are the main tool for building physical maps and analyzing gene synteny, due to their stability and simplicity of handling (Cação et al. 2013). In recent years, improvements in the resolution and accuracy of the fluorescence in situ hybridization (FISH) technique have converted this technique into an indispensable tool for filling the gaps in genome sequencing projects (Gan et al. 2012). Moreover, it has established itself as a method for assembling high-resolution physical maps (Greulich-Bode et al. 2008). Together, BAC-FISH and next-generation sequencing (NGS) represent an efficient approach for anchoring genomic and linkage sequence data onto physical chromosomes (García-Cegarra et al. 2013).

The aim of this paper is to provide new information about the chromosome structure and arrangement of certain genes involved in sex determination and sexual differentiation processes in *S. senegalensis*, which may be of relevance for improving the commercial production of this species. Moreover, the results of this study, together with those previously published (Ponce et al. 2011; García-Cegarra et al. 2013), will enable us to build an integrated and updated genetic map. This information could not only help us to understand the development and evolutionary mechanisms in vertebrates but will also contribute to improving the production of target species for aquaculture.

Material and methods

BAC library

A BAC library was constructed using *S. senegalensis* larvae before mouth opening (3 days after hatching) as starting material. Larvae were washed with DEPC water, frozen in liquid nitrogen, and kept at −80 °C until use. High molecular weight genomic DNA was isolated and, after that, digested with *Bam* HI, before cloning into the CopyControl™ pCC1 BAC (Epicentre Biotechnologies, Madison, USA) and transformed into the host cell DH10B™ (Invitrogen, Life Technologies, Carlsbad, California, USA). The final BAC 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 (average size, 285 kb).

PCR screening of the *S. senegalensis* BAC library

To find and isolate BAC clones bearing targeted gene sequences, the 4D-PCR method was carried out (Asakawa et al. 1997). Briefly, plates were pooled in four dimensions that were used as template DNA. The first and second dimensions identified the plate in which the targeted BAC clone was located. The third and fourth dimensions provided the information about the well coordinates.

The following sex-determining candidate genes were chosen: members of the Sry-related high mobility group box (*sox*) family, particularly *sox3*, *sox6*, *sox8*, and *sox9*; members of the *dmrt* family, particularly *dmrt1*, *dmrt2*, and *dmrt4*; cytochrome P450 aromatase 19a (*cyp 19a1a*); anti-Mullerian hormone (*amh*); follicle-stimulating hormone (*fshb*); luteinizing hormone (*lh*); nanos 3 (*nanos3*); and ATP-dependent RNA helicase DEAD box protein 4 (*vasa*). Specific primers (see Supplementary Material 1) were designed using template sequences from the SoleaDB (Benzekri et al. 2014) and from orthologous sequences of different fish species available in the ENSEMBL database. The *fshb* primers and PCR conditions were the same as those described in García-Cegarra et al. (2013).

BAC clone sequencing and bioinformatic analysis

BAC clones identified by the 4D-PCR method were checked by PCR using specific gene primers, followed by Sanger sequencing. Validated clones were sequenced by 454 Roche Technology. BAC clones were isolated using the Large-Construct Kit (Qiagen, Hilden, Germany), 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 Life Technologies, Carlsbad, California, USA), according to the supplier's recommendations. Pre-amplified products were purified, cloned, and sequenced by the same procedure. The sequencing quality was assessed by the analysis of various parameters, such as the number of reads, the average size of reads, the total length sequenced, the total number of contigs assembled, and the number of large contigs (more than 500 bp in length). The level of *Escherichia coli* contamination and the N50 value were also evaluated. Contig N50 is a weighted median statistic such that 50 % of the entire assembly is contained in contigs or scaffolds equal to or larger than this value.

The functional and structural annotations of the gene sequences identified in each BAC were carried out 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 for gene annotation. Finally, all available information was used to create plausible models and, when possible, functional information was added. Using the Apollo genome editor (Lewis et al. 2002), Signal map software (Roche Applied Science, Penzberg, Germany), and Geneious basic 5.6.5 (http://www.geneious.com/), the results were individually completed and adjusted in the final edition process of the annotation.

Cross-species genome comparisons were carried out at two levels. At the first level, a micro-synteny study was performed using the Genomicus (Louis et al. 2015) platform, which takes the genome information from the ENSEMBL database. For this micro-synteny analysis, the species *Gasterosteus aculeatus* was used as reference genome, with the exception of BAC2K18, for which the reference species selected was *Danio rerio*, because the *lhb* gene could not be found in *G. aculeatus*. The order of the contigs within each BAC of *S. senegalensis* was estimated using the information provided by the Genomicus program. In the schematic figures of the Genomicus program, the blocks that appear colorless correspond to genes that are not represented in the reference species (*G. aculeatus*) in the analyzed region. In addition, an orthology comparison was performed between *C. semilaevis* and *S. senegalensis* using MAFFT alignment (Katoh and Toh 2008) between protein sequences of the candidate genes, in order to confirm that ortholog genes were compared. At the second level, a synteny analysis was performed using the Circos software (Krzywinski et al. 2009); 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. The Circos diagram facilitates the visualization of genome similarity between two species. Thus, the thinner the lines that appear in the diagram, the more chromosome rearrangements that have occurred between the species and, consequently, the greater the genetic distance between the species. The species used in this analysis were available at ENSEMBL: *Tetraodon nigroviridis*, *D. rerio*, *O. latipes* and *G. aculeatus*. In order to compare *S. senegalensis* with the closely related *C. semilaevis*, a cytogenetic map of this species was produced with the Map Viewer tool, using public genome data available at NCBI.

mFISH analysis

Chromosome preparations

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

FISH probes

To prepare FISH probes, BAC clones were grown on LB containing chloramphenicol at 37 °C overnight. BAC-DNA was extracted using the BACMAX™ DNA purification kit

(Epicentre Biotechnologies, Madison, USA), 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 a conventional PCR using four different fluorochromes, i.e. Texas Red (Life Technologies, Carlsbad, California, USA), Spectrum Orange, Fluorescein isothiocyanate (FITC) (Abbott Molecular/ENZO, Illinois, USA), and diethylaminocoumarin (DEAC) (Vysis, Downers Grove, USA), using the protocol described in Liehr (2009). Finally, the probes were precipitated using a protocol with NaAc and ethanol. In addition to the BAC clones considered in this study, the BAC clones studied in previous works (Ponce et al. 2011; García-Cegarra et al. 2013) were also included as FISH probes.

Hybridization and post-hybridization washes

For hybridization, chromosome preparations were pre-treated with pepsin solution at 37 °C 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 Liehr (2009) with some modifications. These modifications involved the labeling of the probes by DOP-PCR instead of nick translation, and the blocking DNA was sonicated genomic DNA from *S. senegalensis* instead of the human-COT1 (Invitrogen, Life Technologies, Carlsbad, California, USA).

The post-hybridization treatment consisted of serial washes of SSC, Tween20 (Panreac, Barcelona, Spain), and PBS. The preparations were then dehydrated with ethanol and counterstained with antifade-DAPI solution (VectorLabs, Burlingame, California, USA). 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).

Phylogenetic analysis

The protein sequences of ten candidate genes (*amh*, *cyp19a1a*, *dmrt2*, *dmrt3*, *dmrt4*, *lhb*, *nanos3*, *sox3*, *sox6*, and *vasa*) were concatenated to carry out the phylogenetic analysis. Twenty-one vertebrate species were included to generate the phylogenetic tree, including *S. senegalensis* (Supplementary Material 2). Additionally, the arthropod *Drosophila melanogaster* was included to root the tree. The sequence alignment was performed with the MAFFT tool (Katoh and Toh 2008) using an iterative method. The PhyML 3.0 program (Guindon et al. 2010) was used to determine the best-fit phylogenetic model and then to run the model. The resulting best-fit model predicted the JTT model, considering a proportion of invariable sites (+I), gamma distribution (+G), and heterogeneous frequencies (+F). The statistic used for model selection was the akaike information criterion (AIC), the value of which was 239,159.76, and the -LnL was −119,521.53. Branch support was tested by the fast likelihood-based method using aLRT SH-like (Anisimova et al. 2011). Finally, the tree was edited in the MEGA6 program (Tamura et al. 2013).

Results

Sequence and micro-synteny analysis

A total of 93 genes were annotated on 13 BAC clones (Table 1). The set of candidate genes were detected in 11 out of 13 BAC clones. The *dmrt1* gene (BAC11O20) and *sox8* gene (BAC10K23) were partially sequenced by Sanger technology (acc. no. KT724725 and KT724726, respectively). Assembled BAC contigs were deposited in the GenBank database (NCBI) under accession numbers AC270096 to AC270104 and AC270124 to AC270125. The complete name of the annotated genes can be found in the Supplementary Material 3. Contamination with *E. coli* was less than 4 %, thus indicating a satisfactory BAC isolation and library preparation (Supplementary Material 4). Only one BAC (BAC6P22) showed a higher contamination level (16 %) considering only 11 out of 63 contigs assembled for annotation on the basis of its similarity to eukaryotic species. The N50 values ranged from 2505 to 47,966 bp (mean 23,201 ± 13,858). The results obtained showed that *dmrt2* and *dmrt3* were co-localized within the same BAC (BAC16E16) although assembled in different contigs, whereas the gene *dmrt1* was found within BAC11O20 (Table 1). The *dmrt4* gene (also named *dmrta1*) was also isolated from a different BAC (BAC21O23) and two genes (*dmrt4* and *fabp2*) were annotated in the only useful contig obtained (Table 1). The sequencing results provided a non-linked arrangement of *sox8* and *sox9* genes in *S. senegalensis*.

The micro-synteny analysis showed that most, but not all, of the candidate genes showed a similar genomic gene organization between *S. senegalensis* and other teleosts (Supplementary Material 5). However, some differences were observed in some cases in regions upstream and/or downstream from the candidate gene, especially between non-closely related species. For the *dmrt* family, micro-synteny analysis confirmed that the *dmrt1-dmrt3-dmrt2* organization was preserved among teleosts, including the closely related species *C. semilaevis*, in which these genes were located in the Z sex-chromosome (Supplementary Material 6). The micro-synteny analysis also showed that *sox8* and *sox9* genes were linked and located near each other in all teleost species

Table 1 Name of the BACs studied, candidate gene used for 4D-PCR, and genes annotated within each BAC (review Supplementary Material 1 for full name of the genes)

Name of BAC	Candidate gene	Genes annotated
BAC2K18	*lh*	*rtn4*, *lhb*, *ube2g1*, *nmcp2*, *tm9sf2*, *cox20*
BAC6P22	*nanos 3*	*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*, *sox6*, *etnk1*
BAC9J21	*sox9*	*dhx15*, *ccdc149b*, *wnk1*, *sh3rf1*, *sstr2*, *trim16*, *fbxl5*, *rasd1*, *cbr4*, *usp3l*, *med9*, *palld*, *glnd*, *sox9*
BAC10K23	*sox8*[a]	*arhgap21*, *apod*, *otos*, *opn3*, *tmtops*, *eps15l1*, *calr*, *valopa*
BAC11O20	*dmrt1*[a]	*nol6*, *aqp3*, *arrdc3*
BAC12N15	*vasa*	*dpp10*, *ddx4(vasa)*,*cnga*, *gpd2*, *ube3a*, *atp1b*, *nr4a2*, *hsf2bp*, *ankrd10*, *tuba1c*, *taar5*
BAC16E16	*dmrt2*	*dmrt2*, *dmrt3*
BAC19H9	*cyp19a*	*gldn*, *dmxl2*, *cyp19*
BAC20D8	*aqp3*	*arrdc3*, *aqp3*, *nol6*, *zbed4*, *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*

[a] The candidate gene has not been found by NGS, but by conventional Sanger sequencing

considered (Supplementary Material 5 and 6). However, *sox3* and *sox6* were neither linked to each other nor to the *sox8/sox9* genes among the species, including *C. semilaevis* (Supplementary Material 5 and 6). Moreover, *sox6* presents two paralog sequences in some of the species analyzed, with inversions in several species, such as *D. rerio*, *Astyanax mexicanus*, and *O. latipes*.

The region surrounding the *amh* gene was highly conserved in all teleosts (Supplementary Material 5). However, for the *nanos3* BAC clone, the results show that the region is more conserved among the species of more recent appearance than among those species considered more ancient, i.e., the region is less conserved in *D. rerio*, *A. mexicanus*, and *Latimeria chalumnae*.

mFISH analysis of BACs

The chromosome mapping of BAC clones listed in Table 1 are depicted in Fig. 1, Table 2, and Supplementary Material 7. All metaphases analyzed showed 21 pairs of chromosomes that correspond with the expected karyotype of *S. senegalensis* (Vega et al. 2002).

The mFISH technique located the 13 BAC clones on 10 different chromosome pairs. Nevertheless, additional secondary signals (four or six) were detected in some BAC hybridizations (Table 2). As a whole, the mFISH resulted in nine BAC clones producing single signals, three localized into two pairs, and just one into three pairs. The *lhb* BAC clone produced multiple signals and could not be assigned to a specific chromosome pair.

Results showed that some BAC clones were co-localized in the same chromosome. The BAC containing *dmrt2* and *dmrt3* genes produced signals on three chromosome pairs: the main signal was on the largest metacentric chromosome pair and co-hybridized with the *dmrt1* BAC clone (Fig. 1g); a second signal was localized on a subtelocentric chromosome pair and co-hybridized with both *aqp3* and *sox8* BAC clones (Fig. 1e); and the third signal was on an acrocentric chromosome pair. The gene *sox9* hybridized on two chromosome pairs: one signal was on a metacentric pair and co-hybridized with the *dmrt4*, *nanos3*, and *thrb* BAC clones (Fig. 1b, h) and the other signal was on an acrocentric chromosome pair and co-hybridized with the *thraa* BAC clone. Finally, the *fshb* BAC clone co-hybridized with the *cyp19a1a* and *sox6* BAC clones (Fig. 1c).

Integrated genetic map

The integrated genetic map is shown in Fig. 2, which summarizes the cytogenetic map, sequence distances in base pairs, and the annotation results. This integrated map shows that the largest metacentric chromosome contains the three important genes for sex determination, i.e., *dmrt1*, *dmrt2*, and *dmrt3*. The second metacentric pair, however, is the chromosome for which the most information has been obtained. In this chromosome up to four BAC clones were co-localized by mFISH, more than 24 genes were annotated and, in some cases, the physical distance could be determined. Other chromosomes with high information density were the first, second, and fourth subtelocentric pairs,

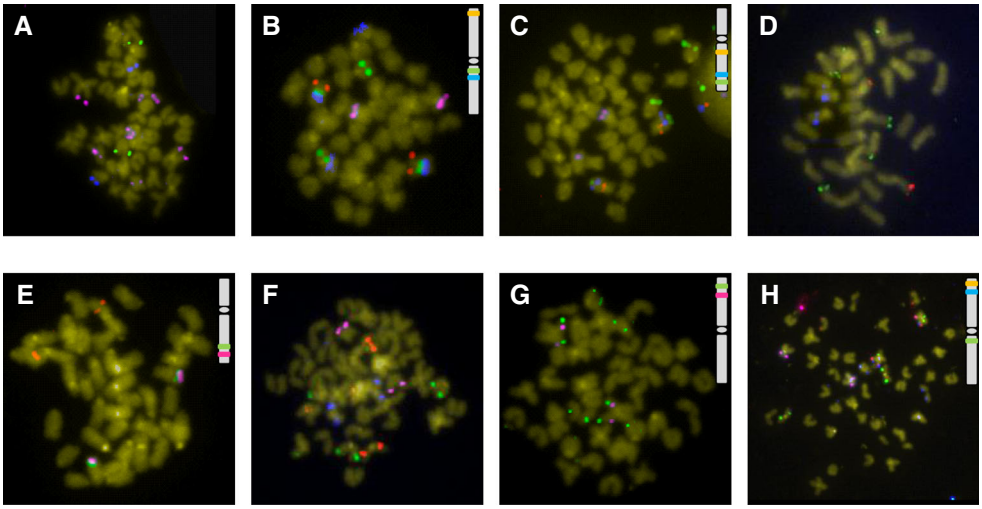

Fig. 1 mFISH of the BACs isolated in the library that contain the following candidate genes: **a** *amh* (*green*), *dmrt2* (*pink*), and *sox3* (*blue*); **b** *sox9* (*green*), *nos3* (*orange*), *vasa* (*pink*), and *dmrt4* (*blue*); **c** *sox6* (*green*), *fshb* (*orange*), *sox8* (*pink*), and *cyp19a1a* (*blue*); **d** *dmrt2* (*green*), *nos3* (*orange*), and *cyp19a1a* (*blue*); **e** *sox8* (*green*), *dmrt1* (*orange*), and *aqp3* (*pink*); **f** *sox6* (*green*), *sox9* (*orange*), *sox8* (*pink*), and *sox3* (*blue*); **g** *dmrt2* (*green*), and *dmrt1* (*pink*); **h** *sox9* (*green*), *nos3* (*orange*), *vasa* (*pink*), and *thrb* (*blue*). In those cases in which two or more probes are co-localized in one chromosome, a diagrammatic representation is included

together with the two acrocentric chromosome pairs that bear the *sox6* and *sox9* genes.

Comparative mapping

The comparative mapping revealed that rearrangements were more common between *D. rerio* and *S. senegalensis* than between any other combination of species (Figs. 3, 4, 5, and 6). Conversely, chromosome gene arrangements were highly conserved between *G. aculeatus* and *S. senegalensis*. Alignment of protein sequences of the candidate genes between *S. senegalensis* and *C. semilaevis* (Supplementary Material 8) confirmed that the same orthologous gene copies were compared between these two species. Sequence comparisons revealed a large conserved region of the *nanos3* BAC clone in chromosome 9 of *G. aculeatus* (Fig. 3). Moreover, several regions of the *aqp3* BAC, *dmrt2/dmrt3* BAC, the *amh* BAC, and the *sox9* BAC clones were localized in the chromosome 8. However, although the majority of the genes within the *sox9* BAC clone were present in chromosome 8, the *sox9* gene could not be identified. Two other candidate genes, *dmrt4* and *sox3*, were co-localized in chromosome 7.

The comparison with *D. rerio* showed more gene re-arrangements, based on the lower number and smaller size of conserved regions (Fig. 4). Again, the largest conserved region was observed in the *nanos3* BAC clone, which is localized in chromosome 1 of *D. rerio*. The *aqp3* and *vasa* BAC clones were partially co-localized in chromosome 6. The *fshb* and *sox6* BAC clones were also partially co-localized in chromosome 7. The genes within the *sox9* BAC clone were distributed in seven different chromosomes in *D. rerio*, thus showing large gene re-arrangements. The *sox9* gene was co-localized with the *vasa* gene in chromosome 10; however, this co-localization is not found in *S. senegalensis*.

Concerning the comparison with *T. nigroviridis* (Fig. 5), the *nanos3*-bearing BAC also presents the largest conserved region and it is localized on chromosome 18. Partial co-localizations which involve several candidate genes were detected including *amh* and *sox3* in chromosome 1, *vasa* and *sox9* in chromosome 3, and *cyp19a1a*, *fshb*, and *sox6* in chromosome 5.

In the comparison between *O. latipes* and *S. senegalensis* (Fig. 6), the largest conserved region was again the *nanos3*-bearing BAC, although the *sox3*-bearing BAC was also highly conserved. A partial co-localization of the *dmrt2*, *sox9*, and *amh* BAC clones was observed in chromosome 4. In addition, the two gonadotropin genes were found in the same chromosome (chr. 15). The most surprising finding is that the *dmrt2* and *dmrt3* genes are not co-localized: instead, the *dmrt3* and *dmrt4* genes were both found in chromosome 18.

The comparative analysis demonstrates that all the genes identified in the BAC clones were distributed in 9 chromosomes in *S. senegalensis*, whereas they appeared distributed in a total of 14, 19, 16, and 17 chromosomes in *G. aculeatus*, *D. rerio*, *T. nigroviridis*, and *O. latipes*, respectively (Figs. 3, 4, 5, and 6, Table 3). This increasing number of BAC-bearing chromosomes is associated with the increasing number of the chromosome complement in the species analyzed (Table 3, column 1). If only the 12 candidate genes are considered, such a trend would disappear, since all the species showed fewer candidate gene-bearing chromosomes. Of these species, *S. senegalensis* and *T. nigroviridis* show the smallest number of sex gene-bearing chromosomes (Table 3, column 2), thus indicating a greater specialization of chromosomes.

Table 2 Number of FISH signals and localization of BAC clones onto *Solea senegalensis* chromosomes

Name of BAC (candidate gene)	Number of signals[a]	Localization of signals	
		Chromosome type	Position within chromosome
BAC2K18 (*lhb*)	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

MT metacentric chromosome, *SMT* sub-metacentric, *STC* subtelocentric, *A* acrocentric, *SC* sub-centromeric position, *I* interstitial, *STL* subtelomeric, *TL* telomeric, *q* long arm, *p* short arm

[a] Number of signals per metaphase plate

[b] From García-Cegarra et al. (2013)

Moreover, *T. nigroviridis* and *O. latipes* are the two species that have the most chromosomes with more than one candidate gene in them (Table 3, column 3).

Phylogenetic analysis

The JTT phylogenetic tree obtained using sequences of 10 concatenated genes showed a good resolution and a robust branch support (Fig. 7). The phylogeny clearly separated the two Classes included, i.e., Sarcoptherygii and Actinoptherygii. Mammals are together in the same clade, and the coelacanth (*L. chalumnae*) is found clustered apart from the remaining Sarcoptherygii species (tetrapods). The ray-finned fish species are grouped together and, among the clades, modern fishes clustered together with a clear separation between fishes belonging to the Otomorpha cohort (*A. mexicanus* and *D. rerio*) and the Euteleosteomorpha cohort (the remaining ray-finned fishes).

Discussion

The *dmrt* genes are transcription factors belonging to the DM domain gene family that are associated with sex determination and differentiation. The gene cluster *dmrt1-dmrt3-dmrt2* appears widely conserved in vertebrates, including teleosts (El-Mogharbel et al. 2007; Brunner et al. 2001; Sheng et al. 2014; Chen et al. 2014). Although our micro-synteny analysis could not clearly establish this cluster, probably because of non-overlapping BAC clones, they were found by the mFISH technique co-localized and near each other in the same chromosome (Fig. 1g). The distance observed between FISH signals produced by *dmrt2*/*dmrt3*-containing BAC and the signal produced by the *dmrt1*-BAC clone could be due to some chromosomal rearrangement. The evolution of these genes is still not clear because of lack of data in basal metazoans. However, using the 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 the domain subsequently expanded in the metazoan lineage (Bellefroid et al. 2013) and, more accurately, during the interval between *Trichoplax* and eumetazoans (Wexler et al. 2014). The cluster *dmrt1-dmrt3-dmrt2* was found in a linkage group (LG) different from that of the *dmrt4* in *G. aculeatus* and *Lepisosteus oculatus* (Supplementary Material 5). This situation has also been observed in *D. rerio* and *O. latipes* (Kondo et al. 2002; Woods et al. 2000). However, in humans, *dmrt1*, *dmrt2*, *dmrt3*, and *dmrt4* genes are linked in chromosome 9 (Kondo et al. 2002). It has been hypothesized that these four *dmrt* genes could have arisen after several rounds of tandem duplications; hence, they indicate an ancestral origin (Kondo et al. 2002). Curiously, the *dmrt1-dmrt3-dmrt2* cluster has been located in the Z sex chromosome of the closely related flatfish *C. semilaevis* (Chen et al. 2014). In *S. senegalensis*, the *dmrt* cluster appears also linked to a histone cluster (Supplementary Material 9). The location of multi-gene families in sex chromosomes has also been reported in some other species (Utsunomia et al. 2014) and, indeed, the 18S rDNA has been accumulated mainly in the X chromosome of the fish species *Hoplias malabaricus* (Cioffi et al. 2010), and even among karyomorphs of the same species (Bertollo et al. 1997).

The evolution of sex chromosomes generally involves the accumulation of repetitive elements by different strategies that lead, in most of the cases, to heteromorphism. Kejnovsky et al. (2009) proposed the repetitive DNA as the initial mechanism involved in the evolution of sex chromosomes. A high density of GATA-motif repeats has been reported in the W

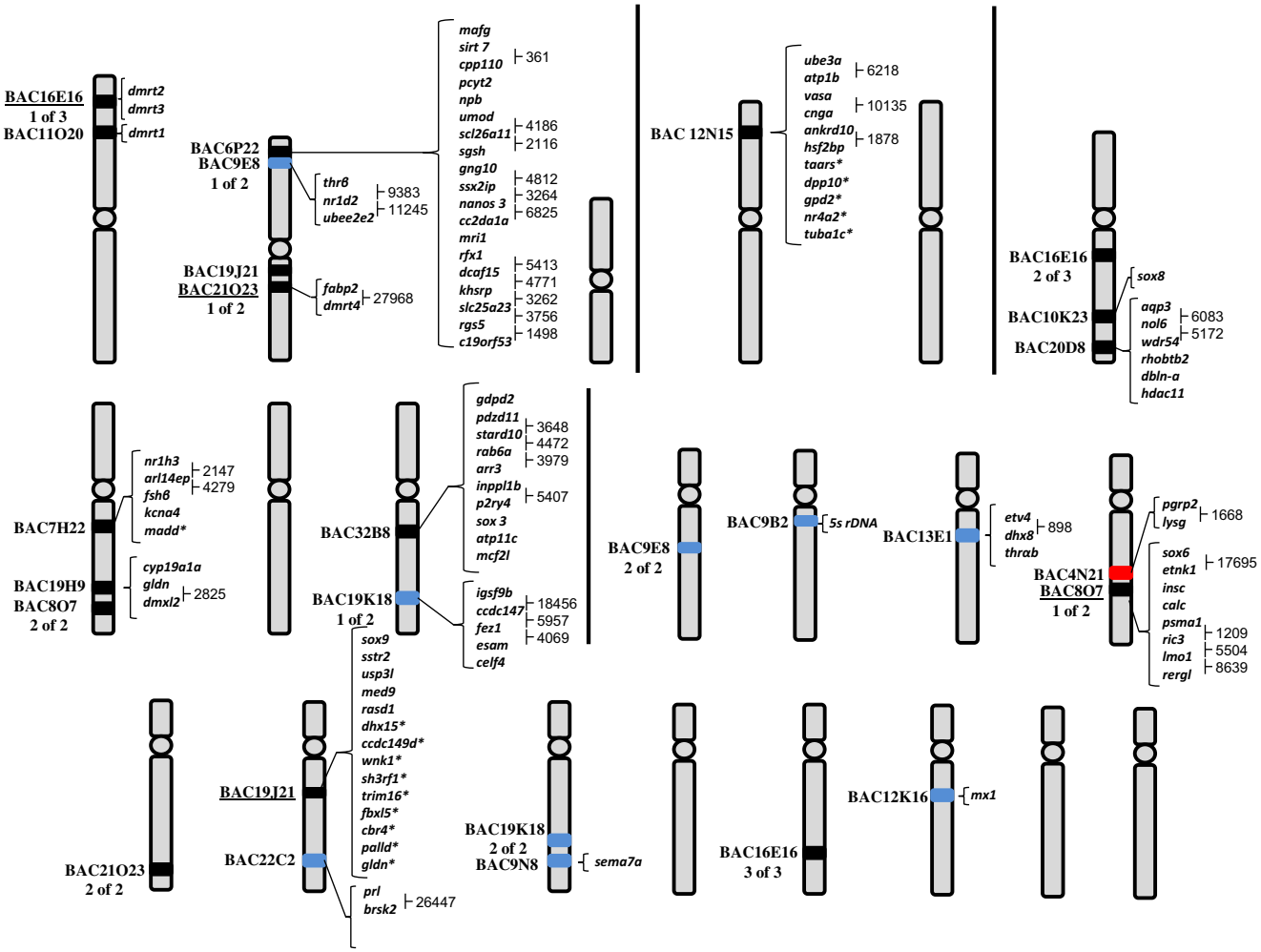

Fig. 2 Integration of cytogenetic and physical maps of *S. senegalensis*. Cytogenetic results are shown in *boxes* within the chromosome diagram; the *red box* refers to the results obtained by Ponce et al. (2011), *blue boxes* those by García-Cegarra et al. (2013) and *black boxes* those by this study. *Underlined BAC names* indicate the main FISH signal of those BACs with more than one signal. The sequencing result of each BAC is shown in *parenthesis*, and the physical distance between genes is represented in bp units. When two contiguous genes come from different contigs, the physical distance cannot be shown. *Asterisk* indicates that contigs cannot be ordered by micro-synteny

chromosome of the female snake *Elaphe radiata* (Jones and Singh 1985) and such repeats have enabled the sex chromosomes in the guppy fish to be identified (Nanda et al. 1990). A high concentration of $(GATA)_n$ repeats at a specific chromosome pair was described in the toadfish *Halobatrachus didactylus* (Merlo et al. 2007). However, previous studies did not find an accumulation of those sequences 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, since it has been postulated that in species with a compact genome, repeated sequences are less frequently present than those in species with a larger genome. Therefore, the quantity of repeated sequences might not be enough to be detected by FISH.

Flatfish genomes are compact, and the number of chromosomes ranges from 21 pairs in Soleidae and Cynoglossidae to 24 in Pleuronectidae and Paralichthyidae (Cerdà and Manchado, 2013). We hypothesize that a Robertsonian fusion between two acrocentric chromosomes could have occurred during the evolution of Pleuronectiformes, giving arise to a large metacentric chromosome in *S. senegalensis*. The cluster *dmrt1-dmrt2-dmrt3* might be located in such acrocentric chromosomes linked to a histone gene family. Although interstitial telomeric $(TTAGGG)_n$ sequences were not found on the metacentric chromosome, its origin in a Robertsonian fusion cannot be excluded because the loss of telomeric sequences can occur after such rearrangements (Cross et al. 2006). These rearrangements could explain the differences in sex-determination systems among closely related species, since they have been proposed as a major driving force for speciation (Heule et al. 2014; Ser et al. 2010). In *S. maximus* and *C. semilaevis*, a ZZ/WZ system for sex determination has been described (Hu et al. 2014). In contrast, an XX/XY system was proposed in *S. senegalensis* (Molina-Luzón et al. 2014). A

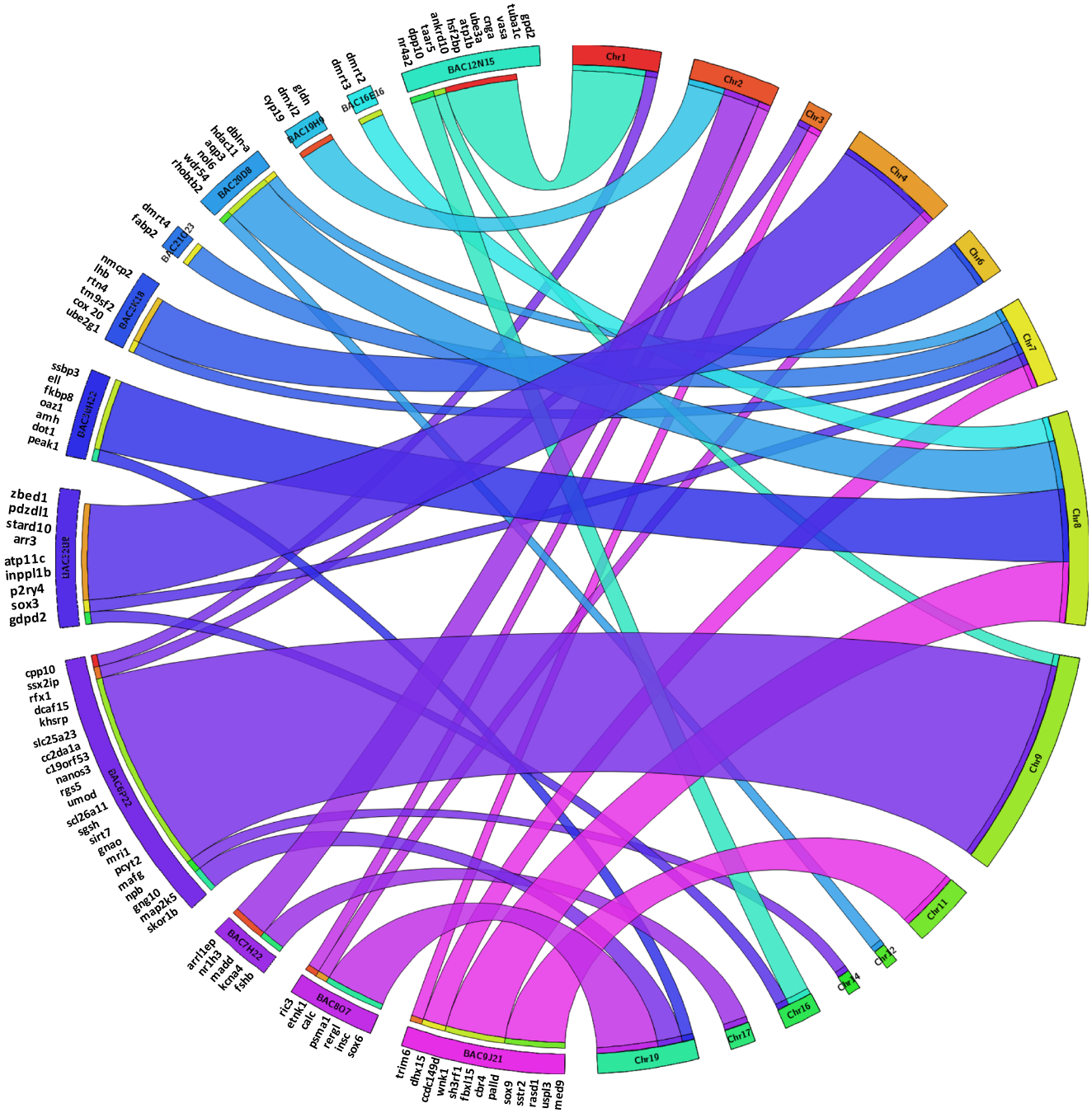

Fig. 3 Circos analysis in the species *G. aculeatus*. On the *left side*, the distribution of the BAC clones of *S. senegalensis* can be observed. Indicated within each BAC are the genes found by annotation, and the corresponding localizations in the *G. aculeatus* genome are denoted by *crossing lines*. The chromosomes of that genome are represented on the *right side* of the figure. BAC clones analyzed are those given in Table 1, in addition to the *fshb*-bearing BAC obtained from García-Cegarra et al. (2013)

recent theoretical model raises the possibility of transitions between the XY/XX and ZZ/ZW systems and environmental sex determination, and some species such as *Xiphophorus maculatus* are at an intermediate stage with both ZW and XY systems occurring in the same population (Pennell et al. 2015).

The *sox8* and *sox9* genes are important in fish reproduction: *sox8* is involved in Sertoli cell development and in spermatogenesis (O'Bryan et al. 2008), whereas *sox9* is initially expressed on the lateral side of the bi-potential genital ridge and up-regulated in the Sertoli cell precursors in the XY male gonad, immediately after the onset of SRY gene expression (Chaboissier et al. 2004). The linkage between *sox8* and *sox9* genes appeared to be highly conserved across teleosts including *C. semilaevis*. However, these two genes are located separately in the chromosomes of *S. senegalensis* (Fig. 1f) and

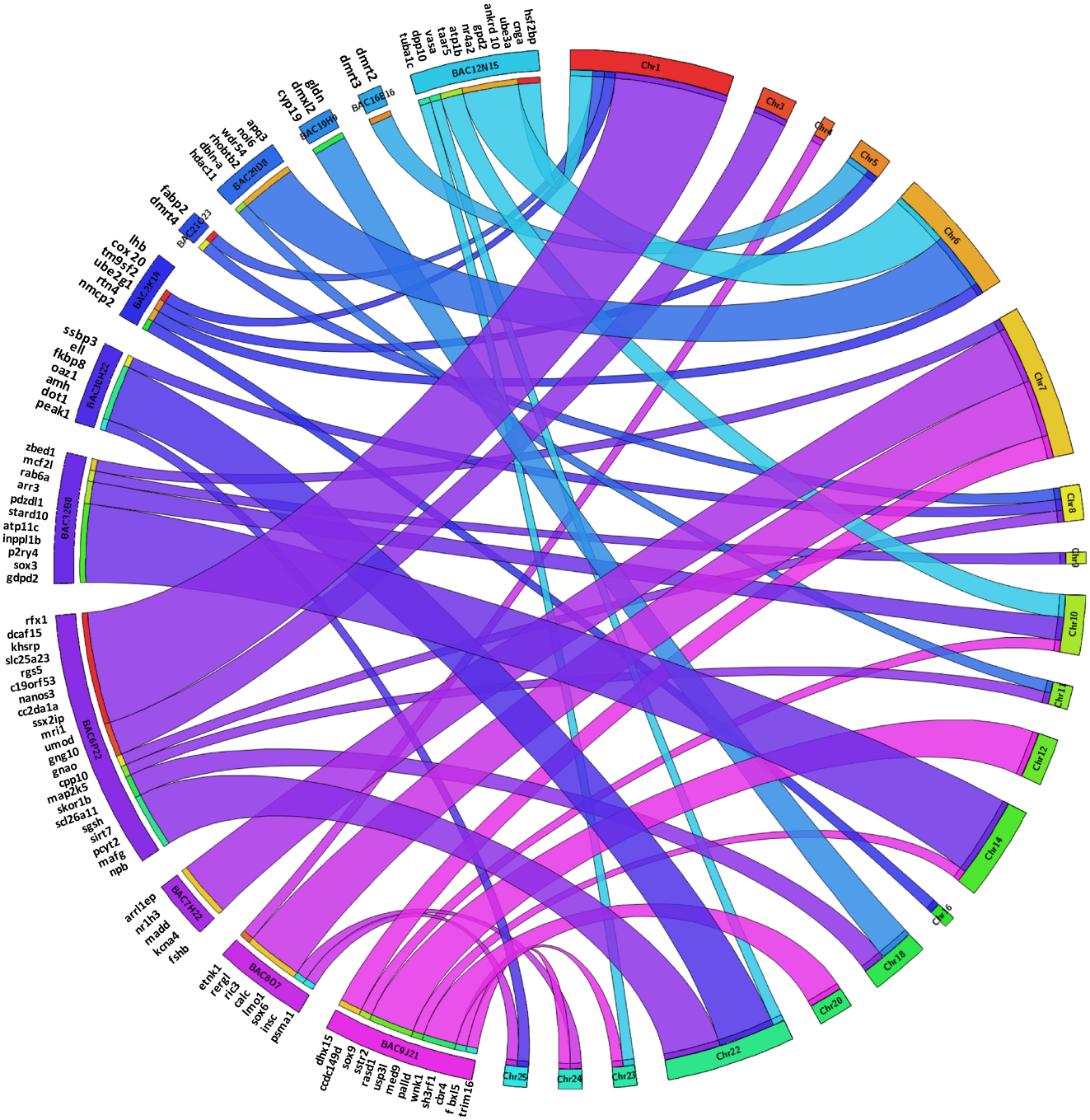

Fig. 4 Circos analysis in the species *D. rerio*. On the *left side*, the distribution of the BAC clones of *S. senegalensis* can be observed. Indicated within each BAC are the genes found by annotation, and the corresponding localizations in the *D. rerio* genome are denoted by *crossing lines*. The chromosomes of that genome are represented on the *right side* of the figure. BAC clones analyzed are those given in Table 1, in addition to the *fshb*-bearing BAC obtained from García-Cegarra et al. (2013)

S. maximus (Viñas et al. 2012), which could be a derived situation within the Pleuronectiformes group. The *sox8* and *sox9* genes belong to the same *sox* subgroup, i.e., *soxE*, which accounts for three *sox* genes that arose from tandem duplications (Heenan et al. 2015). Thus, it is possible that originally, these two genes were linked, although some re-arrangements occurred during Pleuronectiformes evolution. Moreover, *sox3* and *sox6* also appeared distributed separately in the genome, similar to *S. maximus* (Viñas et al. 2012). *The sox3*, *sox6*, and *sox8/sox9* genes belong to different subgroups of *sox* genes (*soxB1*, *soxD*, and *soxE*, respectively) that seem to have arisen by whole-genome duplications (WGDs) followed by sub- and neo-functionalization events (Heenan et al. 2015). Studies in humans and mice with *sox3* suggest a role in the central

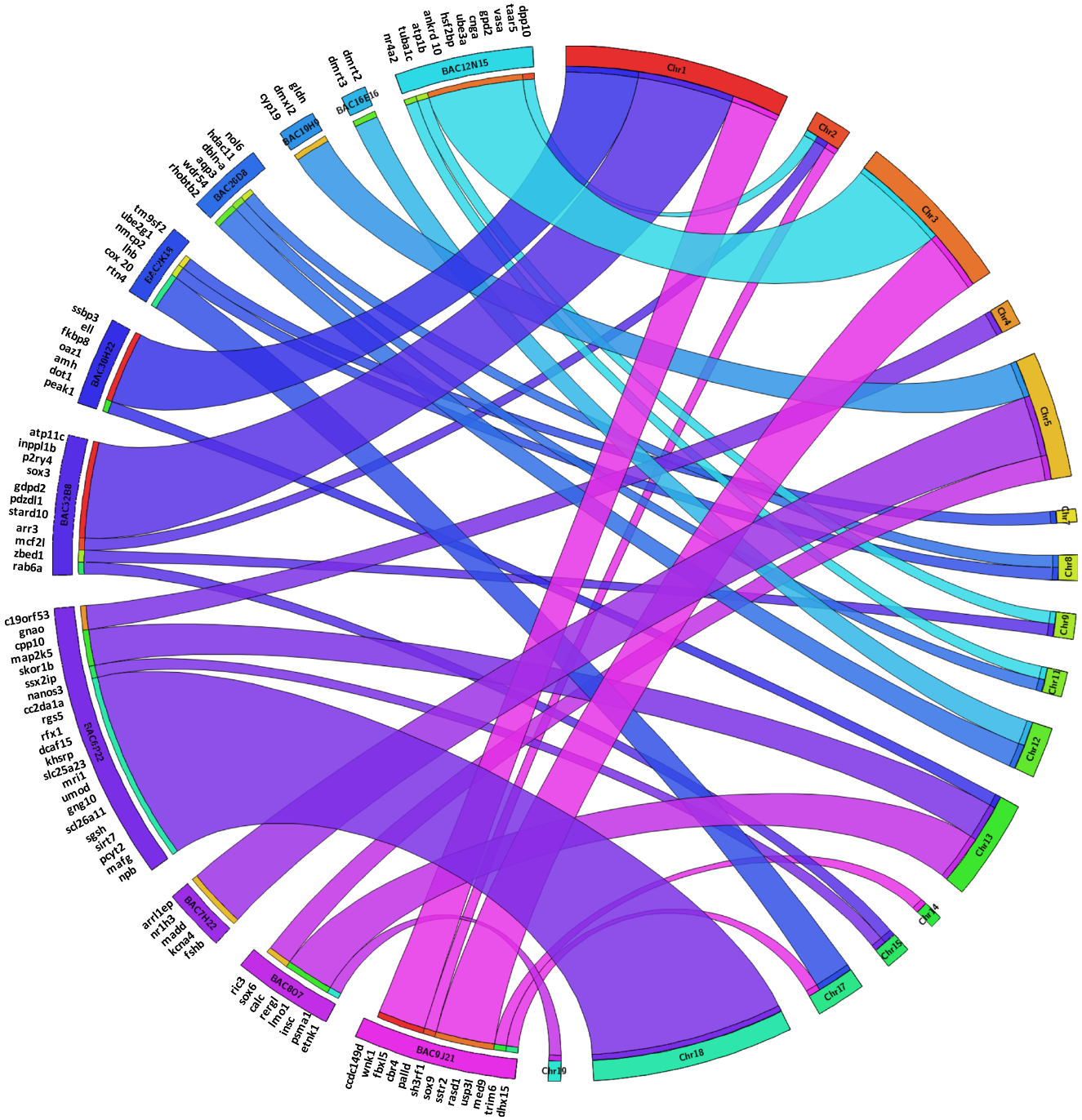

Fig. 5 Circos analysis in the species *T. nigroviridis*. On the *left side*, the distribution of the BAC clones of *S. senegalensis* can be observed. Indicated within each BAC are the genes found by annotation, and the corresponding localizations in the *T. nigroviridis* genome are denoted by *crossing lines*. The chromosomes of that genome are represented on the *right side* of the figure. BAC clones analyzed are those given in Table 1, in addition to the *fshb*-bearing BAC obtained from García-Cegarra et al. (2013)

nervous system and during development (Cheah and Thomas 2015). However, in the fish *Oryzias dancena*, *sox3* has been associated with male sex differentiation (Takehana et al. 2014). Furthermore, *sox6* could be involved in the maturation of sperm in vertebrates (Hagiwara 2011) and, in *O. mykiss*, *sox6* is only expressed in the testis, although it is not the primary sex-determining gene (Alfaqih et al. 2009).

Intriguingly, the secondary hybridization signals found in the *sox6*, *sox9*, *dmrt2*, and *dmrt4* BAC clones suggest some gene duplications dispersed in the genome. Genes involved in transcription and signaling cascades, as well as those encoding for proteins with more than average protein–protein interactions, are examples of genes over-retained after WGD (Hufton et al. 2009). Indeed, teleost fishes have suffered a third round

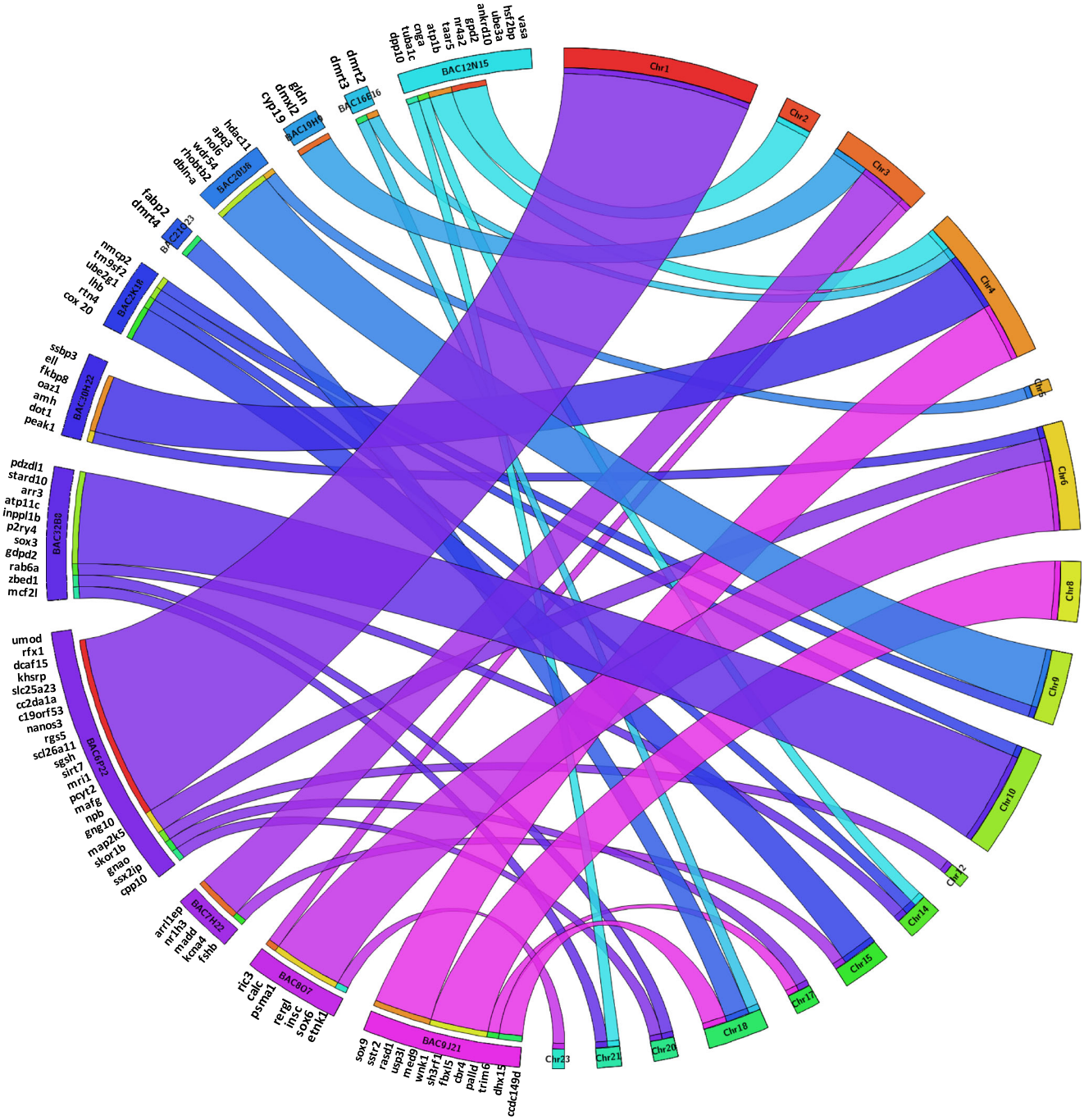

Fig. 6 Circos analysis in the species *O. latipes*. On the *left side*, the distribution of the BAC clones of *S. senegalensis* can be observed. Indicated within each BAC are the genes found by annotation, and the corresponding localizations in the *O. latipes* genome are denoted by *crossing lines*. The chromosomes of that genome are represented on the *right side* of the figure. BAC clones analyzed are those given in Table 1, in addition to the *fshb*-bearing BAC obtained from García-Cegarra et al. (2013)

of genome duplication, termed as teleost-specific whole-genome duplication (TS-WGDs) (Glasauer and Neuhauss 2014), which could explain the presence of the secondary signals found in the most fish species. Genes acting on essential metabolic pathways are present in the four BAC clones previously mentioned as having more than two FISH signals. Indeed, *sox6*, *sox9*, and *dmrt2* present paralog sequences in the majority of the species analyzed by micro-synteny, and the secondary weaker signals might correspond to similar fragments in duplicated regions.

The parts of the genome surrounding both the *amh* and *nos3* genes are fully conserved in teleosts; a conserved group of genes could indicate a functional cluster (Overbeek et al. 1999). Paibomesai et al. (2010) studied the genes surrounding

Table 3 Distribution of candidate genes among the chromosomes of different species

Species	Total chromosomes[a]	Candidate chromosomes[b]	Chromosomes with more than 1 candidate gene	N° missing candidate genes[c]
S. senegalensis 2n = 42	9	7	2	0
G. aculeatus 2n = 42	14	9	2	0
T. nigroviridis 2n = 42	16	7	3	1 (*dmrt4*)
O. latipes 2n = 48	17	8	3	1 (*vasa*)
D. rerio 2n = 50	19	9	2	1 (*lhβ*)

[a] Number of chromosomes which bear all the genes annotated across the BAC clones

[b] Number of chromosomes which bear the candidate genes

[c] Genes which have not found in that species

the *amh* gene in several fish species, as well as in human and mice, and identified functional clusters associated with sexual maturation and cell cycling. The *amh* is an example of the first class of cluster, since it is a member of the transforming growth factor-beta gene family, which mediates male sexual differentiation and participates in the development and maintenance of the male and female gonads (Durlinger et al. 2002a, b). A study conducted in four *nanos* genes determined that *nanos3* was conserved in terms of expression and synteny (Aoki et al. 2009), which could also indicate the existence of a functional cluster in the area surrounding the *nanos3* gene. Both the *nanos* and *vasa* families are involved in the

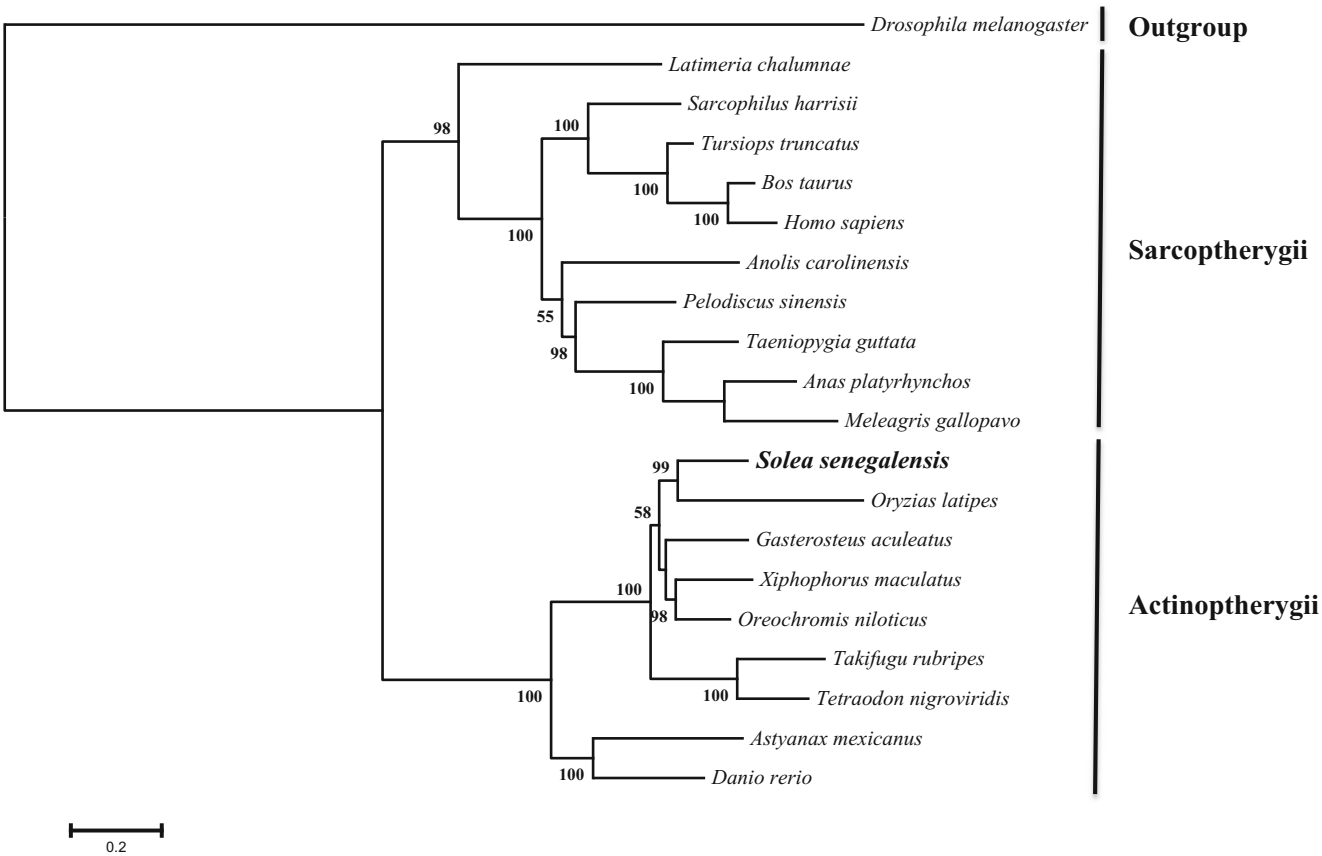

Fig. 7 Phylogenetic tree made from ten candidate genes concatenated (*amh*, *cyp19a1a*, *dmrt2*, *dmrt3*, *dmrt4*, *lhb*, *nanos3*, *sox3*, *sox6*, *vasa*) (see Supplementary Material 2 for accession numbers)

specification of primordial germ cells (PGCs) in sexual reproduction (Cho et al. 2014), but no linkage between these two genes was observed among the species analyzed in this study. In *S. senegalensis*, four vasa transcripts have been described, two of them with an ovary-specific expression (Pacchiarini et al. 2013).

The linkages among *fshb*, *lhb*, and *cyp19a1a* genes are not fully conserved in teleosts, and the linkage between *fshb* and *cyp19a1a* was observed only in *S. senegalensis* and *T. nigroviridis* (Figs. 1c and 5). It is well known that *T. nigroviridis* has a very compact genome (a genome with a similar quantity of genes but of a smaller DNA size) and that property has also been described in flatfish (Zaucker et al. 2014). From an evolutionary point of view, the *S. senegalensis* and *T. nigroviridis* genomes could have evolved in a similar way to optimize the expression of genes such as *fshb* and *cyp19a1a* with similar functions. On the other hand, the linkage between *sox6* and *cyp19a1a* appears to be common in flatfish, since it has been observed in *S. senegalensis* (Fig. 1c), *C. semilaevis* (Supplementary Material 6), *S. maximus* (Viñas et al. 2012), and *T. nigroviridis* (Fig. 5). However, more species need to be analyzed to conclude definitively that this linkage is an ancestral condition in Pleuronectiformes.

Previous cytogenetic studies have also been undertaken in *S. senegalensis* to complete the genetic knowledge and the karyotype of this species (Vega et al. 2002; Manchado et al. 2006; Cross et al. 2006). Ponce et al. (2011) used for the first time the BAC-FISH technique in *S. senegalensis* to localize the BAC containing the lysozyme gene. In other work, a preliminary BAC-based cytogenetic map of *S. senegalensis* was presented with 11 chromosomal markers, which mapped onto 13 chromosomes (García-Cegarra et al. 2013). This study completes those previous works and localizes BAC clones of up to 15 (out of 21) chromosome pairs (Fig. 2). This is an important point because the karyotype of *S. senegalensis* contains 12 pairs of acrocentric chromosomes that are difficult to distinguish because of the similar size that they present. The FISH technique has also been used in the cytogenetic characterization of other flatfish species, such as *S. maximus*; in addition, this technique has been used to consolidate the linkage map produced for this species, thus helping to bring together several previously established linkage groups (Taboada et al. 2014b).

Several authors have reported that the Pleuronectiformes group evolved from Perciformes fishes (Ivankov et al. 2008; Flores and Martínez 2013), so this could be reflected by the very close relationship between *S. senegalensis* and *G. aculeatus* compared with the other species considered in the comparative analysis. Using a concatenated protein sequence provided more robustness to the result, since this approach gives a more accurate tree (Gadagkar et al. 2005). However, a problem could arise from uncertain orthology or hidden paralogy (Thiergart et al. 2014), so special care must be taken in selecting the orthologs. It has been proposed that sex determination signals and mechanisms evolve so rapidly that the master gene rarely stays at the top of the sex determination cascade for very long, although the rest of the genes acting further down in the network are more highly conserved (Heule et al. 2014). The phylogenetic analysis with ten concatenated sex-related genes indicates the possible existence of a similar network among neighbor species; this would give important clues regarding the genes involved in processes of sex determination and sexual differentiation and reproduction studied in this paper. Moreover, the phylogenetic analysis agrees with the comparative mapping, whereby *D. rerio* was the species most distant from *S. senegalensis*. Finally, the phylogenetic analysis supports the relationships previously established between Sarcoptherygii and Actinoptherygii, and among fish species (Betancur et al. 2013).

Conclusions

The present work has followed on from previous research, to complete the karyotype characterization of *S. senegalensis*, focusing on BACs containing genes associated with sex determination and differentiation, using the mFISH technique. Results of co-localizations of candidate genes and synteny studies point to the largest metacentric chromosome of *S. senegalensis* as a sex proto-chromosome. The integrated genetic map showed 15 pairs out of 21 with at least one BAC. This result is important for distinguishing those chromosome pairs of *S. senegalensis* that are similar in shape and size.

Acknowledgments The authors acknowledge their gratitude to the Ensemble team and Genomicus Browser and curators for providing a browser and database that greatly facilitated the use and the analyses of the BACs used in this work. We also thank all the institutions, consortium leaders, coordinators, and supervisors of genome projects that have made genome information available—mainly those not yet published such as *P. formosa*: W. Warren, McDonnell Genome Institute, Washington University, Missouri, USA; but also those already published such as *G. morhua*: K.J. Jakobsen, University of Oslo, Norway; *A. mexicanus*: J.B. Gross, University of Cincinnati, Ohio, USA; *L. chalumnae:* K. Lindbla-Toh, Broad Institute of MIT, Massachusettes, USA; *O. latipes*: Y. Kohara, National Institute of Genetics, Japan; *X. maculatus*: W.C. Warren, Washington University, Missouri, USA ; *L. oculatus*: J.H. Postlethawait, Nicholas State University, Louisiana, USA; *G. aculeatus*: D.M. Kingsley, Broad Institute of MIT, Massachusetts, USA; *F. rubripes*: S. Brenner, Salk Institute, La Jolla, San Diego, California, USA; *T. nigroviridis*: H.R. Collins, Genoscope, France; *O. niloticus*: G.H. Yue, National University of Singapore; and *D. rerio*: D.L. Stemple, Wellcome Trust Sanger Institute, Cambridge, USA. We also thank three anonymous referees for their valuable suggestions, R.F. Snart for English corrections, and A. Arias for a critical reading of the manuscript.

This study has been supported by the Spanish Ministerio de Ciencia e Innovación MICINN-FEDER (projects AGL 2011–25596 and AGL2014-51860-C2-1-P) and by the AQUAGENET project (SOE 2/P1/E287) from the INTERREG IVB SUDOE program. SPB received a fellowship from the UCA.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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