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The gene mapping of Senegalese Sole (*S. senegalensis*)

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Abstract

The Senegalese sole (*S. senegalensis*) is a common flatfish, distributed over the eastern Atlantic coast from the Northern part of Senegal. Senegalese sole aquaculture has aroused great interest in the marine aquaculture in Spain and Portugal because of its relatively rapid growth rate, but it presents limits due to the poor reproduction of captive breeders in many facilities. In fact gonad development during sexual maturation results in major growth reduction, and increased susceptibility to diseases (Felip et al., 2006). Senegalese sole has 42 chromosomes and an XX/XY chromosome system for sex determination, while related species show the ZZ/ZW system. In *S. Senegalensis*, these problems are aggravated because of the lack of knowledge concerning sex.

This study fits into a detailed project of Senegalese sole knowledge, promoted by University of Cádiz, providing new information about the karyotype characterization, the chromosome structure of certain genes involved in sex determination and sexual differentiation processes, which may be relevant for improving the commercial production of this species. This piece of information not only helps to understand the development and evolutionary mechanism in vertebrates, but will also contribute to improving the production of target species for aquaculture.

For this purpose, several bacterial artificial chromosome (BAC) clones that contain candidate genes involved in such processes (*vitellogenin*, *otospiralin*, *R-spondin-1*, *Steroidogenic Factor1* and *Tetkin-2*) were analyzed and compared, where possible, with the same genes in other species.

In this study it was possible to describe a part of those four genes, using 3' RACE method. The BAC-FISH results showed the position of different genes in different chromosome, to improve the knowledge of Senegalese sole karyotype. In particular, the localization of *steroidogenic factor 1* (SF1) in the chromosome 1 that is involved in the sex determination.

I. Introduction

I.1. Taxonomy of the species

Senegalese sole (*Solea senegalensis*) is a common flatfish belonging to Soleidae family, to Pleuronectiformes order, which comprises 570 species, as European flounder (*Platichthys flesus*) and Atlantic halibut (*Hippoglossus hippoglossus*) (Fig.1a, 1b).

A

Kingdom:	Animalia
Phylum:	Chordata
Class:	Actinopterygii
Order:	Pleuronectiformes
Family:	Solidae
Genus:	*Solea*
Species:	*S. senegalensis*

B

Fig. 1: **a)** Adult individual of *S. senegalensis*.; **b)** Taxonomy classification of Senegalese sole.

For the otolith's fossil log, Pleuronectiformes are date back to late Paleocene or early Ecene, approximately 57-53 MA, after this period there are many different branches of Pleuronectiformes, where the biggest family is rappresented by Soleidae (Campbell et al., 2013). This order had a large world distribution and most of its representatives live in seawater, but also there are many in fresh water as *Trinectes maculatus* (hogchocker) and *Synaptura salinarum* (saltpan sole).

I.2 Description of the species

This sole has an oval and asimmetric body (eyes on the right side), blind side of head covered with numerous small hair-like fringes, and upper eye is separated from the dorsal profile of the head by a distance distinctly greater than the diameter of the eye (Florez and Martinez, 2013). Dorsal fin, with 73–86 rays, originates on dorsal profile of head

anterior to the eyes. Pectoral fins are equally well developed on both sides, with 7–10 rays, the fin on eyed-side is asymmetrical in shape. The base of the caudal fin is united by a membrane to the last rays of dorsal and anal fins, but caudal peduncle is still distinct. The lateral line, with its supra-temporal prolongation, describing a smooth curve on head. Colouration of eyed-side varies from greyish-brown to reddish-brown, with large and diffuse dark spots; blind-side is white. The pectoral fin of eyed-side has a black blotch on the distal end; hind part of tail darker than rest of fin. Average adult size is 30–40 cm, but can reach up to 70 cm size (standard length).

Sexual maturity of females occurs at 3 years of age and 32 cm total length. Spawning occurs during the summer between May and August, with a peak in June (Iberian Peninsula, Bay of Biscay). In the spawning period, female are easily recognizable for the ovaric expansion from the head to the tail; male are more difficult to recognize at sight because of small testis, but it is possible by manual detection. Likewise in the maturity is more evident in the female than in the male. Spring is the main spawning period, with some occasional spawning during autumn and winter. Spawning is highly dependent on water temperature, which should be between 15 and 20 °C, the optimum for spawning (Diaz-Ferguson et al., 2007).

Senegalese sole has a very similar external morphology to Dover sole (*S. solea)*, and differentiating between both species needs special attention to be paid to specific external details such as the shape and coloration of the pectoral fin (Lagardere et al., 1979). *S. senegalensis* is a sex separated species, without apparent sexual dymorphism. This fish has a gonochoric life cycle, and sexual maturation occur at similar age and size. The Senegalese sole has a XX/XY sexual determination system, with 21 chromosomal pairs, of which three metamerics (Molina-Luzón et al., 2014). However, in flatfishes is also present a different system, the ZZ/ZW one.

This is a demersal marine species, living on sandy or muddy bottoms, ranging from brackish lagoons and shallow waters to coastal areas up to a depth of 100 m. Adults feed on small benthic invertebrates, mainly polychaetes and bivalve molluscs, and to a lesser extent small crustaceans.

This sole is a benthonic fish, distributed from the north-eastern Atlantic Ocean - Bay of Biscay - to Senegal and from the Strait of Gibraltar to the coast of Tunisia in the Mediterranean Sea (Quero et al., 1986), as shown the map of fig. 2. This species is one of the most important flatfishes in the Tagus estuary (Cabral and Costa, 1999).

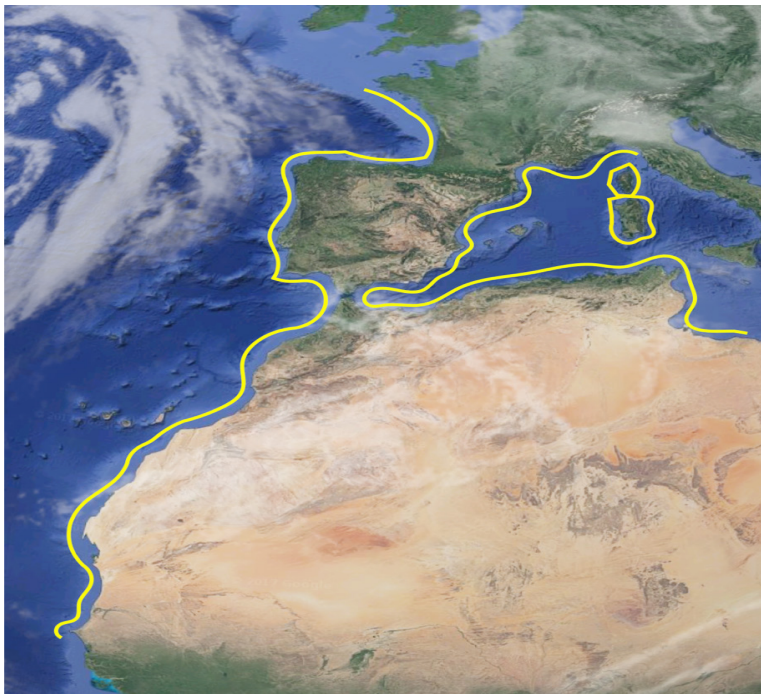


Fig. 2 - Distribution area of *S. senegalensis.*

I.3 Interest in aquaculture

Senegalese sole is a species of increasing interest in aquaculture and is commonly cultured in the Portuguese and Spanish southern coasts (Dinis et al., 1999).

The commercialisation of sole farming has progress in the last few years. In Europe, the dominant species of interest is the Senegalese sole but there is still some commercial and research in the Dover sole (*S. solea*), particularly in more northen countries, notably the Netherlands and the UK. Its importance in aquaculture is due to two main factors: its big food value and the possibility to study the related aquaculture's diversification. For the first reason, its white and lean meat has a great quality, that has also an economic value worth up to three times larger compared the others, e.g. Gilt-head bream (*Sparus aurata*) and European bass (*Dicentrarchus labrax*) (Chairi et al., 2010).

This sole is not actually a new entry in acquacolture: it was introduced in the 80's, when first cultivation studies were focused on the possibility of captivity F1 growth.

The control of reproduction in cultured specimens of Senegalese sole remains a major research challenge for the aquaculture industry, since programming of larval and fry production in hatcheries and the design of genetic breeding programs are fully de-

pendent on wild fish, a situation that is not sustainable in a long-term (Fatsini et al., 2016). The reproduction of this species in aquaculture is currently the biggest problem, in particular the difficulty in F1 reproduction, that is maybe linked with a weak male courtship. Most companies reported problems of poor growth, disease in the nursery and ongrowing stages, and spawning of F1 and F2 stocks. Feed formulation and managment of production system were also reported as problem areas (Felip et al., 2006).

The development of commercial sole farming still has many challenges to face. Also, the research capability that has become established in the key problem areas equips the scientists well to overcome the difficulties (Imsland et al., 2003).

I.4 Sexual determinaton in flatfishes

In contrast to birds and mammals, cold-blooded vertebrates, and among them, teleost fishes in particular show a variety of strategies for sexual reproduction; ranging from unisexuality (all-female species) to hermaphroditism (sequential, serial, and simultaneous, including outcrossing and selfing species) to gonochorism (two separate sexes at all life stages). Sex determination in fish is a labile character in evolutionary terms.

Sex determination mechanisms can be due to two main causes: enviroment and genetic. On one hand, enviromental factors impacting sex determination in fish are oxygen concentration, water pH, growth rate, density, social state, and, most commonly, temperature (Stelkens and Wedekind, 2010). On the other hand, genetic sex determination system has received the most scientific attention, especially about sex chromosomes, which either may be distinguishable cytologically (heteromorphic) or appear identical (homomorphic). In both cases, one sex is heterogametic (with two different sex chromosomes and hence producing two types of gametes) and the other one homogametic (a genotype with two copies of the same sex chromosome, producing only one type of gamete). In teleosts two different types of system exist: XX-XY (male-heterogametic) and ZZ-ZW (female heterogametic). Both systems of heterogamety exist, even found side by side in closely related species, i.e. tilapias, ricefish or sticklebacks (Mank et al., 2006). All the information are reported in the following imagine (fig. 3), by Stelkerns and Wedekind (2010).

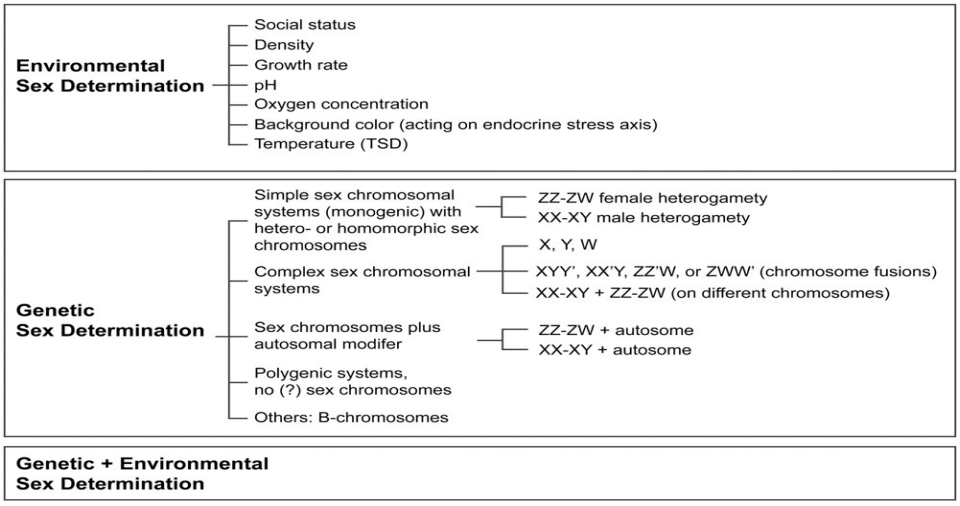


Fig. 3 - Chart of sex determination mechanisms.by Stelkerns and Wedekind (2010).

The sex-determining master gene can differ even between closely related fish species. Flatfishes group is an interesting model for studying the evolution of the sex-determining region and the gonadal differentiation pathway. Sex determination in fish can evolve very rapidly. The suppression of recombination between homologous chromosomes are involved in the evolution of sex chromosomes. The sex determination pathway appears to be less conserved than other developmental process (Penman et al., 2008).

With the arrival of genomics, large amounts of different genetic markers and genomic information are available for scanning genomes to look for their association with sex determination. Quantitative trait loci or marker association approaches have been used to identify the sex-determining regions in some fish species (Martínez et al., 2009). Furthermore, genomic resources in model and aquaculture species incresed to the point of allowing the development of both comparative genomics and candidate gene strategies to identify the sex-determining region in fishes.

I.5 Sole cytogenetics

Cytogenetic studies on fishes have lagged behind those on other vertebrate groups because of the difficulties in obtaining sufficient good-quality chromosome spreads, the relative large number and small size of fish chromosomes, and the general failure to reveal serial chromosome bands in fishes (Hartley and Home 1985; Gold et al. 1990).

Cytogenetic studies on flatfishes have been particularly difficult because of the very

small chromosome size of these species. In fact, the cellular DNA content of flatfishes is the lowest among fishes (only 20% of that in mammals), despite the fact that the chromosome number in flatfishes averages the modal number observed within fishes (Bouza et al., 1994).

As already mentioned, Senegalese sole is included in flatfishes, and its karyotype is composed by 21 chromosome pairs (2n=42), divided in: three metacentric pairs, two submetacentric pairs, four subteleocentric pairs, twelve acrocentric pairs.

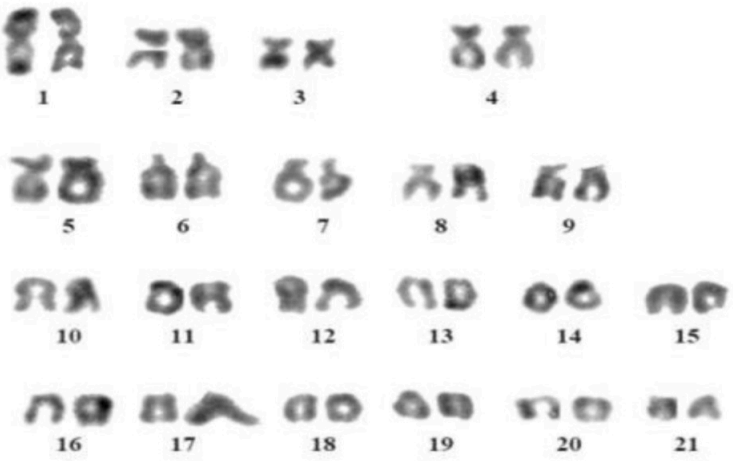


Fig. 4 - Senegalese sole karyotype described by Vega et al. (2002).

During the last few years, research increased in this field, in the overall articulation involving growth, sexual maturity, spawning, egg's morphology and quality, exchange behavior with different salinity and temperature, and so on. In parallel, cytogenetic studies increased also, and improved the knowledge about the karyotype characterization.

The first author that characterized Senegalese sole's was Vega (2002) (fig. 4). Later, in 2006, Manchado et al., localized ribosomial DNA (5S) in two different chromosomic pairs with FISH (Fluorescent In Situ Hybridization) technique (Manchado et al., 2006). Other repeated genomic regions as major ribosomal genes, GATA and telomeric TTAGGG sequences have been mapped too (Cross et al., 2006). In the prospective of a NGS (Next Generation Sequencing) approach, Ponce et al. (2011) localized thelysozyme gene by means of BAC-FISH. From 2013, García-Cegarra et al. made a cytogenetic map, based on BAC clones, from which characterized 13 chromosomic pairs (Portela-Bens et al., 2017).

I.6 Genetic map and BACs libraries

One of the biggest limitations in the contig production is the repetitive DNA. This sequences complicate assembly, because of random DNA fragments, coming from a chromosome or genome region, may be overlapping due to repetitive DNA. The sequencing for the coupled units is the method used to overcome this problem. This system allows to produce short DNA fragments, so genomic DNA is used for producing recombinant library, consisting of longer fragments, generally by 3 to 100 kb.

To obtain data on very long sequences, it is necessary to get sequence data with "paired ends" from DNA fragments very big, long about 100 kb. This process can be implemented with a particular cloning vector, known as Bacterial Artificial Chromosome (BAC). BAC can accommodate very large inserts, up to one hundred DNA kilobases. However, this method has two main disadvantages: fragments rich of AT (DNA fragments with high percentage of nucleotides, containing A and T), and the fact that sometimes many sequences do not work very well in cloning, because they are harmful for *E. coli* and so they can not be cloned in the organism (Russel et al., 2009).

In this context, BAC libraries are useful for producing a quick genetic map for those fish species for which none exists and for which making standard crosses may be a problem (Schulte et al., 2011).

I.7 Genes involved in this study

In this research, the study focused on different genes putative involved in the sexual determination and reproduction of Senegalese sole. This genes are: anti-Müllerian hormone type-2 receptor (*Ahmr2*), vitellogenin (*VTG*), otospiralin (*OTOS*), tektin-2 (*TEKT-2*), R-spondin-1 (*Rspo-1*) and steroidogenic factor-1 (*SF-1*).

I.7.1 Anti-Müllerian hormone type-2 receptor

The anti-Müllerian hormone (*Ahmr*) or Müllerian inhibiting substance (*MIS*), has long been known for its involvement in the sexual differentiation of the male embryo. It is a regulator and marker of ovarian function. It is secreted by the Sertoli cells of the fetal testis and induces regression of the Müllerian duct, the anlagen of the female reproductive tract (van Houten et al., 2010). *Ahmr* is a member of the transforming growth factor

(TGF) superfamily, which includes among others TGF, activin and bone morphogenetic proteins (BMPs). TGF family members signal through a characteristic combination of type I and type II serine/threonine kinase receptors. Upon ligand binding, the type II receptor activates the type I receptor through phosphorylation which in turn phosphorylates and activates the downstream Smad proteins (Massague et al., 2000).

In mammals, *Amhr2* is responsible for the regression of the Müllerian duct in males. Loss-of-function of this gene in male mouse leads to a partial hermaphrodite having a uterus and an oviduct together with the testis (Jamin et al., 2003). However, in medaka, a homozygous mutation in exon 9 of *Amhr2* (Tyr390Cys) results in complete sex reversal in half of the genetic males. However, information on *Amhr* type-2 receptor (*Amhr2*), the specific receptor for *Amhr* binding, is restricted to a couple of fish species (Rocha et al., 2016).

I.7.2 Vitellogenin

Vitellogenin (*VTG*) is the major yolk protein precursor in oviparous vertebrates, including fish. Sex steroids are key hormones involved in the regulation of all reproductive process (i.e. sex differentiation, puberty, gametogenesis and gonad maturation) (Mommsen and Walsh 1988).

Vitellogenin is produced by the liver in response to stimulation by estrogens, principally 17β-estradiol (E2), and is transported by the blood to the ovary. In female fish, the hepatic synthesis of *VTG* is induced by estrogen that is produced by the ovarian follicle during oocyte growth (Tyler and Sumpter, 1996).

Oogenesis in females and spermatogenesis in males are estrogen (estradiol, E2) and androgen (testosterone, T, and 11-ketotestosterone, 11KT) dependent, respectively; while advanced gonadal maturation is mostly, progestin (17,20β-dihydroxypregn-4-en-3-one, 17,20β-P or maturation inducing steroid, MIS) dependent (Mañanós et al., 2008). It is synthesized in the liver in response to stimulation by estrogens, and incorporated into the growing oocytes via specific receptors. Inside the oocyte, *VTG* is cleaved into smaller yolk proteins, phosvitin, lipovitellin and β-component, which are accumulated in yolk globules or granules, providing the main nutrient reserves for the developing embryos and prefeeding larvae (Hiramatsu et al., 2002).

Although males and juveniles are also capable of *VTG* gene expression, they typi-

cally lack sufficient circulating estrogens to stimulate significant production of this protein. However, males and juveniles will synthesize *VTG* if they are administered estrogen (Leonardi et al., 2010). The analysis of plasma *VTG* is used as an indicator of ovarian development in female fish broodstock, because of its high level of synthesis and secretion of *VTG* during vitellogenesis. Numerous bioassays using *vitellogenin* induction in males or juveniles have been developed to study endocrine disruptors (Jones et al., 2000).

I.7.3 Otospiralin

Otospiralin is a small molecule whose precursor is 89 amino acid long (6.4 kDa), it has been identified as an inner ear (cochlea and vestibule) specific molecule. However, compelling evidence from high throughput sequencing projects suggested that *otospiralin* is likely expressed in the central nervous system (Decourt et al., 2009).

This protein is highly conserved in animal kingdom, ranging from fishes to mammals. Its name comes from its expression pattern which was found to be restricted to spiral structures in the cochlea, i.e. non sensory cochlear tissues, spiral limbus, spiral ligament, and spiral ganglion glial cells (Delprat et al., 2002). The conservancy of the protein from mammals through fishes and its specific expression in the inner ear suggests that *otospiralin* is involved in an important function of the inner ear firbocytes (Lavigne-Rebillard et al., 2003).

Otos is a novel gene expressed specifically by spiral ligament fibrocytes (SFLs) that are thought to play an essential role in cochlear fluid and ion homeostasis. (Zhuo et al., 2008).

I.7.4 Tektin-2

Tektins (TEKTs) are composed of a family of filament-forming proteins localized in cilia and flagella. Tektins were originally characterized from sea urchins in which three tektins (A~53 kDa; B~51 kDa; C~47 kDa) form specialized filaments of the axonemal microtubules of cilia, flagella, and centrioles (Norrander et al., 1996). Tektins are microtubule-associated cytoskeletal proteins that are expressed primarily in the male germ cell-lineage in centrioles and basal bodies and within ciliary and flagellar doublet microtubules. Tektins are thought to function in providing for the stability and structural complexity of axonemal microtubules. On the other hand, five types of TEKTs have been

reported in mammals (Yamaguchi et al., 2014).

Five types of mammalian TEKTs have been reported, all of which have been verified to be present in sperm flagella. *TEKT1*, which has been localized in the centrosome in round spermatids as well as in the caudal ends of elongating spermatids (Larsson et al., 2000), has been recently verified to be a constituent of sperm flagella as well as the acrosome (Oiki et al., in press). *TEKT2*, which is identical to Tetkin-t, has been localized in the principal piece of human spermatozoa without detectable immunosignals in the middle piece or the end piece (Wolkowicz et al., 2002). *TEKT3*, which has been cloned as a testis-specific gene, is expressed in spermatocytes and spermatids (Roy et al., 2004). *TEKT4*, which was isolated as a molecule interacting with *Spetex-1*, is located on the rat sperm flagella (Matsuyama et al., 2005). The last one, *TEKT5* is also a constituent of rat sperm flagella (Murayama et al., 2008).

Of mammalian TEKTs, *TEKT2* gene, of a molecular weight of approximately 50kDa, has been found to be required for normal flagellar structure and function. *TEKT2*, which is indispensable for sperm structure, mobility, and fertilization, was present at the periphery of the outer dense fiber (ODF) in the sperm flagella. Tekt2-null spermatozoa display flagellum bending and reduced motility, probably due to a disruption in the dynein inner arm (Tanaka et al., 2004).

I.7.5 R-SPONDIN-1

R-spondin1 (*Rspo-1*) has been characterized as a potential female-determining gene differentially expressed in human (*Homo sapiens*), mouse (*Mus musculus*) and goat (*Capra hircus*). The product encoded by *R-spo1* belongs to the R-spondin family which has four members (R-spondin1-4). All members in R-spondin family have an N-terminal signal peptide leader sequences (SP), two adjiacent cysteine-rich furin-like domains (FU) and a thrombospondin motif (TSP) (Chassot et al., 2008). In mammals, *Rspo-1* by activating the canonical b-catenin signaling pathway, is required for female somatic cell differentiation, germ cell commitment to meiosis, and stem cell survival and differentiation (Chassot et al., 2011, 2008b). *Rspo-1* protein plays a key role in ovarian differentiation because mutations of human *Rspo-1* gene cause complete XX sex reversal (Parma et al., 2006). In zebrafish (*Danio rerio*), *Rspo-1* is expressed more strongly in ovaries than in testes, suggering that it plays an important role in gonad differentiation. However, high *R-spo1* expression was detected in some non-gonadal organs like muscle and kidneys (Zhang et al., 2010).

Comparative studies of *Rspo-1* orthologues in the mouse, chicken, and red-eared slider turtle, three species with different sex-determining mechanisms, have shown that gonadal *Rspo-1* expression is upregulated in female embryonic gonads at the onset of sexual differentiation (Smith et al., 2008), suggesting that *Rspo-1* is an ancient component of the vertebrates ovary-determining pathway. Following expression analyzes suggested that *Rspo-1*/Wnt/b-catenin signaling plays a role in ovarian differentiation in the protandrous black porgy (*Acanthopagrus schlegelii*), medaka (*Oryzias latipes*), rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*). Estrogen induces the upregulated expression of *Rspo-1* gene in the medaka (Zhou et al., 2012).

I.7.6 Steroidogenic Factor-1

Steroidogenic factor-1 (*SF-1*, also known as *NR5A1* or *Ad4BP*) is a member of nuclear orphan receptor family, it has been shown to regulate the expression of enzymes involved in steroid production in vivo (Buaas et al., 2012). *SF-1* is a nuclear hormone receptor that is expressed in the early adrenogonadal primordium from E9.5 (Ikeda et al., 1994). It acts as a master switch to initiate transcription of a series of steroidogenic genes in granulosa cells of mammalian ovary. It has also other functions, particularly during the development of gonads and adrenal glands.

SF-1 expression is first found in the adrenal and gonad anlagen, the adrenogonadal primordium (AGP), and continues in multiple differentiated cell types within steroidogenic organs that are descendant of this embryological structure (Hatano et al., 1996). Foetal and adult testes express high levels of *SF-1* in the testosterone-synthesizing Leyding cell and the non-steroidogenic, germline-supporting Sertoli cell. Analogous to the testes, ovaries express *SF-1* in non-steroidogenic (follicle or granulosa cells), and steroidogenic (stromal and theca cells) cell populations. Similar to the testis and adrenal gland has a transiet steroidogenic foetal population that is replaced by a definitive adult steroidogenic population and both express *SF-1* (Morohashi and Zubair, 2011).

In adults, *SF-1* is constitutively expressed in steroidogenic tissues, including the cortical cells in the adrenal gland, Leyding cells in the testis and theca cells in the ovary. *SF-1* activates the expression of steroidogenic enzymes by binding to shared promoter elements and is believed to be a key regulator of steroid hormone biosynthesis (Ikeda et al., 1994; Morohashi et al., 1993). In fish, *SF-1* is essential in early testicular differentiation and development, it acts as a co-factor and binds to the promoter region of the genes encoding steroidogenic enzymes (Wang et al., 2007; Zhou et al., 2007).

II. Aim of the study

The purpose of this research is to provide more in-depth knowledge of Senegalese sole about its genetic mapping. In particular, the knowledge of putative genes involved in its reproduction process. So far, in fact, there have not been any specific evidences, and this study aims to fill this gap. The achievement of this goals has been divided into two points: genes cloning and sequencing and localization.

1. Genetic cloning and sequencing.

   I. Isolate the different genes of interest (*ahmr2*, *vitellogenin*, *otospiralin*, *tetkin-2*, *r-spondin1* and *steroidogenic factor 1*) expressed in gonads.

   II. Cloning of this genes through to 3' RACE.

   III. Analysis of the sequences obtained, using BLAST, and check the differences between the information for the same gene in different species.

2. Localization of genes in the chromosome.

   Chromosomal localization of five gene of interest (*vitellogenin*, *otospiralin*, *tetkin-2*, *r-spondin1* and *steroidogenic factor 1*) by application of fluorescence in situ hybridization (FISH) based on the use of bacterial artificial chromosome (BAC) clones.

III. Materials and Methods

III.1 Biological samples

The speciments were provided by the Servicio Central de Investigácion en Cultivos Marinos, SC-ICM (Universidad de Cádiz), they belong to the first filial aquaculture generation (F1), while the parental generation individuals (P-generation or F0) were catched in the Bay of Cádiz. The F1 fishes were about 5 years old of both sexes: two females and three males. Once picked up, they were anesthetized with a clove solution. At last, different organs were taken and immediately put in a RNA*later* Stabilization Reagent. Once a biological sample is harvested, its RNA becomes extremely unstable. The RNA*later* solution permeates tissues resulting in immediate stabilization of the RNA and preservation of the RNA expression pattern. So, RNA samples can be saved without risk of RNA degradation, even after multiple freeze–thaw cycles. The tissues taken from the samples were gonads from each sex, so testis and ovary; other tissues, like muscle, liver, heart, spleen, kidney, gill, were also stored.

III.2 RNA extraction

The RNeasy® Lipid Tissue Mini Kit (cat. no. 74804, Qiagen) was used to purify RNA from RNA*later*. It stabilizes samples stored under a wide variety of conditions, which is ideal for downstream processes such as northern blotting and RT-PCR. This technology combines the selective binding properties of a silica-based membrane with the speed of microspin technology. The samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate-containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions and the sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. The tissues, stabilized in RNA*later*, are disrupted in Buffer RLT and homogenized. The disruption and homogenization methods are two distinct steps. The complete disruption of cell walls and plasma membranes of a cell is required to release all the RNA contained in the sample. In order to have significant RNA yields, the complete disruption is necessary. Equally, a complete procedure is necessary also for homogenization, in order to achieve a

reliable outcome. Indeed, the homogenization is necessary to reduce the viscosity of the lysates producted by disruption.

III.3 cDNA semiquantitative expression by PCR

For DNA conversion QuantiTect Reverse Transcription Kit (cat. no. 205311 Qiagen) is used, that operates in two main steps: elimination of genomic DNA and reverse trascription. The purified RNA sample is briefly incubated in gDNA Wipeout Buffer at 42°C for 2 minutes to effectively remove the contaminating genomic DNA. After genomic DNA elimination, the RNA sample is ready for reverse trascription using a master mix prepareted from Quantitrascript Reverse Trascriptase, Quantiscript RT Buffer, and RT Primer Mix. The entire reaction takes place at 42°C and is inactivated at 95°C (table 1). In contrast to other methods, additional steps for RNA denaturation, primer, annealling, and RNase H digestion are not necessary.

Table 1 - DNA conversion protocol provided by QuantiTect Reverse Transcription Kit.

Step	**Temperature**	**Time**
Initial denaturation	42°C	2’
Addition of Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and mix		
Reverse transcription	42°C	15’
RT inactivation	95°C	3’

In general, reverse trascriptase is a multifuncional enzyme with 3 distinct enzymatic activities: a RNA-dependent DNA polymerase, a hibrid-dependent exoribonuclease (RNase H), and a DNA-dependent DNA polymerase. For reverse trascription in vivo, the first 2 activities are utilized to produce single-stranded cDNA: RNA-dependent DNA -polymerase activity (reverse trascription) and RNase H activity. The newly synthesized cDNA are amplificated by polymerase chain reaction (PCR) procedures.

PCR is a molecular biology technique used to amplify a single copy of a few copies of segment of DNA across several orders of magnitude, generating thousands to millions

of copies of a particular DNA sequence. For this procedure was used MyTaq™ DNA Polymerase, that has high performance PCR product and exhibits more robust amplification than other commonly used polymerases. The following table (table 2) presents a 50 µl reaction, describing different reagents and their quantity.

Table 2 - Description of PCR reagents used in the cDNA amplification.

Component	Amount
cDNA	2 µl
MyTaq Red Reaction Buffer	10 µl
Steril H_2O	35,6 µl
Primer Fwd 10 µM	1 µl
Primer Rev 10 µM	1 µl
My Taq HS DNA Polymerase	0,4 µl

The samples were tested with different primers: first with constitutive genes, and later with specifical primers. To test the retrotranscription step, the constitutive gene elongation Factor 1 (eF1) was used. Once all samples had shown expression, they were tested with specific primers. They were designed by the group of Genetics from University of Cádiz, using template sequences from SoleaDB (Benzekri et al., 2014) and from orthologous sequences of different fish species available in the ESEMBLE (https://www.ensembl.org) database.

The table below (table 3) reports different steps, focusing to the three main steps: denaturing, annealing and extending, with their respective number of cycles.

Table 3 - PCR parameters used in gene amplification

Step	Temperature	Time	Cycles
Initial denaturation	94°C	5’	1
Denaturation	94°C	45”	35
Anealling	55/58°C	45”	35
Extension	72°C	1’	35

The PCR amplification products were loaded in 1,5% agarose gel and an electrophoresis was run. To check the amplicon sizes, a pattern ladder (HyperLadder™ 50bp) was used.

III.4 3' RACE

Rapid Amplification of cDNA Ends (RACE) is a procedure for amplification of nucleic acid sequences from messenger RNA template between a defined internal site and either 3' or 5' end of mRNA. PCR requires two sequence-specific primers that flank the sequence to be amplified. However, to amplify and characterize regions of unknown sequences, this requirement imposes a limitation.

The focus of 3' RACE procedure is to amplify cDNA from poly(A) tail, found in mRNA as a generic priming site of PCR. Specific cDNA is amplified by PCR using gene, specific primer that anneals to a region of known exon sequences and an adapter primer that targets the poly(A) tail region. This permits the capture of unknown 3'-mRNA sequences that lie between the exon and the poly(A) tail.

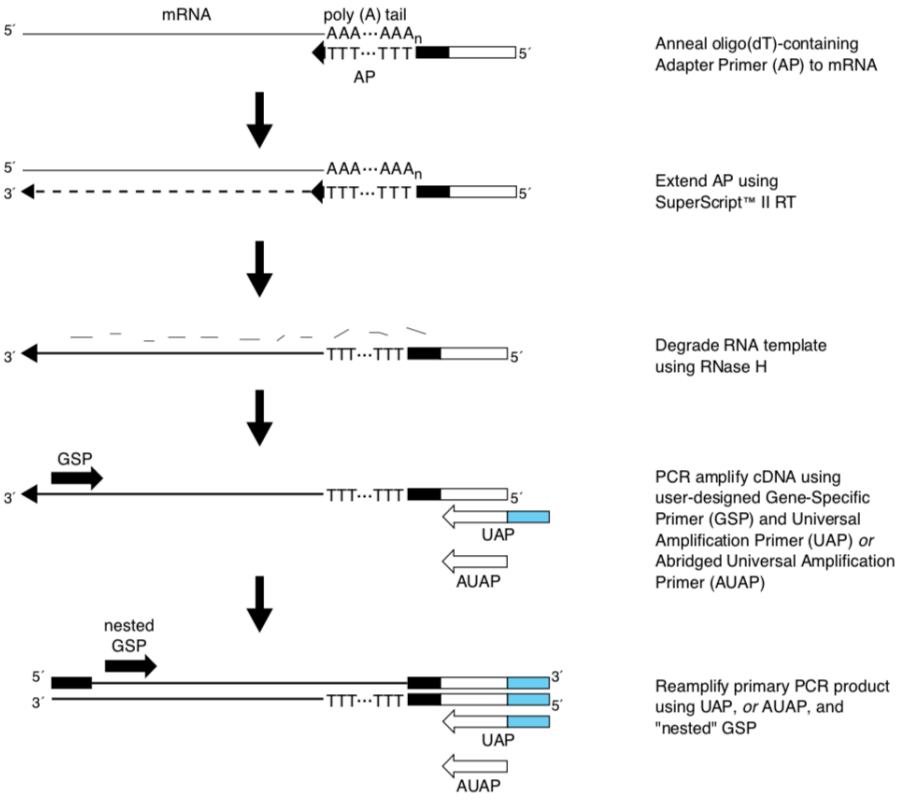


Fig. 5 - Summary of the 3' RACE System procedure.

As reported in the previous picture (fig.5), first strand cDNA synthesis is initiated at the poly(A) tail of mRNA using the adapter primer (AP). After first strand cDNA synthesis, the original mRNA template is destroyed with RNase H, which is specific for RNA:DNA heteroduplex molecules. From now on, amplification is performed, without any ethanol precipitations or intermediate organic extractions. For this step is necessary to use two primers: one is a universal amplification primer that target the mRNA of cDNA complementary to the 3' end of the mRNA; the other is a user-designed gene-specific primer (GSP) that anneals to a site located within the cDNA molecule. The Universal Amplification Primer (UAP) is designed for the rapid and efficient cloning RACE products using the uracil DNA glycosylase (UDG) method. The Abridged Universal Amplification Primer (AUAP) is homologous to the adapter sequence used to prime first strand cDNA synthesis (table 4).

Once the 3' RACE PCR is concluded, the samples are run on agarose gel (1, 5%) with ethide bromure and watched with the specific program.

Table 4 - Description of 3' RACE protocol.

Component	**Volume (µl)**
Steril H_2O	40,6
PCR Buffer	5
Gene-Specific Primer Fwd	1
AUAP Primer Rev	1
My Taq	0,4

III.5 PCR PRODUCT PURIFICATION

The purification was made by QIAquick PCR Purification Kit that provides spin columns, buffers and collection tubes for silica-membrane-based purification of PCR products >100 bp. The QIAquick system uses a simple bind-wash-elute procedure. Binding buffer is added directly to the PCR sample and the mixture is applied to the QIAquick spin column. The binding buffer contains a pH indicator, allowing easy determination of the optimal pH for DNA binding. Nucleic acids adsorb to the silica membrane in the high-salt conditions provided by the buffer. Impurities are washed away and pure DNA

is eluted with a small volume of the low-salt water, ready to use in all subsequent applications.

At first, DNA binding condition were adjusted with a double buffer volume per DNA. With a NucleoSpin Gel and PCR clean-up column, the samples are centrifugated for binding DNA, for washing and drying silica membrane. Last step is to elute DNA in a new collection tube.

III.6 Ligation with P-Gem

The pGEM®-T Easy Vector System is a convenient system for the cloning of PCR products. The high copy number pGEM®-T Easy Vector contain T7 and SP6, RNA polymerase promoters flaking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. The pGEM®-T Easy Vector multiple cloning region is anked by recognition sites for the restriction enzymes *Eco*RI, (*Bst*ZI and *Not*I), providing three single-enzyme digestions for release of the insert (Fig.6).

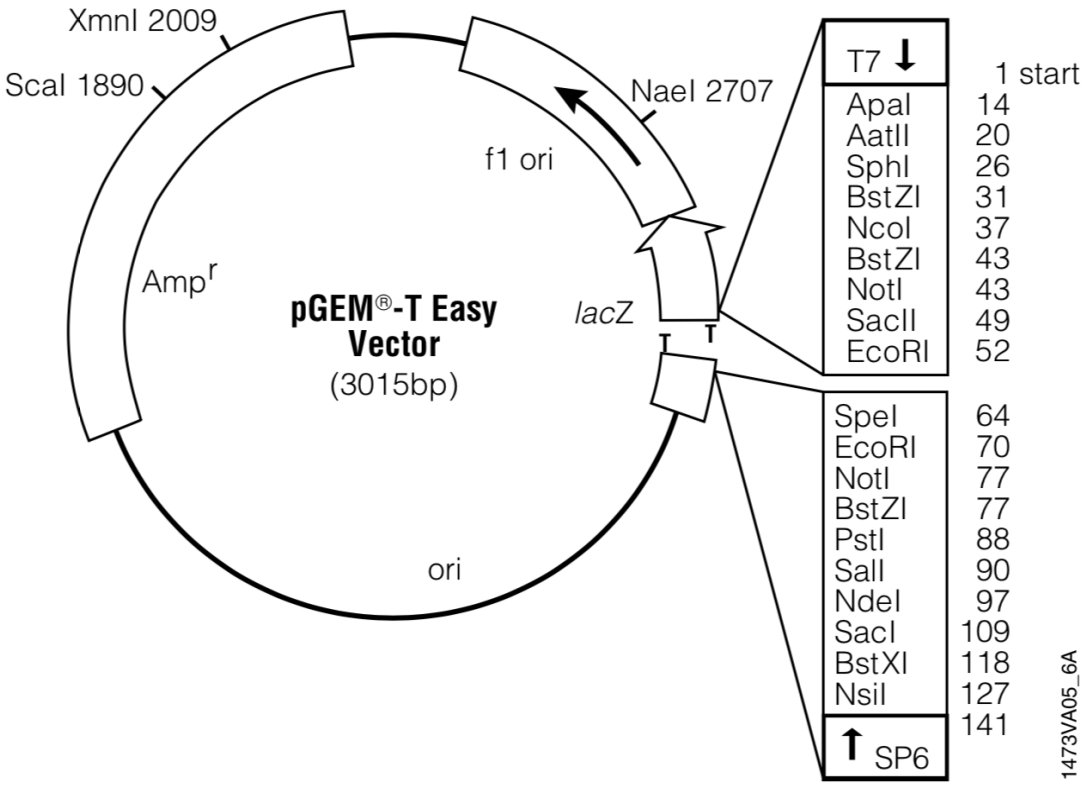


Fig. 6 - Map of pGem -T Easy Vector.

The pGEM system allows ligation reactions, with a specific buffer, which may be incubated for 1 hour at room temperature (the incubation period may be extended to increase the number of colonies after transformation). Generally, an overnight incubation at 4°C produces the maximum number of transformants.

Successful cloning of an insert into the pGEM®-T or pGEM®-T Easy Vector interrupts the coding sequence of β-galactosidase; recombinant clones can be identified by color screening on indicator plates. However, the characeristics of the PCR products cloned into the vectors can significantly affect the ratio of blue: white colonies obtained. Usually clones containing PCR products give white colonies, but blue colonies can result from PCR fragments that are cloned in-frame with the *lac*Z gene.

An aliquot of the PCR reaction should be analyzed on an agarose gel before use the ligation reaction. Use of the crude PCR product may produce successful ligation in some cases, however, the number of white colonies containing the relevant insert may be reduced due to preferential incorporation of primer-dimers or other extraneous reaction products.

The results can foresee different scenarios: low number or no white colonies containing PCR product, PCR product ligation reaction produces white colonies only (no blue colonies) and insufficient clones contain the PCR product of interest. A low number of white colonies is observed when the DNA has rearranged, so the clone of interest should be present and can be identified by screening several clones. A plate with white colonies only should represent the inactivation of ampicillin (allowing ampicillin-sensitive cells to grow), this may be due to an improper preparation of plates or a not working ampicillin; if colonies are not blue, maybe is due to an incompatible plate, so is necessary to check the plates have ampicillin/IPTG/X-Gal and are fresh. The low number of clones containing PCR product should be due to an insufficient A-tailing PCR fragment.

III.7 Topo cloning

Topoisomerase based cloning (TOPO cloning) is a DNA cloning method that does not use restriction enzymes or ligase, and requires no post-PCR procedures. The technique relies on the basic ability of complementary basepairs adenine (A) and thymine (T) to hybridize and form hydrogen bonds (fig. 7). The plasmid (pCR™II-TOPO® vector or pCR™2.1-TOPO® vector) is supplied linearized with a single 3'-thymidine (T) overhangs for TA Cloning® and a topoisomerase I covalently bound to the vector ("activated").

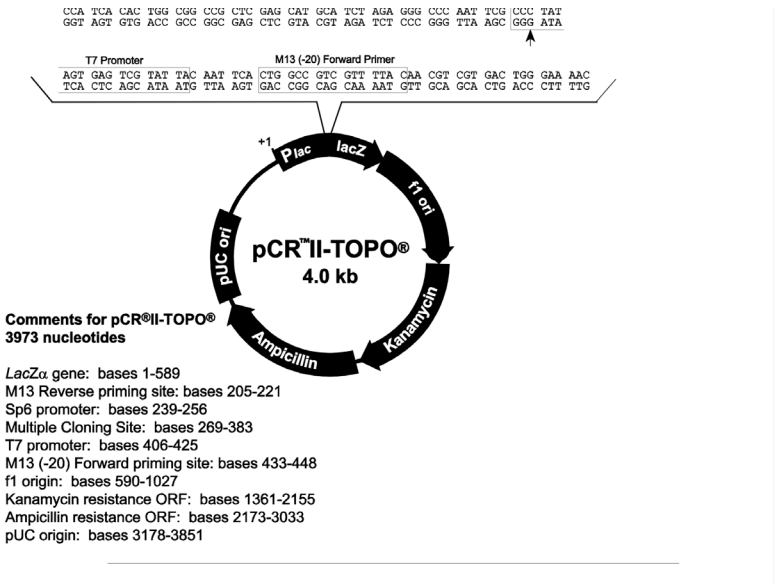


Fig. 7 - Map of pCR™II- TOPO® vector and the sequence surrounding the TOPO Cloning site. Restriction sites are labeled to indicate the actual cleavage site.

Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector has single, overhanging 3' deoxythymidine (T) residues. This allows PCR insert to ligate efficiently with the vector.

TOPO cloning technology, with its high efficiency, allows to streamline the cloning process. In this way, once TOPO cloning reaction is incubated, the obtained result is not a highest number of colonies, but every colony will contain the insert of interest.

Once the cloning is done, the products are transfered on Petri dishes with X-gal, an organic compound with a galattose indole linked inside. For the cultivation of bacterial cells harbouring high-copy plasmids, the incubation temperature is 37°C, with constant shaking (minimum of 12 hours, maximum over night). Cells cultures grown under antibiotic selection (ampicillin) at all times to ensure plasmid propagation. Without this selective pressure, cells tend to lose a plasmid during cell division. Since bacteria grow much faster without the burden of a high-copy plasmid, they take over the culture rapidly and the plasmid yield goes down regardless of cell mass.

III.8 Colony PCR

Colony PCR is used to have a clones screening to verify the effective insertion of

the plasmid's fragment. The procedure starts with picking part of each colony with a steril loop, this material have to be resuspended in steril water to have the PCR sample. The primers for each sample are one gene specific, peculiar and smaller, and the other one of the plasmids T7 or SP6, which have a PCR product bigger, because they are far from their own insert and in the middle there are different targets. Hence, everything is as in a common PCR, which will amplify the fragment inserted in the plasmid. The results are shown in agarose gel with bands corresponding to the gene of interest.

III.9 Plasmid extracion and digestion

NucleoSpin® Plasmid method provides that spin column-based nucleic acid purification precipitates nucleic acid such that it binds a solid matrix and other components flow through. The pelleted bacteria are resuspended and plasmid DNA is liberated from the *E. coli* host cells by SDS/alkaline lysis. Using a buffer, the resulting lysate is neutralized and there are the appropried conditions for binding of plasmid DNA to the silica membrane provided by the commercial kit. The obtained DNA is pelleted and dried by a centrifugation step; the last step is to elute DNA.

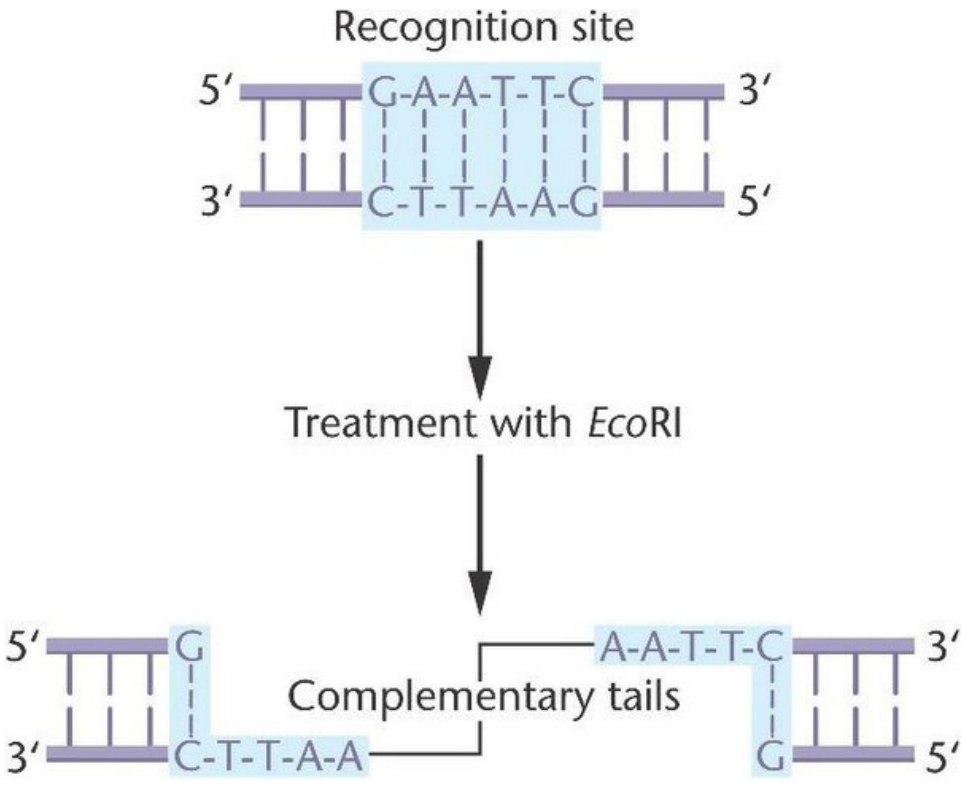


Fig. 8 - Rapresentation of the functionality of *Eco*RI

Restriction enzymes, such as *Eco*RI (fig. 8), are used in a wide variety of molecular genetics techniques including cloning, DNA screening and deleting sections of DNA *in vitro*. Restriction enzymes, like *Eco*RI, that generate sticky ends of DNA are often used to cut DNA prior to ligation, as the sticky ends make the ligation reaction more efficient. *Eco*RI contains the PD..D/EXK motif within its active site like many restriction endonucleases. *Eco*RI can exhibit non-sire-specific cutting, known as star activity. The digestion works at a temperature of 37°C for about one hour and half. To comprove that *Eco*RI worked, it is necessary to run the product on an agarose gel, adding a charge buffer; the result has to show only one stripe.

The last step to verify the effective presence of the plasmid and its consequently digestion consists in an agarose gel run. The presence of a band means the correct execution of the procedure. Once all the samples are ready, they are sent to Biomedal S.L, a biotechnology company which returns them in different sequences.

III.10 Bioinformatic tools

Bioinformatic is an interdisciplinary field that develops methods and software tools for understanding biological data. As an interdisciplinary field of science, it concerns computer science, biology, maths and engeneering to analyze and interpret biological data. This tool has been used for *in silico* analyzes of biological queries using mathematical and statistical techniques. Bioinformatic can range from sequence analysis, to gene and protein expression, to analysis of cellular organization, to network and system biology.

III.10.1 Sequencing

The sequences obtained were analized with bioinformatic tool on National Center for Biotechnology Information (NCBI) platform, who includes GenBank for DNA sequences and PubMed, a bibliographic database for the biomedical literature. In particular, the samples are entrusted to BLAST (Basic Local Alignment Search Tool) sequence alignment program.

BLAST is an algorithm used for calculating sequence similarity between biological sequences such as nucleotide sequences of DNA and amino acid sequences of proteins. BLAST is a powerful tool for finding sequences similar to the query sequence within the

same organism or in different organisms. It searches the query sequence on NCBI databases and serves and post the result back to the person's browser in chosen format. Input sequences to the BLAST are in FASTA format, while output could be delivered in variety of formats such as HTML, XML, formatted to plain text.

III.11 Fluorescence In situ Hybridization

Fluorescence in situ hybridization (FISH) is a powerful tool used in karyotyping, cytogenotyping, cancer diagnosis, species specification and gene-expression analysis. FISH using Bacterial Artificial Chromosomes probes that carry large genomic portions defined by sequence annotation has yelded a "revolution" in the field of cytogenetics because it has allowed the mapping of multiple genes at once, thus rendering constitutive heterochromatin amenable to easy and fast cytogenetics analyzes.

The Genetic group of the University of Cádiz provided to construct a BAC library, 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 from samples, digested with *Bam*HI, cloned into the CopyControl™ pCC1BAC™ (Epicentre Biotechnologies, USA) and transformated into the host cell DH10B™ (Invitrogen, Life Technologies, USA). The library comprised 29,184 positive clones distributed in 384-well plates (76 plates in total). Approximately 99,99% of clones contained Senegalese sole nuclear DNA insert (average size, 285 kb) covering 0,01% empty clones (Garcia Cegarra et al., 2013). The BAC clones refering to this putative genes were provided by Universidad de Cádiz.

III.11.1 Probe labelling

A single colony containing the clone was grown on LB containing chloramphenicol at 37°C overnight to obtain isolated BAC. The BAC was estracted with BACMAX™ Kit (Epicentre), through the instructions. Thanks to the digestion by *Eco*RI, it is possible to extract the insert and consequently to determine its size by electrophoresis. The probes were labeled with nick translation, using DIG BIO-Nick translation Mix (Roche Molecular Biomedicals).

The Nick translation method is based on the ability of DNase I to introduce randomly distribued nicks into DNA at low enzyme concentration presence of $MgCl_2$. In *in*

situ hybridization experiments, the fragment length distribution influences severely the efficiency of hybridization.

The BAC-DNA probes were marked by DIG-Nick Translation. Probes prepared with DIG-Nick Translation Mix for *in situ* probes are specially qualified. For a standard labeling reaction it is necessary to add 1 µg template DNA to sterile, double distilled water and end up with a final volume of 16 µl. After, add 4 µl DIG-Nick Translation Mix, mix and centrifuge briefly; then the samples are incubated for 75-80 minutes at 15°C. The reaction will end by adding 1 µl 0,5 M EDTA (pH 8.0) and heating to 65°C for 10 minutes. The labeled fragments obtained in the standard labeling reaction show a maximum length distribution in the range of 200 to 500 nucleotides. For color *in situ* hybridization the DIG-labeled DNA is detected by an antibody coniugated to a fluorophore (antidigoxigenin-AP conjugate) when metaphase chromosomes are used as hybridization targets.

III.11.2 Two-color BAC-FISH technique

FISH is a tecnique for mapping the location of genes onto chromosomes. The FISH map is a physical map, it shows the physical location of a gene on a chromosome. An advantage of the FISH technique is that it does not require information about other genes to map a single gene. Two-color FISH is a widely used technique for compating relative gene expression patterns (Schumacher et al., 2014). FISH using Bacterial Artificial Chromosomes (BAC) probes that carry large genomic portions defined by sequence annotation has yielded a “revolution” in the field of cytogenetics because it has allowed the mapping of multiple genes at once, thus rendering constitutive heterochromatin amenable to easy and fast cytogenetics analyzes (Accardo at al., 2010).

To perform FISH, first cells that have been arrested in metaphases are treated to make them swell and then they are fixed into the surface of a slide. This also fixes their chromosomes into the slide. Next, the slide is treated so that the chromosomal DNA is denatured into single strands. Then, special DNA probes are flooded into the slide. The DNA probes are small pieces of single-strand DNA with a sequence the genes of interest. Probes are able to hybridize only with their complementary sequence, which will be at the site of the gene of interest on a particular chromosome. The DNA probes are allowed to hybridize with the denatured chromosomal DNA and any excess probes are washed away. Either the DNA probes themselves are fluorescent or they are chemically modified to allow fluorescent labels to attach to them, so that when fluorescently labeled molecules are added to the slide. Some are able to bind the probe DNA that is bound to the site of the

gene on the chromosome. Excess fluorescently labeled molecules are then washed away. The slide is then viewed using a fluorescence microscope. The fluorescently labeled molecules reveal the physical location of the genes of interest.

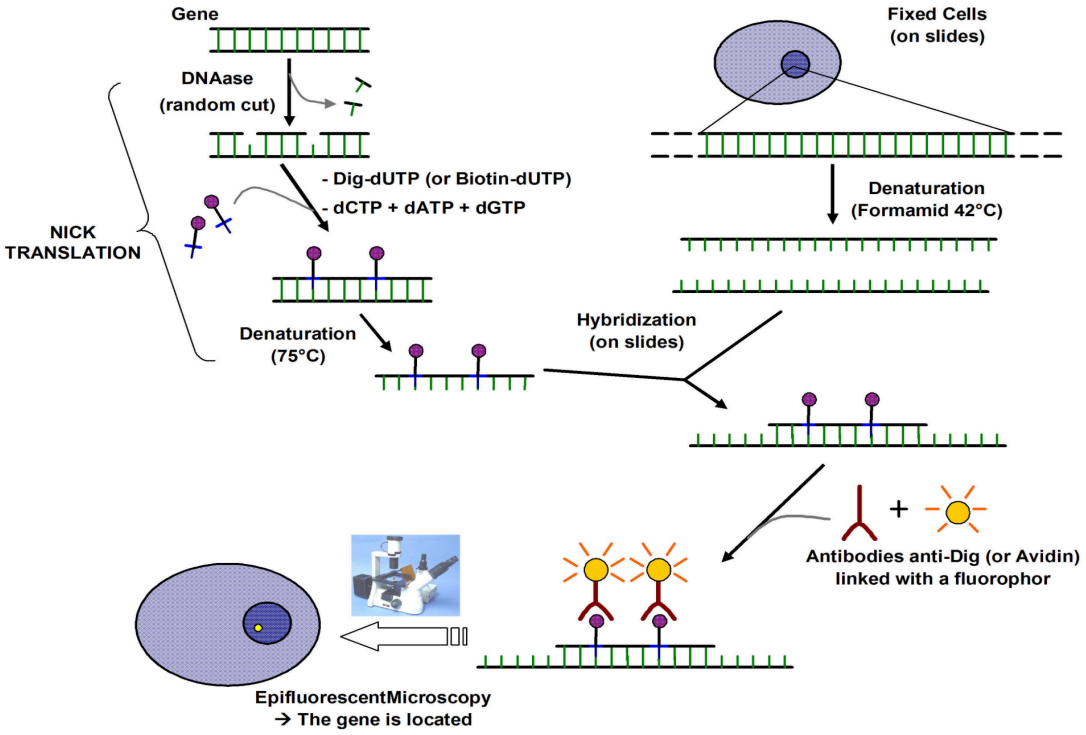


Fig. 9 - FISH mechanisms description.

FISH technique is two days long. At first the DNA was placed on the slide and dehydrated in ethanol (70%, 90% and 100%, each one for 5 minutes). The slides were pretreated with putting the slide in a 37°C bath of a mix of H_2Cl and pepsine, then washed with PBS (phosphate-buffered saline) for 5 minutes. The slides are then ready for the treatment with the hybridation mix composed by dextran sulfate, formamide, SSC (saline-sodium citrate) 20x, SDS (sodium dodecyl sulfate) 5%, and steril water milli-Q, with the two corrispondent probes by each slide. The slides are dried on a hot plate (75°C) and put in a sealed wet box for 24 hours at 37°C.

The day after the slides are dipped in 2x SSC, twice in formamide 20% and again twice in SSC, each time for 5 minutes at 42°C. Then, the slides are treated with 37°C 4x TNFM (composed by SSC 20x, skin milk, tween 20 10%, and steril water), the first time for 30 minutes and the second wash for 5 minutes. Afterwards, the slides are subjected to immunohistochemical detention, articulated in three steps. For the double-probes samples three different mixes are prepared. The first consists in TNFM, Avidin-FITC (1:200), Anti-Digoxigenin (1:200), that will confer green color; the second is a mix of TNFM,

Biotinylated Anti-Avidin (1:100), and Texas red antisheep (1:100), that will shows red colour. The first antibody is put on the slides and let act for 45 minutes at 37°C, in a wet box. When this time is up, the slides are washed with TNFM three times for 5 minutes each time. The second antibody is then put on the slides and left in action for 30 minutes in a wet box at 37°C. Once the treatment of washing is ended (same described above), the slides are ready for the third antibody and for incubation of 30 mins at 37°C. Once finished, the slides are washed as usual in TNFM for three times, hence they are dipped in 4x SSC/0,005% Tween 20 twice for 5 mins each time and the last wash in PBS for 5 mins. The slides are dehydrated in ethanol (70%, 90% and 100%, every step for 3 mins). Once they are dried at room temperature, they are dyed with DAPI and left at 4°C.

In this study, different probes are tested in a few combination to have a general framework and, in addition to the phisical localization, the identification of possible cohybridations. The slides are analyzed for DIG (digoxygenin, a vegetal steroid), that is recognized by Anti-Digoxygenin antibody, and for BIO (biotin, or B7 vitamin), which is linked to Biotinylated Anti-Avidin.

When FISH technique is ended, the slides can be analyzed with a fluorescence microscope, an optical microscope that uses fluorescence instead of reflection and absorption. Nuclei are stained blue with DAPI, and probes (labelled BACs) are located in chromosomes in red (Texas Red) and green (FITC). Each probe shows the primary and the secondary antibody, conjugated to a fluorophore, which binds specifically to the first antibody can be used. The magnification is 100x in oil immersion.

IV. Results

IV.1 3' RACE: cloning and sequencing

Semi-quantitative PCR results were positive in every sample for all genes except for *anti-Müllerian hormon receptor type-2*. Indeed, it was tested for all the available cDNAs, but it has not show any expression neither in female nor in male gonads. Starting from this result, the research focused on different genes, since it was not possible to continue with *ahmr-2*. Therefore, other genes (*Otospiralin*, *Vitellogenin*, *Tetkin-2*, *R-spondin-1*, *Steroidogenic Factor-1*) were examined to verify the expression in ovary and testis. They resulted positive, consequently they were analized with 3' RACE method.

3' RACE PCR showed positive results for all genes, as fig.10 shows, the anealling temperature is 55°C.

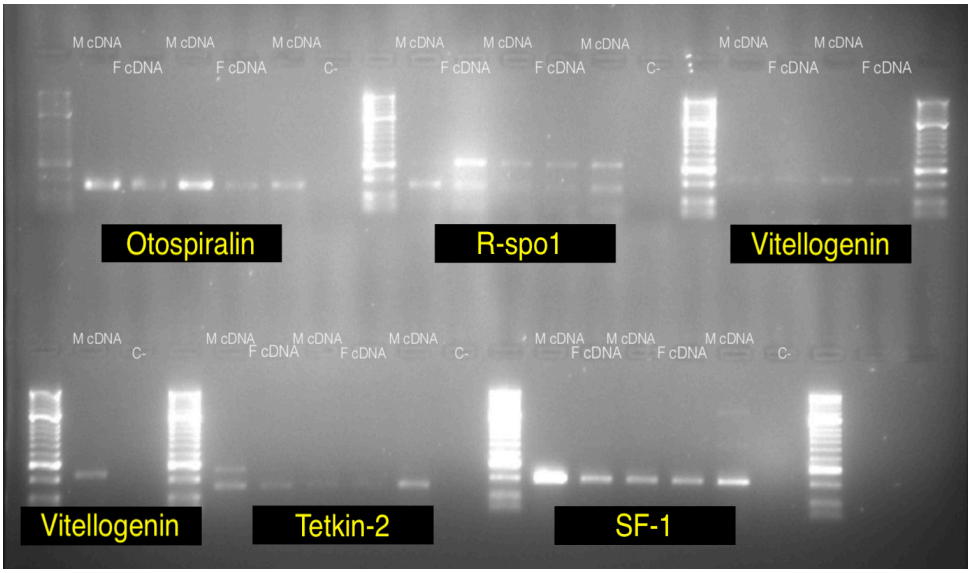


Fig. 10 - 3' RACE PCR results for five cDNA of both sexes.

The following observations are approximate and do not represent a sure data, however they should be compared with their constitutive gene, using multiplex PCR (with couple of primers) and electrophoresis (semiquantitative PCR) or qPCR. As proved in the picture above, regarding *Otos*, both sexes show the same stripe (about 180 bp, 40 ng/BAND), but with a stronger signal in the male than in the female. *R-spo-1* indicates a strong double stripes, respectively in correspondence to 180bp and 400 bp, possibly also

a weak stripe at 50 bp; differences between male and female are not pointed out. For what concerns *VTG*, it has a very weak signal both for male and female; the stripes are just less than 300 bp. *Tetk-2* shows a stripe at about 180 bp, with a weaker signal in the female than in the male, moreover in male samples show a tenue second stripe at about 280 bp. At last, *SF-1* has a strong signal in correspondence to 200 bp, harder in male than female.

Once the cDNAs had been well comproved, they were purificated and the majority resulted with a good degree of purity at the Thermo Scientific NanoDrop™. Part of PCR products (2 µl) were loaded in NanoDrop™, to verify the absence of any contaminants. The results did not show any contamination by protein (values <1,8) neither by RNA (values ≥2), indeed, according with Nanodrop™ bulletin, the samples values are sited between 1,72 and 1,94 at 260/280 nm. So, the PCR product were well purified and it was possible to continue.

In the colony PCR few samples per gene had a positive results with TOPO cloning method. All genes were tested for 10 different colonies, and most of them gave positive results. In the two picture below it is possible to see the good results, in two versions. On the right side a colony PCR of *otospiralin*, on the upper side the female samples and on the lower the male ones. On the left side, it is present a colony PCR, but this time of another gene, *SF-1*.

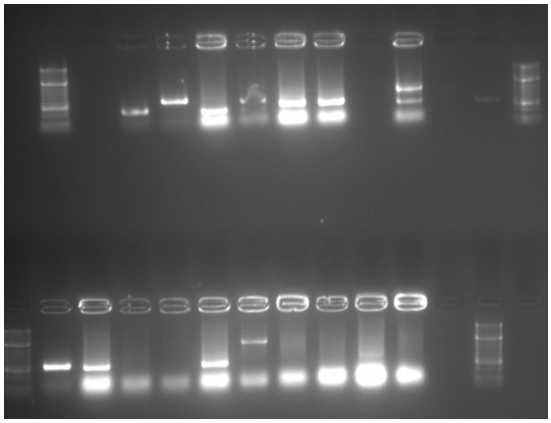


Fig. 11 - Colony PCR for *Otospiralin* in both sexes

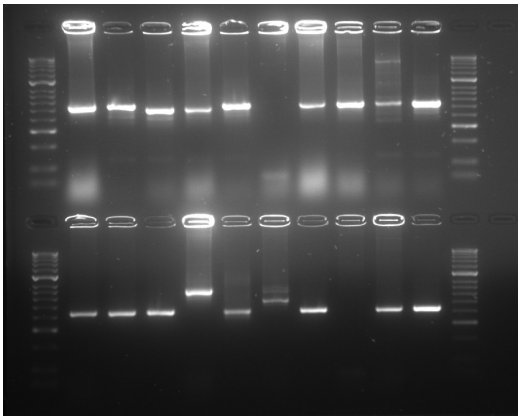
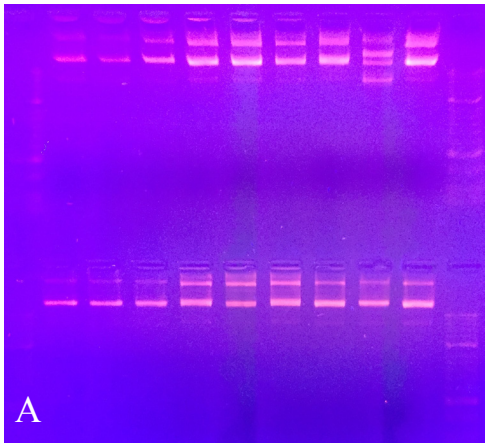


Fig 12 - Colony PCR for *SF1* in female and male

As reported in the picture (fig. 11), *otospiralin* presents for female roughly the same results, about 500 bp, but two different results for male: 400 bp for the majority of samples and about 600 bp for two of them. As the right picture shows (fig. 12), *SF1* is more complex. It is possible to see two different bands at 100 bp and 300 bp, sometimes single, sometimes together, for the female; for the male is the same, with only one sample with a second at about 1000bp. For *R-spondin-1* for both sexes the results show the same band

at about 500 bp. For what concerns *Tektin2*, it does not show, as *R-spo-1,* any differences between the two sexes, indeed it presents bands at about 450 bp. However, *Vitellogenin*, that had a weak signal in the semi-quantitative PCR did not show any expression in any sample, neither male nor female. This study will proceed taking into consideration only the four genes that had showed expression.

A limited number of colonies, 10 per genes, for both sexes (total of 20 samples for each gene), were chosen among different plates. Plasmids were extracted from those samples, and later they were digested. The images below show plasmids of *Otospiralin* extracted (fig. 13a) and digested by restriction enzymes (fig. 13b).



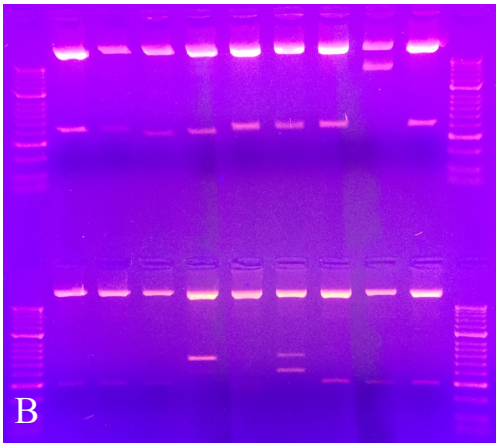


Fig. 13 **a)** *Otos* plasmid extraction for both sexes.; **b)** Electrophoresis of digested *Otos* clones.

IV.2 Data analysis

Once the plasmids had been extracted, it was possible to analyze the sequences by each gene, in both sexes.

Four putative *S. senegalensis* genes were characterized: *Otospiralin*, *R-spondin-1*, *Steroidogenic factor-1* and *Tetkin-2*. In table 5 is possible to observe the results. Columns show the different genes, their positive results, the different individuals, the insert size and the kind of vector used. Indeed, for each gene the total number of samples were 20, divided in 10 per sex, of which the following resulted positive: 17 (6 male, 7 female) for *otos*, 9 (3 male, 6 female) for *tetk-2*, 18 (10 male, 8 female) for *R-spo1* and 13 (5 male, 8 female) for *SF1*. As explained at the beginning, this study analyzed a total of five individuals of Senegalese sole, in particular three male and two female. Reffering to the chart, the different individuals (i1, i2, i3) were tested in the same way for each gene: the three males were tested in four sample; for the remaining two female soles four results are pro-

vided. The vectors utilized are differents among genes, in fact for *otos* and *R-spo1* TOPO were used, while for the other two genes (*tetk-2*, *SF1*) P-Gem method was performed.

Table 5 - Results of analyzed sequences. To each gene corresponds the number and the size of the clones, in the three different individual (i1, i2, i3) analyzed by sex.

Gene	Number of clones	Sex	Individual			Insert size (bp)	Vector
			i1	i2	i3		
Otos	17	Male	4	4	2	300	TOPO
		Female	2	5			
Tetk-2	9	Male	1	2		300	P-Gem
		Female	2	4			
R-spo-1	18	Male	2	4	4	200	TOPO
		Female	4	4			
SF-1	13	Male		3	2	300	P-Gem
		Female	4	4			

The sequences were analyzed using VecScreen and identified by comparison with existing and predicted sequences, using the blast tool at http://www.ncbi.nlm.nih.gov. Table 6 shows only the most representative of the few positive sequences (all collected data are reported in appendix). The following listed results for each gene had a E-value < 2.50, and a minimun of 80% of query cover.

For what concerns *otospiralin* its sequence is provided by a female sole, and it has a high homology with the same gene of few species: *Ictalurus punctatus* (XM_017453699.1), *Larimichthys crocea* (XM_010752467.2) *Lates calcarifer* (XM_018700082.1), *Pygocentrus nattereri* (XM_007252345.3), *Astyanax mexicanus* (XM_007252345.3), *Sinocyclocheilus rhinocerous* (XM_016535256.1), *Cyprinus capio* (XM_019097833.1), *Hyppocampus comes* (XM_019866153.1), *Xiphophorus maculatus* (XM_014471265.2), *Nothobranchius furzeri* (XM_015955475.1), *Amphiprion ocellaris* (XM_023276427.1), *Acanthochromis polyacanthus* (XM_022193894.1), *Stegastes partitus* (XM_008291895.1), and *Larimichthys crocea* (XM_019267163.1).

Also in this *tetkin-2* case the query corresponds to the effective expectation. The sequence reported in table 6, belonging to the first female individual analized, had a strong homology with *tetkin2* of different species, among which: *Lates calcarifer,* (XM_018699637.1), *Labrus bergylta* (XM_020642313.1), *Oryzas latipes*

(XM_011481325.3), *Hyppocampus comes* (XM_019866153.1), *Oreochromis niloticus* (XM_013272565.2), *Maylandia zebra* (XM_012924116.2), *Haplochromis burtoni* (XM_005943560.2), *Neolamprologus brichardi* (XM_006794391.1), *Cynoglossus semilaevis* (XM_008331085.2) and *Fundulus heteroclitus* (XM_021310052.1).

Table 6 - Putative genes analyzed and their sequences.

Putative gene	**Sequence** (5' ⟶ 3')
Otos	TTCATATCCCAGCGTGTTTCCGATGGGCTGGTGGGCGAAGAAAGTGC-GTGCCATCTGGTTTATCTGGTTGTAGGCGCCGATGGATCTGAAGTAT-TCGATGTAGTTCCAGAAGTCAGAGGTGGAGTAAGGCCAGTAGGGCATA-GCTGCTGGTCGTCGTAAAGGGC
Tetk-2	TTTGTTAACGAGGTCCATCAGCTGGAGGCGACAATTCTGGCTCTGAA-ACAAAAGCTGTCTGAAGCTCAACACTCCCTGCAGAAAATGAAGCTCCA-TCATTACCATATGCTGCAGGATCTTTCCACAAAAAAGGAAGCTTTAA-ACCTGGAACAACGAAGCATGAAG
R-spo-1	GGCTGTGACCGATGCTCTGAGTACAATGGCTGCATTAAATGTAAGCC-CAAGCTCTTCATCTTCCTGGAGCGCTATGACATCCGTCAGATTGGCGT-GTGCCTCGCCTCCTGCCCAATGGGGTACTTTGGGATGAGGAACCCA-GAGGGCAACAACAGAT
SF-1	TTGGAGGAGAGTCTGGTCCGCCATGCGACACATGAGGCTGAAGGT-GCTGGGCTTGTCCAGTCGGGCCCGGCCGCCCTGCTCCTGCTGCAGGT-GCGCAACAATCTTGTTCTGCACCGCGAGTTCGTCCGGGTCACAGCGCA-GCAGCTCCAGCACGAGCGGCGGCAGGTTGGGCGGCTGCGGTGAGGTG-GACGGGTACATGTCTG

About *R-spo-1*, its data analysis are reffering to a male sequence, that had as results high homology with: *Cynoglossus. semilaevis* (XM_017037568.1), *Labrus. bergylta* (XM_020633559.1), *Hyppocampus comes* (XM_019866153.1), *Boleophthalmus pectinirostris* (XM_020940482.1), *Oryzas latipes* (XM_011481325.3), *Stegastes partitus* (XM_008291895.1), *Seriola lalandi dorsalis* (XM_023415234.1), *Seriola dumerili* (XM_022764284.1), *Oreochromis niloticus* (XM_013272565.2), *Lates calcarifer* (XM_018700082.1), *Larimichthys crocea* (XM_019267163.1), *Notothenia coriiceps* (XM_010767095.1), *Paralichthys olivaceus* (XM_020092797.1).

Regarding *Steroidogenic Factor 1*, the refering sequence is provided by the most complete information obtained of the second male analyzed. The results showed high homology with the same gene for *Monopterus albus* (XM_020589621.1), *Oryzas latipes* (XM_011481325.3), *Oreochromis niloticus* (NM_001279486.1), *Oncorhynchus kisutch* (XM_020489714.1), *Salvelinus alpinus* (XM_023979023.1), *Salmo salar*

(XM_014143572.1), *Paralichthys olivacetus* (XM_020102351.1). The BLAST results showed also a remarkable homology with chromosome XIV of *Larimichthys crocea* (XM_010747181.2) and for *Esox lucius* nuclear receptor subfamily 5, receptor A (XM_010876204.3).

IV.3 BAC-FISH

As described in previous articles (Vega et al., 2002; Garcia Cegarra et al., 2013), *S. senegalensis* karyotype presents 21 chromosome pairs, composed of 3 metacentric, 2 submeta-subtelocentric, 4 subtelocentric, and 12 acrocentric chromosome pairs. In the following table (table 7) are summarized and reported the two-colour FISH combinations tested:

Table 7 - Combination of BAC-FISH two colours analized.

BIO / DIG	***TETK***	***VGT***	***SF-1***	***RSPO-1***
TEKT				
VGT	X			
SF-1	X	X		
RSPO-1	X	X	X	
OTOS	X	X	X	X

Two-color FISH was carried out in order to value the position of each BAC (containing genes of interest) in the chromosomic complement of Senegalese sole and to check eventual colocalization among them. The two-color FISH technique located all the 5 BAC clones on 5 different chromosome pairs. As a whole, the BAC-FISH resulted in five BAC clones, producing single signals. None of the BAC clones were co-localized in the same chromosome.

BAC clones provided a consistent hybridization signal in different chromosomes for each gene, as reported in the table below (table 8). *Tetkin-2* showed its signal in the middle of the long arm of a telocentric chromosome. The gene *Vitellogenin* were located on an acrosome chromosome, near to the centromere. For what concern *R-spondin-1* and *Otospiralin*, they showed only one signal in different submetacentric chromosomes, both on the respective long arm. Finally, *Steroidogenic factor 1* has localized in metacentric

chromosome, approximately in the telomeric portion of one of the arms (Fig. 16).

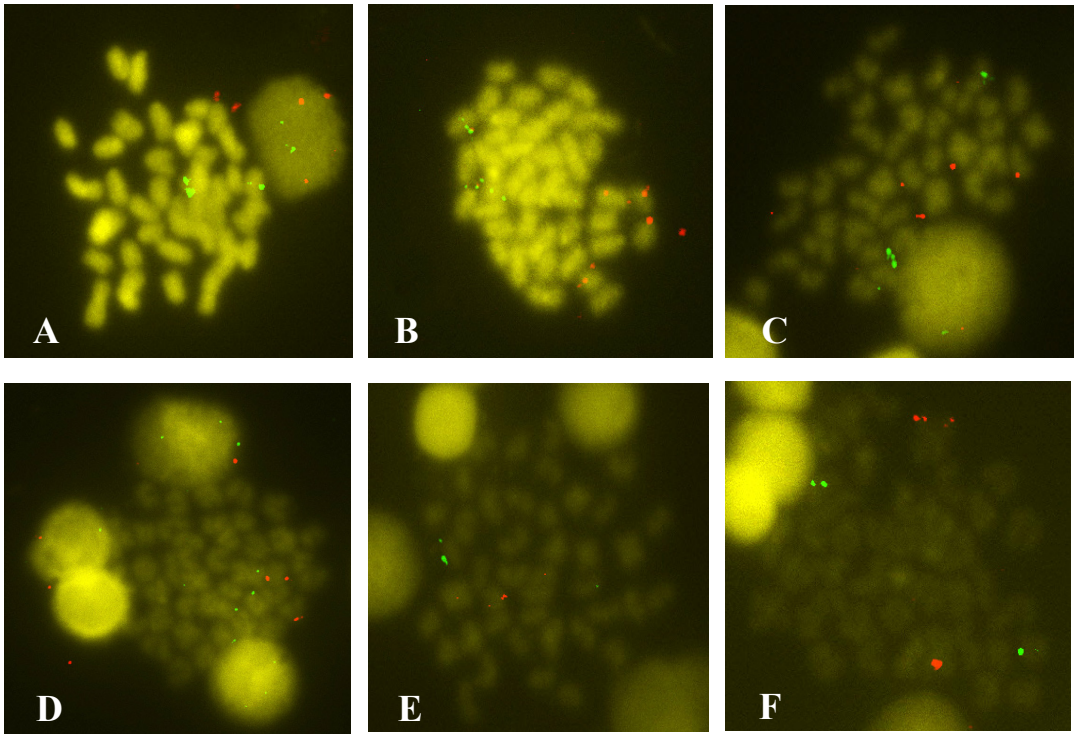


Fig. 16 - FISH of the BACs isolated in the library that contain the following candidate genes: **a)** *Tetk2* (green), *Vgt* (red); **b)** *Vgt* (green), *SF-1* (red); **c)** *R-spo*1 (green), *Otos* (red); **d)** *SF-1* (green), *R-spo1* (red); **e)** *Tetk2* (green), *R-spo1* (red); **f)** *Tetk2* (green), *Otos* (red).

Table 8 - Number of FISH signals and localization of BAC clones onto *S. senegalensis* chromosomes. *MT* metacentric chromosome, *SMT* sub-metacentric, *STC* sub-telocentric, *A* acrocentric, *SC* sub-centromeric position, *I* interstitial, *STL* subtelomeric, *TL* telomeric, *q* long arm, *p* short arm.

Candidate gene	**Number of signal**	**Localization of signals**	
		Chromosome type	**Position within chromosome**
Vitellogenin	1	A	I
Otospiralin	1	SMT	TL
R-spondin-1	1	SMT	qI
SF-1	1	MT	qTL
Tetkin-2	1	STC	qTL

V. Discussion

V.1 Cloning and sequencing

Four putative genes involved in sex determination and sex-differentiation cDNAs were isolated from gonads by 3'-RACE strategy. The resulting sequences permitted a partial knowledge of genes' characterization.

Otospiralin is a gene that has limited references; it has initially identified as an inner ear specific molecule (Delprat et al., 2002), ranging from fish to mammals (Lavigne-Rebillard et al., 2003). Previous works focused on its expression using q-PCR, in situ hybridization and western blotting (Decourt et al., 2009). Nevertheless, further investigations are required to determine the precise function of *otospiralin*. Results from the evidences of sequencing process show a good homology with predicted *otos* of *L. calcarifer* (91%), *L. crocea* (88%), *S. rhinocerous, S. partitus* and *X. maculatus* (87%), *A. ocellaris* and *A. polycanthus* (85%), *N. furzeri* (84%), *A. mexicanus* (81%), *I. punctatus*, *P. nattereri*, *C. carpio* and *H. comes* (80%).

In the last few years, more and more researches focused on *Rspo1* expression and clonation are being carried out. In particular, Smith et al. (2008) observed and compared earth turtle, chicken, mouse and human sequences and expressions. This gene shares gretest homology with the predicted chicken sequence (91% amino acid similarity), followed by human (84% similarity) and mouse (81%). *Rspo1* showed a conserved pathway leading to folliculogenesis and germ cell development. In the female mouse and chicken gonads, *Rspo1* is mainly located in the germ cells just before the onset of meiosis. In the medaka (*O. latipes*), *Rspo1* mRNA, together with a germ-cell marker (*Vasa*) and a meiosis marker (*Spo11*), it was specifically upregulated just at the time of meiosis initiation (Zhou et al., 2012). A research about Nile tilapia (*Oreochromis niloticus*) suggests that the *Rspo1* signaling pathway is involved in ovarian development by opposing the testis developmental pathways, and it also plays a key role in testicular development and spermatogenesis (Wu et al., 2016). *Rspo1* shows greatest homology with the predicted sequences of *L. calcarifer*, *S. lalandi dorsalis*, *S. dumerili* (96%), *L. crocea* , *P. olivaceus* (94%), *L. bergylta* (93%), *C. semilaevis, S.. partitus*, *O. niloticus*, *N. coriiceps* (92%), *O. latipes* (90%), *B. pectinirostris* (83%) and *H. comes* (75%). The BLAST results showed

a high homology also with those cited species, so it is likely that for *S. senegalensis* also *Rspo1* would have this role.

Steroidogenic factor-1 is a tissue-specific orphan receptor located in the nucleus (Lynch et al., 1993). *SF1* mRNA has also been detected in gonad of common carp adult and can regulate the translation of SF-1 protein (Tang et al., 2010). The present study effectively *SF1* in *S. senegalensis*, in the BLAST comparation, it shows a high homology for *M. albus*, *O. niloticus*, *P. olivaceus* (87%), *O. latipes* (85%), *O. kisutch* (82%), *S. salar* (79%) and *S. alpinus* (77%). Previous studies provided evidence about *SF1* role as promoter of zebrafish (Tchoudakova et al., 2001) and goldfish (Tong and Chung, 2003) *cyp19a1a* in teleost gonads. It could be interesting to analize all sequences of these two genes.

In mammals, *Tetkin-2*, which is identical to Tetkin-t, has been located in the principal piece of human spermatozoa without detectable immunosignals in the middle piece or the end piece (Iguchi et al. 1999, 2002; Wolkowicz et al. 2002). *Tetkin-2* were characterized in mouse and had revealed high homology with two mouse genes. Nevertheless, these genes have not been characterized yet (Yamaguchi et al., 2014). In this research, NCBI BLAST analyzes showed a meaningfull homology from *S. senegalensis* sequence with *L. calcarifer*, *O. niloticus* (84%), *M. zebra*, *N. brichardi*, *H. burtoni* (83%), *Cynoglossus semilaevis*, *L. bergylta* (82%), *Fundulus heteroclitus* (78%), *O. latipes* (75%), *H. comes* (74%).

V.2 BAC-FISH

The karyotype of *S.senegalensis* displays a wide range of variation in chromosome size and morphology. In previous studies, several repetitive sequences have been localized by FISH; these repetitive sequences include 5S and 45S rDNA, telomeric $(TTAGGG)_n$ and $(GATA)_n$ repeats (Cross et al., 2006; Manchado et al., 2006). The BAC-FISH analysis showed the presence of each gene in a different chromosome.

Vitellogenin gene expression has been studied in many organisms, including fishes, amphibians and mammals (Thorpe et al., 2003; Mosconi el al., 2002; Vomsaal et al., 1997). It is synthesized in the liver for vertebrates and in the gonad tissues for invertebrated (mussels, clams, and gastropods), and is under the control of estradiol-17β and other neuropeptidic precursors from the nervous system (Gagné, 2014). *Vitellogenin* has been verified in an acrocentric chromosome: this is an important detail 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.

Otospiralin is a short protein expressed in fribrocytes of the inner ear (Lavigne-Rebillard et al., 2003), but it is also expressed in other tissues including brain, muscle, liver and gonads (Decourt et al., 2008). The conservation of the protein from mammals throught fishes and its specific expression suggests that *otospiralin* is involved in important functions. In previous pubblications about *Otos* in human using FISH technique, it has been localized in the telomeric portion of a submetracentric chromosome long arm, that was identified as chromosome 2 (Lavigne-Rebillard et al., 2003). Likewise, in this study, this gene was localized in a submetacentric chromosome. Indeed, *Otospiralin* was found in the telomeric portion of the long arm. This would confirm the presence of this gene in this kind of chromosome, despite the different species. Thus, it is reasonable to imagine this gene to have a very robust conservation through evolution.

R-spondin1 is a potential female-determining gene in human (*Homo sapiens*) and mouse (*Mus musculus*). Its differential expression in these mammals is correlated with signaling for sex determination (Chassot et al., 2008). For the purpose of studing sex determination in fish, *Rspo1* was cloned and analyzed in zebrafish (*Danio rerio*) (Zhang et al., 2010). This gene has found in a submetacentric chromosome. In *S. senegalensis* karyotype there are only two pairs of submetacentric chromosome (Vega et al., 2003), so if *Otos* is located in one of those two pairs, surely *Rspo1* will occupy the other one.

Steroidogenic factor 1 (SF1), also called Ad4BP, a nuclear receptor encoded by gene NR5A1, regulates the transcription of an array of genes involved in reproduction, steroidogenesis, and male sex differentiation (Rey et al., 2016). *SF-1* expression is found in steroidogenic cells of adrenal cortex and gonads, as well as in neurons of ventromedial nucleus of the hypothalamus (Ramos-Lobo et al., 2017). *SF-1* is located in a metacentric chromosome pair, likely chromosome 1. This would means that *SF-1* is on a possible sexual protochromosome, as explained in Portela-Bens et al. (2017). Indeed, in this pubblication the authors hypothesized that chromosome 1 is too big because of a Robertsonian fusion between two acrocentric chromosomes, that could have occurred during the evolution of Pleuronectiformes, giving arise to a large metacentric chromosome in *S. senegalensis*.

Tektins are composed of a family filament-forming proteins located in cilia and flagella. Four types of tetkins have been reported, in particular *tetkins-2* have been verified to be present in sperm flagella (Murayama et al., 2008). This gene has been localized in

one of the four pairs of subtelomeric chromosome.

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), and Portela-Bens et al. (2017), which localized 15 BAC clones. This study completes those previous works and localizes BAC clones of up to 5 (out of 21) chromosome pairs. The FISH technique has also been used in the cytogenetic characterization of other flatfish species, such as *S. maximus* (Taboada et al., 2014).

VI. Conclusions

The present work has followed on from previous research published by the group of Genetic belonging to University of Cádiz, to complete the karyotype characterization of *S. senegalensis*, focusing on one hand on cloning and sequencing of four putative genes involved in sex determination and, on the other hand, on BACs containing genes associated with sex determination and differentiation, using two-colour FISH technique.

Cloning and sequencing performed well but, with only 3' RACE results available, it is not possible to know the full-lenght sequences of this four putative genes yet. Nevertheless, the obtained results showed for each gene a great homology with predicted gene sequences of other bony fish.

Results of localization of candidate gene in different chromosomes allow a wider knowledge of Senegalese sole genetic. In particular, candidate gene *SF1* was found in the largest metacentric chromosome of *S. senegalensis* as a sex proto-chromosome. This results is important for focusing further the research about those chromosome pairs of *S. senegalensis*.

VI.1 Future perspectives

This study opens up to further developments: first of all to finish the clonation of these four putative genes (*Vtg*, *Otos*, *R-spo1*, *Sf-1* and *Tetk-2*) involved in sex determination, using 5' RACE technique. Then, it would be useful to know the expression of these genes through qPCR and identify any differences between sexes. Then, if it is possible, compare these data with the expression of the same genes in other species. In addition to the proposed cytological localization with BAC-FISH, the histological knowledge could be expanded too. Once these goals about *S.senegalensis* are reached, it would be interesting to create species-specific DNA marker, as already done in turbot(Casas et al., 2011).

To have a wider vision about the aquaculture F1 generation problem, it would be also interesting to investigate in a comparison between the wild generation (F0) and the aquaculture one (F1). It will then be possible to realize the differences in genetic expression of the two generation, and even to carry out a transversal research involving not only genetics, but also ecological field, keeping in mind the different enviromental conditions which they are subjected to.

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VIII. Appendix

In the following tables all the positive clonations are collected, divided by gene.

Table A - 3' RACE sequences of *Otospiralin* (M: male, F, female; i1: individual 1, i2: individual 2, i3: individual 3)

Clone Otos	**Sequence** (5' ⟶ 3')
31 A (M, i1)	CTTTACGACGAACCAGCAGCTATGCCCTACTGGCCTTACTC-CACCTCTGACTTCTGGAACTACATCGAATACTTCAGATCCA-TCGGCGCCTACAACCAGATAAACCAGATGGCACGCACTTTCTT-TGCCCACCAGCCCATCGGAAACACGCTGGGATATGAAGGGCGA-ATTC
31 B (M, i1)	TTGGCCTGGTGGGCAAAGAAAGTGCGTGCCAGCGGGTTTAC-CTGGTTGTAAGCGCCTAGGGATCTGATGTACTCAATGTGATTC-CAGAAGTCAGACGTTGAGTAGCGCCAGTAGTACACAGCTTCTG-GTT
32 A (M, i1)	CAGGGCTATGCGCTACTGGCCTTACTCCCCTCTGACTTCTGGA-ACTACATCGAATACTTCCATCCATCGGCGCCTACAACCAGATA-AACCACATGGCACGCACTTTCTTTGCCCACCAGCCCATCGGAA-ACACGCTGGGATATGAAGGGCGAATTCGT
32 B (M, i1)	CTTCATATCCCAGCGTGTTTCCGATGGGCTGGTGGGCAAACAA-AGTGCGTGCCCTCGGGTTTATCTGGTTGTAGGCGCCGATGGA-TCTGAAGTATTCGATGTAGTTCCNNAAGTCAGAGGTGGAGTA-AGGCCAGTAGGGCATAACTGCTGGGTCGTCTTAAAGGGCGAAT-TCGCGG
33 A (M, i2)	AACCAGCAGCTATGCCCTACTGGCCTTACTCCACCTCTGACT-TCTGGAACTACATCGAATACTTCAGATCCATCGGCGCCTACA-ACCAGATAAACCAGATGGCACGCACTTTCTTTGCCCACCAGCC-CATCGGAAACACGCTGGGATATGAAGGGCGAATTCG
33 B (M, i2)	CTTCATATCCCAGCGTGTTTCCGATGGGCTGGTGGGCAAAGAA-AGTGCGTGCCATCTGGTTTATCTGGTTGTAGGCGCCGATGGA-TCTGAAGTATTCGATGTAGTTCCAGAAGTCAGAGGTGGAGTA-AGGCCAGTAGGGCATAACTGCTGGTTCGTCGTAAAGGGC
34 A (M, i2)	CCCAGCGTGTTTCCGATGGGCTGGTGGGCAAAGAAAGTGCGT-GCCATCTGGTTTATCTGGTTGTAGGCGCCGATGGATCTGAA-GTATTCGATGTAGTTCCAGAAGTCAGAGGTGGAGTAAGGCCA-GTAGGGCATAGCTGCTGGTTCGTCGTAAAGGGCGAA

34 B (M, i2)	ACGACGAACCAGCAGCTATGCCCTACTGGCCTTACTCCAC-CTCTGACTTCTGGAACTACATCGAATACTTCAGATCCATCGGC-GCCTACAACCAGATAAACCAGATGGCACGCACTTTCTTTGCC-CACCAGCCCATCGGAAACACGCTGGGATATGAAG
35 A (M, i3)	GAACCAGCAGCTATGCCCTACTGGCCTTACTCCACCTCTGACT-TCTGGAACTACATCGAATACTTCAGATCCATCGGCGCCTACA-ACCAGATAAACCAGATGGCACGCACTTTCTTTGCCCACCAGCC-CATCGGAAACACGCTGGGATATGAAGGGCGAAT
35 B (M, i3)	TTCATATCCCAGCGTGTTTCCGATGGGCTGGTGGGCAAAGAA-AGTGCGTGCCATCTGGTTTATCTGGTTGTAGGCGCCGATGGA-TCTGAAGTATTCGATGTAGTTCCAGAAGTCAGAGGTGGAGTA-AGGCCAGTAGGGCATAGCTGCTGGTTCGTCGTAAAGGGCG
37 A (F, i1)	GACGACCAGCAGCTATGCCCTACTGGCCTTACTCCACCTCT-GACTTCTGGAACTACATCGAATACTTCAGATCCATCGGCGC-CTACAACCAGATAAACCAGATGGCACGCACTTTCTTCGCCCAC-CAGCCCATCGGAAACACGCTGGGATATGAAGGG
37 B (F, i1)	TTCATATCCCAGCGTGTTTCCGATGGGCTGGTGGGCGAAGAA-AGTGCGTGCCATCTGGTTTATCTGGTTGTAGGCGCCGATGGA-TCTGAAGTATTCGATGTAGTTCCAGAAGTCAGAGGTGGAGTA-AGGCCAGTAGGGCATAGCTGCTGGTCGTCGTAAAGGGC
38 B (F, i1)	CAGCTATGCCCTACTGGCCTTACTCCACCTCTGACTTCTGGA-ACTACATCGAATACTTCAGATCCATCGGCGCCTACAACCAGA-TAAACCAGATGGCACGCACTTTCTTTGCCCACCAGCCCATCG-GAAACACGCTGGGATATGAAGGGCGAATTCGC
39 A (F, i2)	CATATCCCAGCGTGTTTCCGATGGGCTGGTGGGCAAAGAAAGT-GCGTGCCATCTGGTTTATCTGGTTGTAGGCGCCGATGGATCT-GAAGTATTCGATGTAGTTCCAGAAGTCAGAGGTGGAGTAAGGC-CAGTAGGGCATAGCTGCTGGTTCGTCGTAAAGGGCGAATT
39 B (F, i2)	CGACGAACCAGCAGCTATGCCCTACTGGCCTTACTCCACCTCT-GACTTCTGGAACTACATCGAATACTTCAGATCCATCGGCGC-CTACAACCAGATAAACCAGATGGCACGCACTTTCTTTGCCCAC-CAGCCCATCGGAAACACGCTGGGAT
40 A (F, i2)	ACGACGAACCAGCAGCTATGCCCTACTGGCCTTACTCCAC-CTCTGACTTCTGGAACTACATCGAATACTTCAGATCCATCGGC-GCCTACAACCAGATAAACCAGATGGCACGCACTTTCTTTGCC-CACCAGCCCATCGGAAACACGCTGGGATATGAAGGGC
40 B (F, i2)	TTCGCCCTTCATATCCCAGCGTGTTTCCGATGGGCTGGTGG-GCAAAGAAAGTGCGTGCCATCTGGTTTATCTGGTTGTAGGCGC-CGATGGATCTGAAGTATTCGATGTAGTTCCAGAAGTCAGAG-GTGGAGTAAGGCCAGTAGGGCATAGCTGCTGGTTCGTCGTAA-AGGGCGAATTC

Table B - 3' RACE sequences of *Rspo1*

Clone Rspo1	**Sequence** (5' ⟶ 3')
21 A (M, i1)	GGCTGTGACCGATGCTCTGAGTACAATGGCTGCATTAAATGTA-AGCCCAAGCTCTTCATCTTCCTGGAGCGCTATGACATCCGTCA-GATTGGCGTGTGCCTCGCCTCCTGCCCAATGGGGTACTTTGGGA-TGAGGAACCCAGAGGGCAACAACAGAT
21 B (M, i1)	GTTGCCCTCTGGGTTCCTCATCCCAAAGTACCCCATTGGGCAG-GAGGCGAGGCACACGCCAATCTGACGGATGTCATAGCGCTCCA
23 A (M, i2)	AAGGCTGTGACCGATGCTCTGAGTACAATGGCTGCATTAAAT-GTAAGCCCAAGCTCTTCATCTTCCTGGAGCGCTATGACATCC-GTCAGATTGGCGTGTGCCTCGCCTCCTGCCCAATGGGGTACTT-TGG
23 B (M, i2)	TTCATCTGTTGTTGCCCTCTGGGTTCCTCATCCCAAAGTACCC-CATTGGGCAGGAGGCGAGGCACACGCCAATCTGACGGATGTCA-TAGCGCTCCAGGAAGATGAAGAGCTTGGGCTTACATTTAATGCA-GCCATTGTACTCAGAGCATCGGTCACAGCCTTTCGAAGGGCGAATT
24 A (M, i2)	AGGCTGTGACCGATGCTCTGAGTACAATGGCTGCATTAAATGTA-AGCCCAAGCTCTTCATCTTCCTGGAGCGCTATGACATCCGTCA-GATTGGCGTGTGCCTCGCCTCCTGCCCAATGGGGTACTTTGGGAT
24 B (M, i2)	CGCCCTTCATCTGTTGTTGCCCTCTGGGTTCCTCATCCCAAA-GTACCCCATTGGGCAGGAGGCGAGGCACACGCCAATCTGACGGA-TGTCATAGCGCTCCAGGAAGATGAAGAGCTTGGGCTTACATTTA-ATGCAGCCATTGTACTCAGAGCATCGGTCACAGCCTTTCGAAGG-GCGAATTC
25 A (M, i3)	AAAGGCTGTGACCGATGCTCTGAGTACAATGGCTGCATTAAAT-GTAAGCCCAAGCTCTTCATCTTCCTGGAGCGCTATGACATCC-GTCAGATTGGCGTGTGCCTCGCCTCCTGCCCAATGGGATACTT-TGGG
25 B (M, i3)	GAATTCGCCCTTCATCTGTTGTTGCCCTCTGGGTTCCTCATCC-CAAAGTATCCCATTGGGCAGGAGGCGAGGCACACGCCAATCT-GACGGATGTCATAGCGCTCCAGGAAGATGAAGAGCTTGGGCTTA-CATTTAAT
26 A (M, i3)	AAGGCTGTGACCGATGCTCTGAGTACAATGGCTGCATTAAAT-GTAAGCCCAAGCTCTTCATCTTCCTGGAGCGCTATGACATCC-GTCAGATTGGCGTGTGCCTCGCCTCCTGCCCAATGGGGTACTT-TGGG
26 B (M, i3)	TTCATCTGTTGTTGCCCTCTGGGTTCCTCATCCCAAAGTACCC-CATTGGGCAGGAGGCGAGGCACACGCCAATCTGACGGATGTCA-TAGCGCTCCAGGAAGATGAAGAGCTTGGGCTTACATTTAATGCA-GCCATTGTACTCAGAGCATCGGTCACAGCCTTTCGAAGGGCG

27 A (F, i1)	GAAAGGCTGTGACCGATGCTCTGAGTACAATGGCTGCATTAAA-TGTAAGCCCAAGCTCTTCATCTTCCTGGAGCGCTATGACATCC-GTCAGATTGGCGTGTGCCTCGCCTCCTACCCAATGGGGTACTT-TGGGAT
27 B (F, i1)	TCATCTGTTGTTGCCCTCTGGGTTCCTCATCCCAAAGTACCC-CATTGGGTAGGAGGCGAGGCACACGCCAATCTGACGGATGTCA-TAGCGCTCCAGGAAGATGAAGAGCTTGGGCTTACATTTAATGCA-GCCATTGTACTCAGAGCATCGGTCACAGCCTTTCGAA
28 A (F, i1)	CGAAAGGCTGTGACCGATGCTCTGAGTACAATGGCTGCATTAAA-TGTAAGCCCAAGCTCTTCATCTTCCTGGAGCGCTATGACATCC-GTCAGATTGGCGTGTGCCTCGCCTCCTGCCCAATGGGGTACTT-TGGGAT
28 B (F, i1)	TCATCTGTTGTTGCCCTCTGGGTTCCTCATCCCAAAGTACCC-CATTGGGCAGGAGGCGAGGCACACGCCAATCTGACGGATGTCA-TAGCGCTCCAGGAAGATGAAGAGCTTGGGCTTACATTTAATGCA-GCCATTGTACTCAGAGCATCGGTCACAGCC
29 A (F, i2)	AAGGCTGTGACCGATGCTCTGAGTACAATGGCTGCATTAAAT-GTAAGCCCAAGCTCTTCATCTTCCTGGAGCGCTATGACATCC-GTCAGATTGGCGTGTGCCTCGCCTCCTGCCCAATGGGGTACTT-TGGG
29 B (F, i2)	ATCTGTTGTTGCCCTCTGGGTTCCTCATCCCAAAGTACCCCAT-TGGGCAGGAGGCGAGGCACACGCCAATCTGACGGATGTCATAGC-GCTCCAGGAAGATGAAGAGCTTGGGCTTACATTTAATGCAGC-CATTGTACTCAGAGCATCGGTCACAGCCTTTCGAA
30 A (F, i2)	GAAAGGCTGTGACCGATGCTCTGAGTACAATGGCTGCATTAAAT-GTAAGCCCAAGCTCTTCATCTTCCTGGAGCGCTATGACATTTC-GTCAAATTGGCGTGTGCCTCGCCTCCTGCCCAATGGGGTACTT-TGGGATGAG
30 B (F, i2)	TCATCTGTTGTTGCCCTCTGGGTTCCTCATCCCAAAGTACCC-CATTGGGCAGGAGGCGAGGCACACGCCAATCTGACGGATGTCA-TAGCGCTCCAGGAAGATGAAGAGCTTGGGCTTACATTTAATGCA-GCCATTGTACTCAGAGCATCGGTCACAGCCTT

Table C - 3' RACE sequences of *Steroidogenic Factor 1*

Gene SF1	**Sequence** (5' → 3')
13 A (M, i2)	AATTCGCCCTTATCCAGACATGTACCCGTCCACCTCGCCGCA-GCCGCCCAACCTGCCGCCGCTCGTGCTGGAGCTGCTGCGCTGT-GACCCGGACGAACTCGCGGTGCAGAACAAGATTGTTGCGCACCT-GCAGCAGGAGCAG
15 A (M, i3)	ATTCGCCCTTATCCAGACATGTACCCGTCCACCTCACCGCAGCC-GCCCAACCTGCCGCCGCTCGTGCTGGAGCTGCTGCGCTGTGACC-CGGACGAACTCGCGGTGCAGAACAAGATTGTTGCGCACCTGCA-GCAGGAGCAGGGCGGCCGGGCCCGACTGGACAAGCCCAGCACCT-TCAGCCTCATGTGTCGCATGGCGGACCAGACTCTCTTCTCCA-AGGGCG
15 B (M, i3)	CCTTGGAGAAGAGAGTCTGGTCCGCCATGCGACACATGAGGCT-GAAGGTGCTGGGCTTGTCCAGTCGGGCCCGGCCGCCCTGCTCCT-GCTGCAGGTGCGCAACAATCTTGTTCTGCACCGCGAGTTCGTC-CGGGTCACAGCGCAGCAGCTCCAGCACGAGCGGCGGCAGGTTGG-GCGGCTGCGGTGAGGTGGACGGGTACATGTCTGGATAAGGGCGA-ATTCGCGG
16 A (M, i3)	CCTTATCCAGACATGTACCCGTCCACCTCACCGCAGCCGCCCA-ACCTGCCGCCGCTCGTGCTGGAGCTGCTGCGCTGTGACCCGGAC-GAACTCGCGGTGCAGAACAAGATTGTTGCGCACCTGCAGCAGGA-GCAGGGCGGCCGGGCCCGACTGGACAAGCCCAGCACCTTCAGC-CTCATGTGTCGCATGGCGGACCAGACTCTCTTCTAAG
16 B (M, i3)	GAAGAGAGTCTGGTCCGCCATGCGACACATGAGGCTGAAGGT-GCTGGGCTTGTCCAGTCGGGCCCGGCCGCCCTGCTCCTGCT
18 A (F, i1)	CTTATCCAGACATGTACCCGTCCACCTCACCGCAGCCGCCCAAC-CTGCCGCCGCTCGTGCTGGAGCTGCTGCGCTGTGACCCGGACGA-ACTCGCGGTGCAGAACAAGATTGTTGCGCACCTGCAGCAGGA-GCAGGGCGGCCGGGCCCGACTGGACAAGCCCAGCACCTTCAGC-CTCATGTGTCGCATGGCGGACCAGACTCTCC
18 B (F, i1)	TTGGAGGAGAGTCTGGTCCGCCATGCGACACATGAGGCTGAAG-GTGCTGGGCTTGTCCAGTCGGGCCCGGCCGCCCTGCTCCTGCT-GCAGGTGCGCAACAATCTTGTTCTGCACCGCGAGTTCGTCCGG-GTCACAGCGCAGCAGCTCCAGCACGAGCGGCGGCAGGTTGGGCG-GCTGCGGTGAGGTGGACGGGTACATGTCTG
19 A (F, i2)	CCCTTATCCAGACATGTACCCGTCCACCTCACCGCAGCCGCCCA-ACCTGCCGCCGCTCGTGCTGGAGCTGCTGCGCTGTGACCCGGAC-GAACTCGCGGTGCAGAACAAGATTGTTGCGCACCTGCAGCAG

19 B (F, i2)	TTGGAGGAGAGTCTGGTCCGCCATGCGACACATGAGGCTGAAG-GTGCTGGGCTTGTCCAGTCGGGCCCGGCCGCCCTGCTCCTGCT-GCAGGTGCGCAACAATCTTGTTCTGCACCGCGAGTTCGTCCGG-GTCACAGCGCAGCAGCTCCAGCACGAGCGGCGGCAGGTTGGGCG-GCTGCGGTGAGGTGGACGGGTACATGTCTGGATAAGGGCGAAT-TCGC
20 A (F, i2)	GACATGTACCCGTCCACCTCACCGCAGCCCCCAACCTGCCGCC-GCTCGTGCTGGAGCTGCTGCGCTGTGACCCGGACGAACTCGCG-GTGCAGAACAAGATTGTTGCGCACCTGCAGCAGGAGCAGGGCG-GCCGGGCCCGACTGGACAAGCCCAGCACCTTCAGCCTCATGT-GTCGCATGGCGGACCAGACTCTCTTCTCCAAGGGCGA
20 B (F, i2)	TTGGAGAAGAGAGTCTGGTCCGCCATGCGACACATGAGGCTGA-AGGTGCTGGGCTTGTCCAGTCGGGCCCGGCCGCCCTGCTCCT-GCTGCAGGTGCGCAACAATCTTGTTCTGCACCGCGAGTTCGTC-CGGGTCACAGCGCAGCAGCTCCAGCACGAGCGGCGGCAGGT-TGGGGGCTGCGGTGAGGTGGACGGGTACATGTCTGGATAAGGGC-GAATTCGC

Table D - 3' RACE sequences of *Tetkin-2*

Clone Tetkin	**Sequence** (5' → 3')
1 B (M, i1)	CTTCATGCTTCGTTGTTCCAGGTTTAAAGCTTCCTTTTT-TGTGGAAAGATCCTGCAGCATATGGTAATGATGGAGCT-TCATTTTCTGCAGGGAGTGTCTGAG
4 A (M, i2)	GTTAACGAGGTCCATCAGCTGGAGGCGACAATTCTGGCTCT-GAAACAAAAGCTGTCTGAAGCTCAGTG
4 B (M, i2)	CATGCTTCGTTGTTCCAGGTTTAAAGCTTCCTTTTTTGTG-GAAAGATCCTGCAGCATATGGTAATGATGGAGCTTCATTT-TCTGCAGGGAGTGT
7 A (F, i1)	TTCATGCTTCGTTGTTCCAGGTTTAAAGCTTCCTTTTT-TGTGGAAAGATCCTGCAGCATATGGTATGATGGAGCT-TCATTTTCTGCAGGGAGTGTTGAGCTTCACACAGCTTTT-GTTTCAAAGCCAGAATTGTCGCCTCCAGCTGATGGACCTC
7 B (F, i1)	TTTGTTAACGAGGTCCATCAGCTGGAGGCGACAATTCTG-GCTCTGAAACAAAAGCTGTCTGAAGCTCAACACTCCCTGCA-TAAAATGAAGCTCCATCATAACATATGCTGCAGGATCTTTC-CACAAAAAAAGAAGCTTTAAACCTGGAACAACGAAGCATGA-AGGGCG
8 A (F, i1)	TTTGTTAACGAGGTCCATCAGCTGGAGGCGACAATTCTG-GCTCTGAAACAAAAGCTGTCTGAAGCTCAACACTCCCTGCA-GAAAATGAAGCTCCATCATTACCATATGCTGCAGGATCTT-TCCACAAAAAAGGAAGCTTTAAACCTGGAACAACGAAGCAT-GAAG
8 B (F, i1)	GCCCTTCATGCTTCGTTGTTCCAGGTTTAAAGCTTC-CTTTTTTGTGGAAAGATCCTGCAGCATATGGTAATGATGGA-GCTTCATTTTCTGCAGGGAGTGTTGAGCTTCAGACAGCTTT-TGTTTCAGAGCCAGAATTGTCGCCTCCAGCTGATGGACCTC-GTTAACAAAGGGCGAATTCGCGGC
10 A (F, i2)	CGAATTCGCCCTTTGTTAACGAGGTCCATCAGCTGGAGGC-GACAATTCTGGCTCTGAAACAAAAGCTGTCTGAAGCTCAGT-GAGTTTATTCCTAGTTCAAAAAACTAATGTATATGCTGTTA-CACCCACAGGAATTAGGACAAACATAATCCTAAGTATCAA-ATTTTACTTCTCGTCACTCAGACACTCCCTGCAGAAAATGA-AGCTCCATCATTACCATATGCTGCAGGATCTTTCCACAAAA-AAGGAAGCTTTAAACCT
10 B (F, i2)	CTTCATGCTTCGTTGTTCCAGGTTTAAAGCTTCCTTTTT-TGTGGAAAGATCCTGCAGCATATGGTAATGATGGAGCT-TCATTTTCTGCAGGGAGTGTCTGAGTG