Molecular analysis of gonad development in medaka (*Oryzias latipes*) and *Oryzias celebensis*

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von
Nils Klüver
aus
Bremen

Würzburg, 2007

Angefertigt am Lehrstuhl für Physiologische Chemie I,
Biozentrum der Universität Würzburg
In der Arbeitsgruppe und unter der Leitung von Prof. Dr. Dr. Manfred Schartl

Eingereicht am: .....

Mitglieder der Promotionskommission:

Vorsitzender: .....

Gutachter : Prof. Dr. Manfred Schartl

Gutachter: Prof. Dr. Ricardo Benavente

Tag des Promotionskolloquiums: .....

Doktorurkunde ausgehändigt am: .....

Table of contents

Zusammenfassung

Ein besseres Verständnis des Prozesses der Geschlechtsbestimmung lässt sich über Untersuchungen von Organen und Zellen welche bei der Bildung der undifferenzierten Gonaden involviert sind erlangen. Bei Fischen zeigt sich besonders ein breites Spektrum an Vielfältigkeit bei den Mechanismen der Geschlechtsbestimmung. Doppelgeschlechtigkeit (Zwitterwesen) oder getrennte Geschlechter und die Geschlechtsbestimmung in Abhängigkeit von Umweltfaktoren bis hin zur genetischen Bestimmung des Geschlechtes existieren. Hormone und abiotische Faktoren, wie Temperatur und pH-Wert, beeinflussen die Entwicklung der Echten Knochenfische (Teleostei) und deren Fortpflanzung. Diese Faktoren unterliegen besonders dem Einfluss durch Umweltverschmutzung oder der Veränderung des Klimas. Die Echten Knochenfischen sind mit ungefähr 25000 Arten die artenreichste Gruppe der Wirbeltiere und somit ein geeignetes Forschungsobjekt für die Untersuchung der Entwicklungsprozesse im Verlauf der Geschlechtsbestimmung und Geschlechtsdifferenzierung.

Kürzlich wurde im Medaka (*Oryzias latipes*), dem Japan-Reiskärpfling, das Gen *dmrt1bY* (auch als *dmy* bezeichnet), einem Mitglied der *Dmrt* Gen-Familie, als männliches Geschlechtsbestimmungs-Gen identifiziert. *Dmrt1bY* ließ sich bisher nur in der nahverwandten Art *Oryzias curvinotous* isolieren und ist nicht in anderen Arten der Gattung *Oryzias*, wie z.B. in *Oryzias celebensis*, vorhanden. Somit stellt *dmrt1bY* nicht das generelle geschlechtsbestimmende Gen in den Echten Knochenfischen dar.

Im Rahmen meiner Doktorarbeit habe ich die Gonadenentwicklung bei *O. latipes* und der nahverwandten Art *Oryzias celebensis* untersucht. Die Keimzell-Spezifizierung ist bei *O. latipes* vermutlich abhängig von maternal zytoplasmatisch gespeicherten Komponenten, dem so genannten Keimplasma. *Nanos* und *vasa* sind keimzellspezifische Gene, deren Transkripte maternal für die frühe embryonale Entwicklung bereitgestellt werden. Eine gleichmäßige Verteilung der *nanos* mRNA während der frühen Embryogenese konnte ich beim Medaka nachweisen. Dies wurde auch schon für *olvas* Transkripte beschrieben. Die Verteilung von *nanos* und *vasa* mRNAs im Zebrafisch ist hingegen asymmetrisch. Dies lässt vermuteten, dass zwischen Zebrafisch und Medaka Unterschiede in der Keimzell-Spezifizierung bestehen.

In *O. celebensis* konnte ich des Weiteren *vasa* isolieren und mittels *in-Situ*-Hybridiserung dessen keimzell-spezifische Expression nachweisen. *Vasa* lässt sich in *O. celebensis* folglich als Keimzell-Marker verwenden.

Die Untersuchung der Keimzellwanderung in *O. celebensis* zeigte hohe Ähnlichkeiten zu der bereits Beschriebenen im Medaka. Die Keimzellwanderung in Wirbeltieren ist abhängig von stromal cell-derived factor 1 (Sdf-1), einem chemotaktisch wirkendem Zytokinin. Im Medaka existieren zwei *sdf-1* Gene, *sdf-1a* und *sdf-1b,* die während der embryonalen Entwicklung im Seitenplattenmesoderm (LPM) exprimiert werden. Die Expression der beiden Gene unterscheiden sich jedoch zeitlich und auch örtlich im LPM. Dies lässt vermuten, dass sich im Verlauf der Evolution eine frühe und eine späte keimzellspezifische Funktion zwischen *sdf-1a* und *sdf-1b* aufgeteilt hat.

In „höheren" Wirbeltieren wurden schon verschiedene Gene, z.B. *Wt1*, *Sox9* und *Amh*, in dem Prozess der Gonadenentwicklung beschrieben. Die Expressionsmuster von *wt1* und *sox9* Co-Orthologen und *amh* habe ich während meiner Arbeit untersucht. Im Medaka und in *O. celebensis* wird *wt1a* im LPM transkribiert und ähnelt der von *sdf-1a* im Medaka. Die Expression von *wt1b* erfolgt hingegen nur in der Region der Vorläufer-Niere. Im weiteren Verlauf der Embryogenese ließen sich *wt1a* Transkripte erstmalig in somatischen Zellen des Gonaden-Vorläufers nachweisen. *Wt1a* spielt vermutlich eine Rolle in der Entwicklung der bipotentialen Gonade. Die funktionelle Analyse von *wt1* Genen im Medaka zeigte, dass durch eine konditionale Co-Regulation zwischen *wt1a* und *wt1b* die Keimzellen überleben bzw. erhalten bleiben.

Die Expression von *sox9b* im Medaka und in *O. celebensis* ließ sich in somatischen Zellen des Gonaden-Vorläufers nachweisen. Zusätzlich werden *amh* und *amhrII* ebenfalls in somatischen Zellen beider Geschlechter exprimiert, daher kann man eine wichtige Rolle dieser Gene während der Gonadenentwicklung und in der adulten Gonade annehmen.

Die Expression von *dmrt1* in *O. celebensis* konnte ich, in etwa der Hälfte der beobachteten Embryonen, bereits schon früh in der embryonalen Entwicklung (6 Tage nach der Befruchtung) nachweisen. Das Transkriptionsmuster von *dmrt1* in *O. celebensis* ist ähnlich der Expression von *dmrt1bY* im Medaka. Inwieweit diese Expression in *O. celebensis* spezifisch für Männchen ist wird zurzeit noch untersucht.

Die erhaltenen Ergebnisse zeigen neue Einblicke in die Genexpressionsmuster der Gonadenentwicklung von Medaka und *O. celebensis* und weisen neue Möglichkeiten für weitere Forschungen auf. Des Weiteren konnte ich im Verlauf der Gonadenentwicklung keine Unterschiede in der Genexpression von *wt1a* und *sox9b* zwischen Medaka und *O. celebensis* nachweisen. Dies deutet an, dass die genetischen Mechanismen der Gonadenentwicklung zwischen den beiden nahverwandten Arten sehr ähnlich sind.

Summary

The process of sex-determination can be better understood through examinations of developing organs and cells, which are involved in the formation of undifferentiated gonad. This mechanisms show in fish a broad variety, ranging from hermaphroditism to gonochorism and environmental to genetic sex determination. Hormones and abiotic factors such as temperature and pH can influence teleost development and reproductive traits. These factors are vulnerable to pollutants and climate changes. Therefore, it is important to examine gonad development and sex-determination/differentiation in teleost fish. Teleost fish are the largest known group of vertebrates with approximately 25,000 species and are used for such kind of examinations as model organisms.

Recently, in *Oryzias latipes* (medaka), *dmrt1bY* (or *dmy*), a member of the *Dmrt* gene family, has been described as testis-determining gene. However, this gene is not the universal master sex-determining gene in teleost fish. Although *dmrt1bY* is present in the most closely related species of the genus, namely *Oryzias curvinotous*, it is absent from other *Oryzias* species, like *Oryzias celebensis*, and other fish.

During my thesis, I studied gonad development in medaka and in the closely related species *Oryzias celebensis*. Germ cell specification in medaka seems to be dependent on maternally provided cytoplasmatic determinants, so called germ plasm. *Nanos* and *vasa* are such germ cell specific genes. In zebrafish they are asymmetrically localized in the early embryo. I have shown that *nanos* mRNA is evenly distributed in the early embryo of medaka. A similar pattern has been already described for the medaka *vasa* homolog, *olvas*. This suggests differences in PGC specification in zebrafish and medaka. Further, the *vasa* homolog was isolated and the expression pattern examined in *O. celebensis*. The results show that it can be used as a germ cell specific marker. Additionally, the primordial germ cell migration in *O. celebensis* was followed, which is similar to medaka PGC migration.

Primordial germ cell migration in vertebrates is dependent on the chemokine stromal cell-derived factor 1 (Sdf-1). Medaka has two different *sdf-1* genes, *sdf-1a* and *sdf-1b*. Both genes are expressed in the lateral plate mesoderm (LPM). During late embryonic development, I could show that *sdf-1a* is expressed in newly formed somites and not longer in the LPM. *Sdf-1b* expression persisted in the posterior part of the lateral plate mesoderm in the developing gonad. In terms of early and late functions, this suggests subfunctionalization of *sdf-1a* and *sdf-1b*.

In “higher” vertebrates, genes that are involved in the process of gonad development have been studied in detail, e.g. *Wt1*, *Sox9*, and *Amh*. I have analyzed the expression pattern of *wt1* and *sox9* co-orthologs and *amh*. In both, the medaka and *O. celebensis*, *wt1a* transcripts were localized in the LPM and its expression was similar to *sdf-1a* gene expression in medaka. *Wt1b* expression was restricted to the developing pronephric region. During later embryonic development, *wt1a* is specifically expressed in the somatic cells of the gonad primordium in both sexes. This is the first time that in fish *wt1* gene expression in developing gonads has been described. Therefore, this result suggests that *wt1a* is involved in the formation of the bipotential gonad. Furthermore, I have analyzed the gonad specific function of the *wt1* co-orthologs in medaka. I could show that a conditional co-regulation mechanism between Wt1a and Wt1b ensures PGC maintenance and/or survival.

The expression of *sox9* genes in medaka and *sox9b* in *O. celebensis* were detected in the somatic cells of the gonad primordium of both sexes. Additionally, I have shown that *amh* and *amhrII* in medaka are expressed in somatic cells of the gonad primordium of both sexes. This suggests that *sox9b*, *amh* and *amhrII* are involved in gonad development and have specific functions in the adult gonad.

In *O. celebensis* I could detect an expression of *dmrt1* already six days after fertilization in half of the embryos, which is similar to the *dmrt1bY* expression in medaka. Whether the expression of *dmrt1* is male specific in *O. celebensis* is currently under investigation.

Altogether, the obtained results provide new insights into gene expression patterns during the processes of gonad development. Furthermore, no differences in the expression pattern of *wt1a* and *sox9b* during gonad development between the medaka and *O. celebensis* could be detected. This might indicate that the genetic mechanisms during gonad development are similar in both species.

1. Introduction

One of the biggest investments of the biology of animals and plants is dedicated to reproduction and sex. In all sexually reproducing organisms, the embryo develops from the zygote, which is usually generated by the fusion of a male and a female gamete at fertilization. Nevertheless, how are male and female gametes determined during development? In sexual reproducing animals, a fundamental distinction is between germ cells and somatic cells, which together form the gonad in male and female into testis and ovary, respectively. Thus, germ cell specification and sex determination are key processes in gonad development. The following chapters are mainly focused on gonad development in vertebrates.

1.1. Gonad development

Ovary and testis can develop from a single primordium and therefore this process of gonad development is an interesting model system to study organogenesis. The process of gonad development can be divided into two phases. It begins with the formation of the indifferent gonad, which is identical in males and females. The next phase is the development of a testis or an ovary, which is triggered by a process called sex determination. As already mentioned the gonad consists of two major cell lineages, germ cells and the somatic gonadal mesoderm that surrounds the germ cells. Germ cell specification is dependent on either germ cell determinants or inductive signals. However, both cell lineages are important for gonad development.

1.1.1. Germ cell specification and migration

Primordial germ cells (PGCs) are usually established early during embryonic development. Two modes of PGC specification have been described: The first one is the inheritance of germ cell determinants, which are already present in the oocyte, like in *Caenorhabditis elegans* and *Drosophila*. The determinants are assembled in the so called germ plasm that contains unique cytoplasmatic organelles and is asymmetrically provided to the egg (Houston and King 2000; Rongo et al. 1997; Seydoux and Strome 1999). In *C. elegans* the germ lineage is separated from the somatic lineages through a series of four asymmetric divisions and thereby the germ plasm ends up in one daughter cell (P-cell). In Drosophila, the components of the germ plasm are already assembled at the posterior pole of the oocyte during oogenesis. During the initial stages of embryogenesis, the fly embryo divides by nuclear rather than cellular divisions. Those nuclei that enter the germ plasm become PGCs (Santos and Lehmann 2004a). In an alternative mechanism, PGCs are formed in response to inductive signals, as probably in all mammals. Here, no germ plasm has been identified and specification of germ cells occurs

relatively late in embryonic development. In mice it is initiated by signals that induce expression of Blimp1, a key regulator of the germ cell, in a few cells and thereby represses in these cells the somatic program and promotes the germ cell fate (Hayashi et al. 2007).

Generally, PGCs develop at some distances from the prospective gonad region and then have to migrate through diverse tissues until they reach it. In recent years several genes have been identified which are essential for germ line development and are evolutionary conserved, as nanos and vasa. Nanos is an RNA binding zinc finger protein that was first identified in Drosophila. Its function is not required for PGC specification. However, nanos-deficient PGCs develop abnormally and fail to incorporate into the gonad. In zebrafish, *nanos* is required for the migration of PGCs and for the maintenance of the germ line (Koprunner et al. 2001). The *vasa* gene encodes a DEAD-box RNA helicase and has been identified in *Drosophila* as maternal effect gene, which is required for germ cell specification (Hay et al. 1988; Schupbach and Wieschaus 1989). Its vertebrate homologue is also expressed in the germ line. In zebrafish and chicken *vasa* can be detected from the two-cell stage onwards and thereby the germ line can be followed throughout development (Knaut et al. 2000; Tsunekawa et al. 2000). In mice zygotic *vasa* is not expressed until PGCs begin to colonize the somatic gonadal mesoderm.

Genetic analyses in zebrafish (*Danio rerio*) have revealed new insights into the mechanisms that guide germ cells to the embryonic gonad in vertebrates. Zebrafish PGCs are specified in four clusters at the embryo's vegetal margin (Fig. 1). During gastrulation, the four clusters move dorsally and align with the anterior and lateral trunk mesoderm. At each side of the embryo two clusters group and migrate to the posteriorly located gonad primordium (Raz 2002).

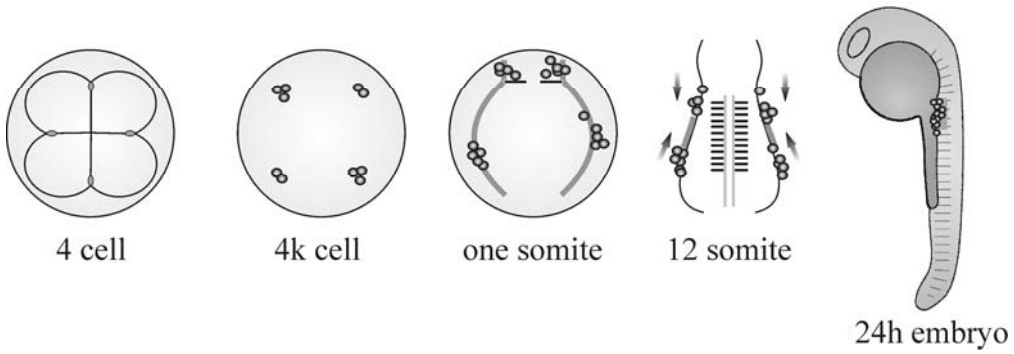


Fig. 1: Schematic summary of PGC specification and migration in zebrafish. At the four-cell stage the germ plasm containing *nanos1* and *vasa* RNAs, can be detected along the first two cleavage planes (arrowheads). In the 4k-cell stage, PGCs (grey dots) have been specified in four clusters and begin to divide. PGCs move dorsally and align with the anterior and lateral trunk mesoderm. On both sides of the developing embryo PGCs migrate (arrows) along the Sdf-1a localization (grey lines). The Sdf-1a/Cxcr4 activity mediates guidance and PGCs cluster in the gonad primordium. Modified from Raz, 2003.

Recent studies in mouse, chicken and fish have shown that the PGC migration is guided by the chemokine stromal cell-derived factor 1 (*sdf-1*), which is expressed in somatic cells along the migration pathway. The receptor of Sdf-1, *cxcr4* is expressed in germ cells (Ara et al. 2003; Knaut et al. 2003; Kurokawa et al. 2006; Molyneaux et al. 2003; Stebler et al. 2004). Additionally the Hydroxymethylglutaryl coenzyme A reductase (HMGCoAR), a key enzyme in cholesterol biosynthesis, has been shown to be required for PGC migration in Drosophila and zebrafish (Santos and Lehmann 2004b; Thorpe et al. 2004).

1.1.2. Formation of the bipotential gonad

The details of the cellular processes during the formation of the gonad primordium vary among vertebrates. In mammals, the indifferent gonads arise as paired structures within in the intermediate mesoderm, which is known as the urogenital ridge. Sexual development is coupled with kidney development as the gonad arises from the central region of the urogenital ridge, the mesonephros (Gilbert 2006). In mice, the gonad emerge on the ventromedial surface of the mesonephros at around 10.5 day post coitum (dpc) and can be stained with antibodies against laminin and E-cadherin but so far no gonad specific function has been described for these molecules (Upadhyay et al. 1981; Wilhelm et al. 2007). Within the gonad primordia, the male and female duct systems are already present. The Wolffian ducts are the progenitors of the male duct system. Closely attached to the Wolffian ducts is female ductual system, the Müllerian duct. In mammals, depending on the sex, only one of the two duct systems is going to develop further (Gilbert 2006).

Genes, which are important for the formation of the indifferent gonad, have been identified mostly by mutation analysis in mice and humans. One of the genes is the Wilms' tumor suppressor 1 (*Wt1*), which is widely expressed throughout the urogenital ridge including kidney and gonad (Fig. 2). Wt1 encodes a zinc finger transcription factor that is inactivated in a subset of Wilms' tumors (Gessler et al. 1990; Klamt et al. 1998). Mutations in WT1 in humans lead to anomalies of kidney and gonad development and cause a variety of syndromes (Barbaux et al. 1997; Gessler et al. 1990; Klamt et al. 1998). During gonad development, *Wt1* is expressed in the coelomic epithelial cell layers and somatic gonadal precursors. In $Wt1^{-/-}$ mutant mice the gonadal primordium can be observed until 11dpc. Thereafter it degenerates by increased apoptosis (Kreidberg et al. 1993). WT1 can function as a transcriptional activator as well as a repressor. The complexity of WT1 functions includes that the *wt1* gene encodes several protein isoforms. Of particular interest is the evolutionary conserved alternative splice

site that results in the insertion or exclusion of three amino acids (KTS) between zinc fingers three and four (Kent et al. 1995).

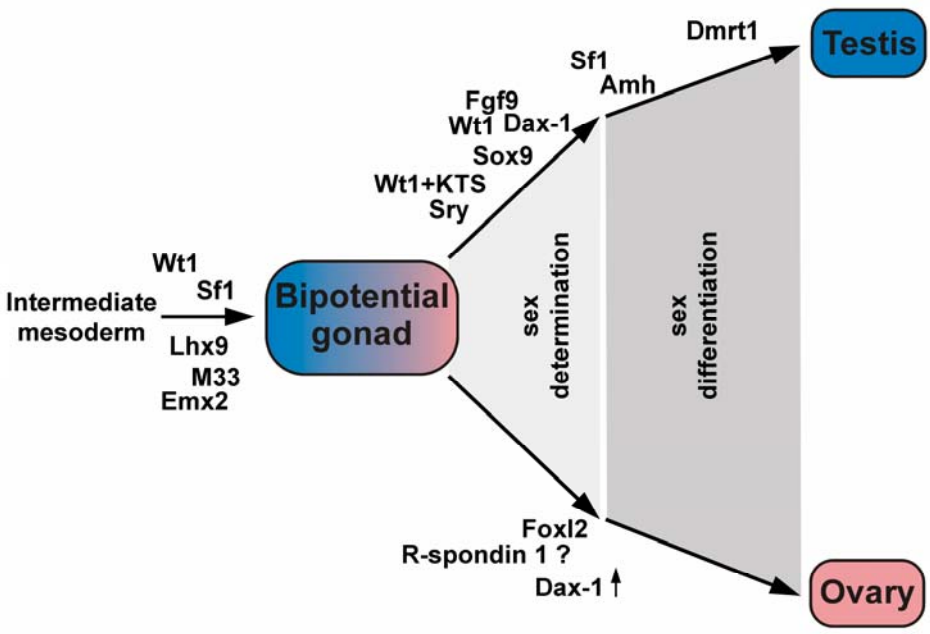


Fig. 2: Bipotential gonad formation and sex determination in mammals. Several genes are known to be necessary for the early bipotential gonad development (*Wt1*, *Sf1*, *Lhx9*, *M33*, and *Emx2*). *Wt1*+KTS is involved in the activation of *Sry* expression in the XY gonad. *Sry* is the male sex determining gene and XY gonad follows testis differentiation. During the early steps of testis development *Sox9* expression is initiated. *Fgf9* and *Dax-1* are co-expressed together with *Sry*, *Sox9*, and *Wt1* in pre-Sertoli cells. *Sox9* is involved in the activation of Amh that leads to the degeneration of the Müllerian duct. Downstream pathways promote the testis development, including *Dmrt1*. In the XX gonad, *Dax-1* is much higher expressed than in XY gonad. One of the first female specific genes that are expressed in XX gonads is *Foxl2*. *R-spodin-1*, at least in humans, is suggested to be involved in female gonad development and maybe the female-determining gene.

The complexity of WT1 functions includes that the *wt1* gene encodes several protein isoforms. Of particular interest is the evolutionary conserved alternative splice site that results in the insertion or exclusion of three amino acids (KTS) between zinc fingers three and four (Kent et al. 1995). Studies in mammalian systems revealed that the + KTS and – KTS isoforms serve distinct functions within the nucleus and differ in theirs binding affinity to nucleic acids. – KTS isoforms are generally more active in transcriptional regulation, whereas + KTS products have been shown to co-localize with splicing factors (Haber et al. 1991; Hammes et al. 2001). In male and female gonads of mice, which lacked the –KTS isoform, had reduced size and were poorly differentiated. Mice lacking +KTS isoforms showed a complete male to female sex reversal, which thereby highlights the important function in sex determination (Hammes et al. 2001).

Another gene is the steroidogenic factor 1 (*Sf1*) which has been shown to be activated by WT1(-KTS) in vitro (Wilhelm and Englert 2002). *Sf1* is expressed in the developing gonad,

hypothalamus, and the pituitary gland indicating an essential role in the development of the hypothalamic-pituitary-gonadal axis. Sf1-mutant mice show gonads that arrest in the indifferent stage. Additional genes, which have been postulated to be involved in the formation of the bipotential gonad in mammals, are *Lhx9*, *Emx2*, and *M33* (Fig. 2).
In other vertebrates like teleosts fishes, somatic cells of the gonadal anlagen form without a significant contribution from the mesonephros-equivalent tissue (pronephros and interregnal organ). The gonad of teleosts fishes may represent an evolutionary prototype for that of the vertebrate gonad. Genes that are involved in these processes are discussed later in this chapter.

1.2. Sex determination

Most sexual organisms have two sexes. In many cases, genes on sex chromosomes determine sex [genotypic sex determination (GSD)]. GSD occurs in different constitutions. Types of the GSD are the XX/XY male heterogamety, as in mammals, and the ZZ/ZW female heterogamety, as in birds. Additionally, the X0 sex-determination system, a variant of the XX/XY system, is observed in certain invertebrates including *Drosophila* and *C. elegans*. Females have two copies of the sex chromosome (XX) but males have only one (X0) in *Drosophila*. Whereas in *C. elegans* XX is a hermaphrodite and the X0 is a male. Reptiles, amphibians, and fish exhibit different mechanisms of sex determination including GSD of both XX/XY and ZZ/ZW systems and variations thereof, environmental sex determination such as temperature-dependent sex determination (TSD) like in reptiles, or social variables (the size of an organism relative to other members of its population) (Charlesworth 2002).

1.2.1. Sex determination in mammals

Most mammals have an XX/XY sex determining system and the key components of the sex-determining cascade have been identified, albeit their position in the hierarchy and their modes of interactions are still under investigation. The genetic sex of mammals is established at fertilization. The development of testes is associated with a single Y-linked gene locus, named as testis determining factor (TDF) in humans and Tdy in mice. The search for the TDF or Tdy in conserved sequences on the Y chromosomal parts has lead to the identification of the male sex-determining gene, SRY. Evidences came from mice in which Sry is deleted in XY female mice and transgenic XX male mice carrying a genomic fragment containing the *Sry* gene (Gubbay et al. 1990; Koopman et al. 1990). In mice, the indifferent stage of the gonad changes rapidly at around 10.5 dpc, when *Sry* is expressed in the center of XY genital ridges. *Sry* encodes a member of the high mobility group (HMG)-protein family. This nuclear protein family is characterized by their HMG-box, a DNA-binding domain, and are grouped

into two classes. HMG-1 and HMG-2 are members of the first class and bind to DNA in a sequence-independent manner. SRY belongs to the second class and these proteins bind DNA sequence specifically. SRY binds via the HMG-box to the minor groove of the DNA and induces a sharp bend. Interestingly, all mutations found in SRY that causes male to female sex reversal are located within the HMG box (Harley et al. 1992; Jager et al. 1992; Pontiggia et al. 1994; Schmitt-Ney et al. 1995). The regulation of *Sry* expression has to be tightly controlled. Mice with a "weak" *Sry* allele, that expresses *Sry* too late, show ovotestis formation. Additionally, *Sry* expression has to reach a certain threshold to induce testis development (Bullejos and Koopman 2005; Eicher 1982; Nagamine et al. 1999). Recently, WT1 (+KTS), GATA4/FOG2 and the insulin receptor family have been implicated in the transcriptional or post-translational regulation of Sry. All null mutants of these mouse models show a reduced *Sry* expression to about 25% of wild-type levels, resulting in XY sex reversal (Hammes et al. 2001; Nef et al. 2003; Tevosian et al. 2002). The question is still if these effects are direct or indirect.

Immediately after *Sry* expression, all known major autosomal genes of the sex determination pathway are expressed but the identification of a SRY target gene is still one of the biggest challenges in the field. One of the best candidates up to date is *Sox9*, a member of the Sry-related homeobox (SOX) family of transcription factors. SOX9 plays an essential role in cartilage development and is sufficient for male sexual development as XX mice over-expressing *Sox9* develop as males but are sterile (Healy et al. 1999; Vidal et al. 2001; Wagner et al. 1994; Zhao et al. 1997). However, *Sox9* is expressed in the bipotential gonad of both sexes. Immediately after the onset of *Sry* expression, *Sox9* expression is upregulated in male gonad and downregulated in the female gonad (Kent et al. 1996; Morais da Silva et al. 1996). The expression of *Sox9* persists in the Sertoli cell lineage after birth, whereas *Sry* is just expressed until 12.5 dpc (Fig 2). SOX9 is important for the induction of anti-Müllerian hormone (*Amh*) gene expression (Arango et al. 1999). Amh is a glycoprotein and a member of the transforming growth factor-beta (TGF-beta) superfamily. During testicular differentiation, AMH is secreted by Sertoli cells. Binding to the AMH type II receptor (AMHRII) at the mesenchyme surrounding the Müllerian ducts results in the regression of the Müllerian ducts (Roberts et al. 2002). Male $Amh^{-/-}$ mice retain their Müllerian ducts but develop normal testis, which indicates that Amh has no essential role during male development (Behringer et al. 1994). Additionally, the downstream pathway of male sex determination includes several other genes. *Ffg9* and *Dax-1* have been identified to be expressed together with *Sry*, *Sox9*, and *Wt1* in pre-Sertoli cells. Thus leads to the differentiation of Sertoli cells that associate with

germ cells and nurture their development into sperm. These downstream events in testis determination and differentiation have been suggested to occur probably in all mammals (Wilhelm et al. 2007).

Remarkably, four mammalian species, so far, have been identified that determine male sex without the *Sry* gene. In two species of the mole vole (*Ellobious lutescens* and *E. tancrei*) and in two subspecies of the Japanese spinous country rat (*Tokudaia osimensis osimensis* and *Tokudaia osimensis spp.*) male have functional testes but do not have an apparent Y chromosome or the Sry in their genome (Fredga 1983; Sutou et al. 2001). Interestingly, other species of *Ellobious* and *Tokudaia* seem to express *Sry*.

A puzzling phenomenon in mammalian sex determination is female-to-male sex reversal. A recent study has identified mutations in *R-spondin 1* in XX males. This finding might place R-spondin 1 in the exceptional position of being the female-determining gene in mammals (Parma et al. 2006; Wilhelm 2007). The role of *R-spondin 1* during sex determination has to be investigated.

1.2.2. Sex determination in fish

Interestingly, orthologous genes that are downstream of SRY in the mammalian sex determination cascade have been identified in non-mammalian vertebrates. However, nonmammalian vertebrates have a tremendous variety of sex determination mechanisms, especially in fish. Sex determination in fish is a very flexible process in terms of evolutionary patterns. In fish species, all kinds of sex determination systems have been observed, ranging from hermaphroditism to gonochorism and from environmental to genetic sex determination (for review see (Devlin and Nagahama 2002)). Fish are the largest known group of vertebrates with approximately 25,000 species (Nelson 1994). It is possible that among genera and families sex determination varies and almost all-different forms of genetic sex determination can be found in fish, including XX/XY male heterogamety, ZZ/ZW female heterogamety, and multiple sex chromosomes. Sex chromosomes are found in approximately 10% of fish species examined to date. Sex-linked phenotypic traits and protein and molecular markers have been identified in several fish systems (Devlin and Nagahama 2002). Unlike in mammals, several fish species are known to be hermaphrodites. These fishes become male and female at the same time (synchronous), are female first and become male subsequently (protogynous), or vice versa (protandrous). Age, social factors or temperature can control such a sex changing (Devlin and Nagahama 2002).

In zebrafish, no sex-linked markers and no sex chromosomes have been identified. During gonad development in juvenile zebrafish, all individuals first develop undifferentiated ovary-

like structures. In males, the oocytes all disappear from the gonads by 30 days post hatching (dph), and spermatocytes develop with testicular differentiation (Maack and Segner 2004; Takahashi 1977). This induced oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation has been recently described to be the effect of *amh* which possibly down-regulates *cyp19a1a*, a key enzyme in steriodogenesis that converts androgens to estrogens (Rodriguez-Mari et al. 2005; Wang and Orban 2007). Additionally, a role of PGCs during sex determination has been suggested. Germ cell depleted zebrafish embryos develop into phenotypically males (Slanchev et al. 2005). The detailed genetic program that is initiated after germ cell depletion is so far not known.

The platyfishes and swordtails of the genus *Xiphophorus* represent one of the extensively studied groups in fish and show diversity of genetic sex determination. In *Xiphophorus maculatus* three different sex chromosomes (W, X, and Y) have been identified and females have WY, WX or XX genotypes, whereas males are either YY or XY (Kallman 1984). The sex determining region on the X and Y chromosomes in *X. maculatus* are under investigation to isolate the sex determining gene(s) (Schultheis et al. 2006).

The teleost fish medaka (*Oryzias latipes*) has got a lot of attention for studying gonad development. The medaka is the first non-mammalian vertebrate in which a male sex-determining gene has been identified. This gene is named *dmrt1bY* or *DMY* (Matsuda et al. 2002; Nanda et al. 2002).

1.4. Sex determination in the medaka

The medaka is a small, egg-laying freshwater fish native to East Asia and has been established as a model organism for a wide range of biology, including ecotoxicology, carcinogenesis, developmental genetics and sex determination (for detailed review see (Wittbrodt et al. 2002)). The medaka genome size is about 700 megabases, which is half of the zebrafish genome, and the medaka genome draft is available at the ensembl database (http://www.ensembl.org) (Kasahara et al. 2007). The sex of medaka is genetically determined by a XX-XY male heterogametic system and crossing-over between the sex chromosomes is possible (Aida 1921). Classical genetic approaches and candidate gene analyses have led to the identification of *dmrt1bY* in a short region on the Y chromosome as a male sex determining gene (Matsuda et al. 2002; Nanda et al. 2002). In mammals, *Dmrt1* is a downstream gene in the cascade of genes involved in testis differentiation. In birds, the *Dmrt1* gene is located on the Z chromosome but is absent from the W, emphasize its possible function as a male sex determining gene (Nanda et al. 1999). *Dmrt1* (doublesex and Mab-3-

related transcription factor 1) is a putative transcription factor that contains a conserved DM-domain. The DM domain was originally described as a DNA-binding motif shared between *doublesex* (*dsx*) from Drosophila and *mab-3* from *C. elegans*, which are well-characterized as sex differentiation genes (Zarkower 2001). *Dmrt1bY* arose through a recent duplication event of the autosomal segment from the linkage group 9 (LG9), which contains Dmrt1 (Fig. 3). This process created a chromosomal region of 258 kb on one of the LG1 homologs which thereby became the proto-Y chromosome and the other without insertion the proto-X chromosome (Kondo et al. 2006; Kondo et al. 2004; Nanda et al. 2002). It has been hypothesized that all sex chromosomes originated from a pair of autosomes (Muller 1914; Ohno 1967). This duplication event that led to the formation of the *dmrt1bY* gene have occurred ~ 10 million years ago (mya). Compared to other vertebrates the medaka Y chromosome is the youngest Y known so far (Kondo et al. 2004).

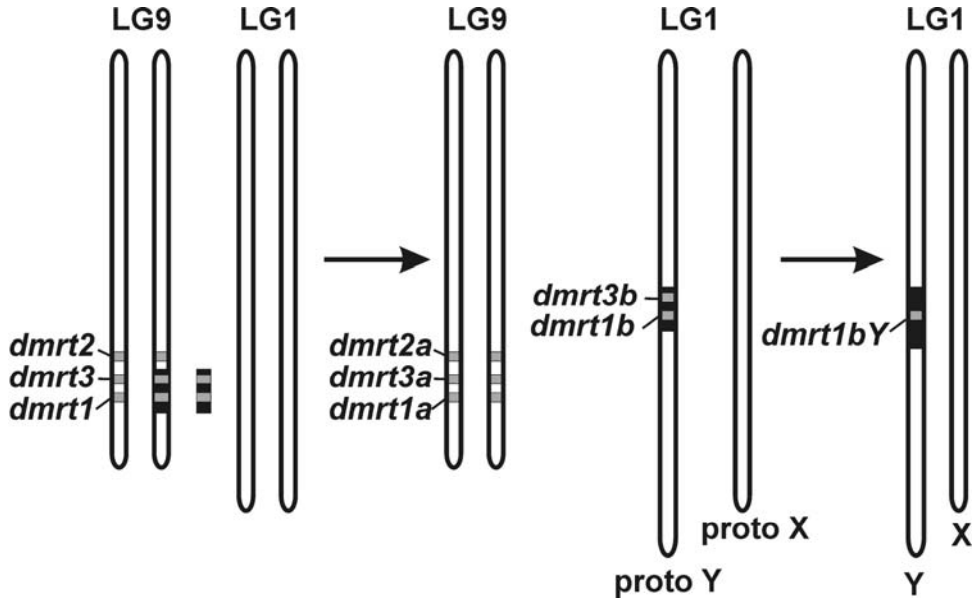


Fig. 3: Evolution of the medaka Y-chromosome. *Dmrt1bY* arose through a recent duplication event of a segment from LG 9, containing the *dmrt1* gene and some neighbouring genes like *dmrt3*. This process led to the insertion of the duplicated segment in one chromosome on LG1, which thereby became the proto-Y chromosome. Genes from the duplicated segment on the proto-Y chromosome except *dmrt1bY* get non-functional by accumulation of mutations. Modified from Schartl, 2004.

Dmrt1bY is also found in the most closely related species of the genus, namely *Oryzias curvinotous*, but is absent from other *Oryzias* species, like *Oryzias celebensis*, and other fishes. This suggests that *dmrt1bY* is a recently evolved sex determining gene specific to some species in the genus *Oryzias* (Kondo et al. 2003; Volff et al. 2003a). The involvement of *dmrt1bY* in the process of sex determination has been confirmed by the identification of several naturally occurring sex-reversed mutants, in which *dmrt1bY* was either truncated or expressed at reduced levels (Matsuda et al. 2002; Otake et al. 2006). This shows that a certain

threshold level of Dmrt1bY is needed to induce male development, which has been as well described for Sry in mammals (Bullejos and Koopman 2005; Eicher 1982; Nagamine et al. 1999). Recently, a gain-of-function approach has shown that a 117-kb genomic DNA fragment that carries *dmrt1bY* is able to induce testis differentiation and subsequent male development in XX medaka (Matsuda et al. 2007).

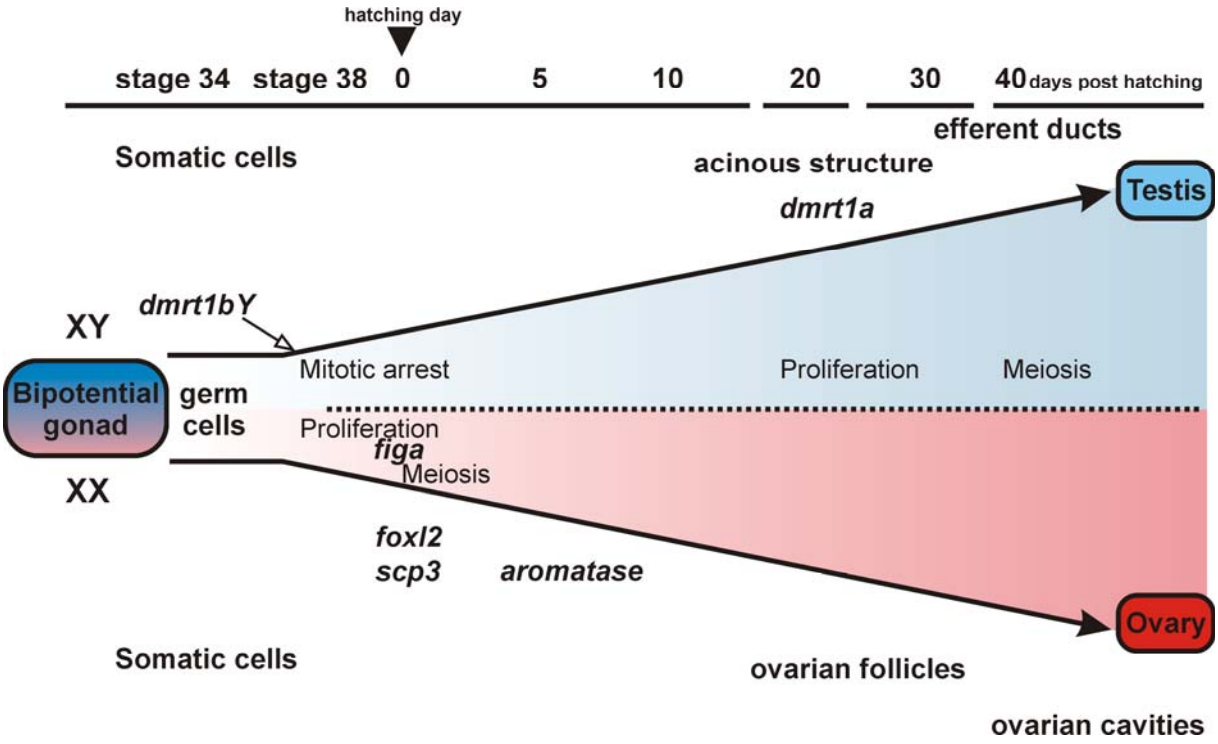


Fig. 4: Sex determination and differentiation in medaka. Sex-specific genes expressed in the XY and XX gonads are indicated in bold/italic letters. Morphological events in germ cells and somatic cells are depicted in the inner field and outer field, respectively. *DmrtbY* is involved in repressing germ cell proliferation in males (open arrow). Modified from Matsuda, 2005.

During gonad development, *dmrt1bY* is expressed specifically in the somatic cells surrounding the PGCs in the early gonad primordium in XY embryos, before morphological sex differences are visible (Kobayashi et al. 2004). The first morphological difference between males and females is the numbers of PGC around stage 38 (Hamaguchi 1982; Satoh and Egami 1972). After the PGC have reached the gonadal region in XX females the PGC start to proliferate, whereas in males the PGC arrest in mitosis (Kobayashi et al. 2004; Satoh and Egami 1972). So far two functions of *dmrt1bY* are thought. Firstly, *dmrt1bY* is involved in repressing the germ cell proliferation in XY embryos, which have been shown in the *dmrt1bY* mutants and by loss-of- and gain-of-function studies (Matsuda et al. 2007; Paul-Prasanth et al. 2006). Secondly, *dmrt1bY* is necessary for Sertoli cell differentiation. In some XX recipient and XY donor chimeras of medaka have developed into males, suggesting that somatic cells induce sex reversal of the XX gonad (Shinomiya et al. 2002). During the period

of gonad development, several genes have been already described to be expressed in a sex specific manner (Fig. 4). *Foxl2* is a member of the winged helix/forkhead family of transcription factors and is known to be involved in ovarian development. In medaka *foxl2* is expressed in somatic cells surrounding germ cells of XX embryos around hatching stage when germ cells enter meiosis in XX gonads (Nakamoto et al. 2006). Additionally at that stage the synaptonemal complex protein 3 (*scp3*) is expressed in a female specific manner up to 50 days post hatching (dph) in the germ cells (Paul-Prasanth et al. 2006). During testis differentiation, *dmrt1bY* is permanently expressed in the Sertoli cell lineage and at 20dph *dmrt1bY* and the autosomal copy *dmrt1a* are co-expressed in the Sertoli cells. Commonly it is thought that genes trigger the sexual development and thereby initiate a genetic cascade but the primary signals vary among animals (Fig 5).

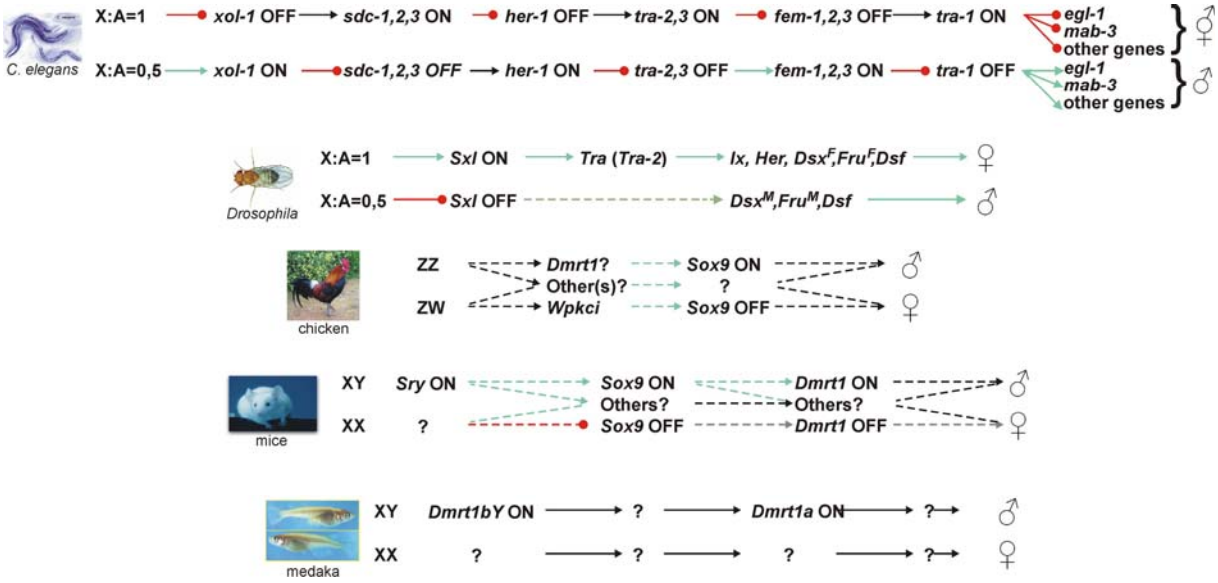


Fig. 5: Variation on top of primary signals in metazoan sex determination. The genetic pathways are less conserved between the animals with the exception of the DM genes *mab-3*, *Dsx* and *Dmrt1*. Green arrow, positive regulation; red arrow, negative regulation; grey arrow, conditions permissive for gene expression. Dotted line assumed regulatory relationship. Modified from Koopman and Loffler, 2003.

1.5. Whole genome duplication in teleost fish

Teleosts do not only show the greatest variety of SD-mechanisms, they are also peculiar with respect to other mechanisms generating genotypic variety. Half of all vertebrate species are teleost fish (Nelson 1994). Teleosts have diverged from the tetrapod lineage about 450 million years ago (Amores et al. 1998; Taylor et al. 2003; Wittbrodt J 1998). Their genome evolution and diversification are important subjects for the understanding of vertebrate evolution. Recent phylogenomic studies suggest that, during the evolution of vertebrates, several rounds

of whole genome duplications (WGD) have occurred. Especially in the teleost fish lineage it has been shown that, many genes in the genome are present as duplicates, while only one copy is present in tetrapods (Meyer and Van de Peer 2005). The teleost specific WGD is widely accepted and has occurred ~ 350 million years ago (Kasahara et al. 2007; Meyer and Schartl 1999; Meyer and Van de Peer 2005; Naruse et al. 2004). Susumu Ohno has postulated that gene and genome duplication are important mechanism for the evolution of phenotypic complexity, diversity, and the origin of novel gene functions (Ohno 1970). The teleost specific WGD has been suggested to be responsible for the diversification in the teleost lineage (Meyer and Van de Peer 2005; Postlethwait et al. 2004; Taylor et al. 2003).

In general, an entirely redundant copy is unlikely to be maintained in the genome for a long time and several fates of gene duplicates have been described including fates that are responsible for the maintenance of gene duplicates (Fig. 6). In most cases, one of the gene copies accumulates mutations, which render the gene non-functional (nonfunctionalization). Alternatively, the partitioning of the ancestral gene function between the gene duplicates can occur (subfunctionalization). The third possibility is that one gene acquires a new advantageous function (neofunctionalization). The latter two scenarios are associated with the maintenance of gene duplicates during evolution (Bullejos and Koopman 2005; Force et al. 1999; Lynch and Force 2000; Taylor and Raes 2004). Additionally, neofunctionalization can follow subfunctionalization. This has been described as subneofunctionalization (He and Zhang 2005; Rastogi and Liberles 2005).

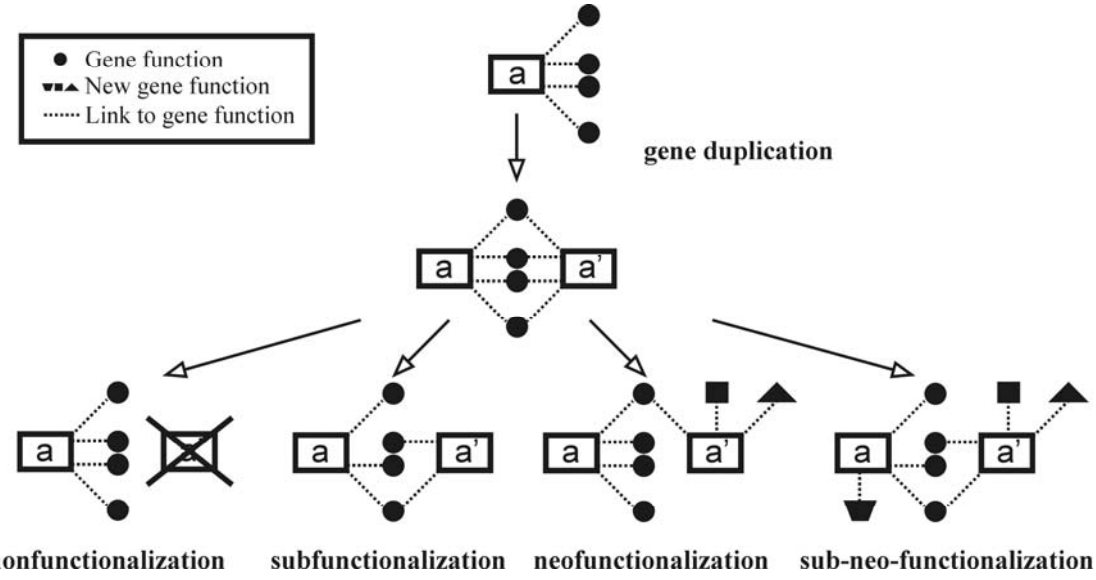


Fig. 6: Evolutionary fates of duplicated genes. Duplicate genes are depicted by open boxes and different gene functions are shown by solid circles. New gene functions that have been acquired are indicated by solid geometric symbols. Dotted lines link genes with their functions.

For genes that are involved in the process of gonad development in higher vertebrates duplicated genes have been identified in teleost fish species. For example, gene duplicates of *sdf-1*, *sox9* and *wt1* have been described in teleosts (Bollig et al. 2006; Chiang et al. 2001; Cresko et al. 2003; Klüver et al.; Klüver et al. 2005; Nakamoto et al. 2005). Additionally, in recent studies, *amh* has been identified in fish and no gene duplicate is present in the genome (Klüver et al. 2007; Miura et al. 2002; Rodriguez-Mari et al. 2005; Yoshinaga et al. 2004). This suggests that after the teleosts specific WGD one copy of the duplicated genes accumulated mutations and became non-functional. In teleosts *amh* expression is restricted to the somatic cells of the gonad (Klüver et al. 2007; Miura et al. 2002; Rodriguez-Mari et al. 2005; Yoshinaga et al. 2004). Compared to mammals, teleost fish do not have a Müllerian duct, which suggests *amh* in teleosts has different functions. Only basal ray-fin fish, like sturgeon, develop Müllerian ducts and these do not degenerate in males (Wrobel 2003). However, the involvement of these genes in gonad development in teleosts is not understood.

1.6. Aim of the PhD thesis

While the sex determining genes are known in mammals, *Drosophila* and *C. elegans* little is known in other animals. In medaka, the genetic cascade of sex determination has yet not been clarified, whereas the male sex-determining gene, *dmrtbY*, has been identified. Therefore, medaka is a well-suited model organism to study sex determination in fish and to analyze genes that are involved in the process of gonad development. The aim of my PhD thesis was to identify and analyse genes in medaka that are involved in gonad development in higher vertebrates and to compare the findings with the situation in other vertebrates, including teleosts. Of particular interest is the situation of *dmrt1bY* that became the male sex determining gene in medaka, whereas in other vertebrates *dmrt1* is a downstream factor. In *Oryzias celebensis*, a closely related species, *dmrt1bY* is absent. Therefore, I additionally analyzed genes during gonad development in *O. celebensis* and compared these findings with results of medaka to elucidate the genetic mechanism of testis development in these two closely related species. Through my studies I wanted to increase the knowledge about the function (and malfunction) of genes involved in gonad development. I also wanted to contribute to an understanding of the evolution of sex determination systems, which present an amazing variety of mechanisms, especially in fish.

[1]own contributions are underlined and listed in Appendix B

2. Results and Discussion

2.1. Primordial germ cell specification and migration in the medaka *Oryzias latipes*

2.1.1. Primordial Germ cell specification

Critical to our understanding of sex-determination processes, the formation of the gonad primordium is the first step towards gonad determination and subsequent sex differentiation. The precursors of the somatic cells originate from cells of the developing gonadal primordium. The germ cells derive from a very particular and specialized cell-type, the PGCs. Interestingly, in the medaka the first morphological difference between the sexes is the number of PGCs. Females show an increased PGC proliferation around stage 38, however, in males the PGC are mitotically arrested (Kobayashi et al. 2004; Satoh and Egami 1972). Later in development PGC give rise to gametes that ensures sexual reproduction of the animal. During development the PGCs in medaka express *olvas*, the medaka *vasa* homolog, at late gastrulation and start to migrate to the gonadal region through the lateral plate mesoderm (LPM) (Shinomiya et al. 2000). The *olvas* mRNA is maternally deposited and the transcripts are uniformly distributed (Herpin et al. 2007; Shinomiya et al. 2000). The zygotic *olvas* expression starts around early blastula (Tanaka et al. 2001). During late gastrulation, some cells located around the posterior region embryonic shield seem to retain or increase *olvas* expression while in other cells *olvas* transcripts diminish (Herpin et al. 2007). However, in zebrafish the *vasa* mRNA is enriched at the marginal positions of the first two cleavage planes and this suggest that the zebrafish PGC specification appears to be depend on inheritance of germ plasm in which several RNA molecules reside (Raz 2002). This raises the question if teleost fishes follow different or the same modes of PGC specification.

Recently, it has been shown by duplicating the medaka embryonic axis with the injection of β-catenin mRNA into one marginal blastomere at the eight- or 16-cell stage the average numbers of PGCs in the embryos were just as in control embryos (Herpin et al. 2007). Additionally, this study confirmed no differences in PGC numbers by disturbing early axial mesoderm specification through BMP pathway modulation. These results indicated that PGCs are not specified in response to inductive signals. *Nanos* and *olvas* germ cell specific RNAs are maternally deposited. In particular, I have shown that during early stages of embryonic development medaka *nanos* mRNA was evenly distributed and did not aggregate at the first two cleavage planes (Fig 7). This has also been shown for *olvas* transcripts (Herpin et al. 2007). This is in contrast to zebrafish *vasa* mRNA localization (Herpin et al. 2007; Yoon et al.

1997). The mRNA localization of *nanos* and *olvas* did not provide direct support for medaka PGC specification through inheritance of germ plasm.

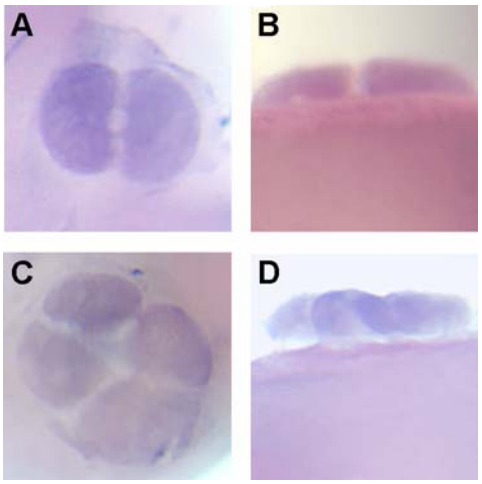


Fig. 7: *Nanos* mRNA localization during medaka embryonic development. (A, B) *Nanos* mRNA localization at the two-cell stage. **(C, D)** *Nanos* mRNA localization at the four-cell stage.

Evidences for the mode of PGC specification through maternally provided material came from the identification of germ plasm like structures in the early medaka embryos, which were observed in the cytoplasm of the four-cell stage. The presumptive germ plasm structure was composed of irregularly shaped amorphous inclusion with a granular fine structure (Herpin et al. 2007) as it is described for zebrafish (Knaut et al. 2000). Further, different GFP-fusion constructs were injected into one- to two-cell stage medaka embryos and detected that the zebrafish vasa-GFP-fusion RNA was stabilized in the PGCs. Additionally, this was also the case for the GFP-*zfnos* 3'UTR RNA. However, injecting GFP-*zfvasa* 3'UTR did not result in selective GFP expression or RNA stabilization but in zebrafish it becomes localized in the PGCs. Recently, in zebrafish it has been shown that specific regions in the *nanos* 3'UTR compensate for microRNA-mediated repression in PGCs and allow germline-specific expression. The repression is mediated via the microRNA (miRNA)-430 (Mishima et al. 2006). Several miRNAs have been identified in the medaka (Ason et al. 2006). So far, no similar miRNA mechanism in PGCs has been described in medaka but we can assume that it exists. As already mentioned above, in medaka, the olvas-GFP protein is rapidly restricted to the PGCs. On the contrary the olvas-GFP RNA was not preferentially localized in the PGCs. This suggests posttranscriptional mechanisms like enhanced translation, somatic inhibition of translation, or soma-specific protein destabilization. Altogether, this verifies possible species-variability in the mechanism of cis-acting RNA sequence elements or RNA-interacting protein sequences. It is likely that medaka and zebrafish use a similar conserved mechanism. In summary, we could show that very early during development in medaka, PGC specification

is dependent on the presence of a germ plasm regardless of surrounding somatic tissue induction and the germ line is already distinct from other cell lineages at early gastrulation stage.

2.1.2. PGC migration in medaka

In medaka PGC migration was first followed through *olvas* expression (Fig. 8). *Olvas* mRNA is maternally deposited and diminishes during late gastrulation (Fig. 8A-C). However, some cells located around the posterior region between stages 15 and 16 retain *olvas* transcription (Fig. 8D). At the four-somite stage (stage 20), germ cells migrate along the LPM and are detectable on both sides of the embryonic axis (Fig. 8E). Recent studies in zebrafish, chicken, mouse and in medaka have shown that PGC migration towards the developing gonad is guided by Sdf-1 and its receptor Cxcr4 (Ara et al. 2003; Doitsidou et al. 2002; Knaut et al. 2003; Kurokawa et al. 2006; Molyneaux et al. 2003).

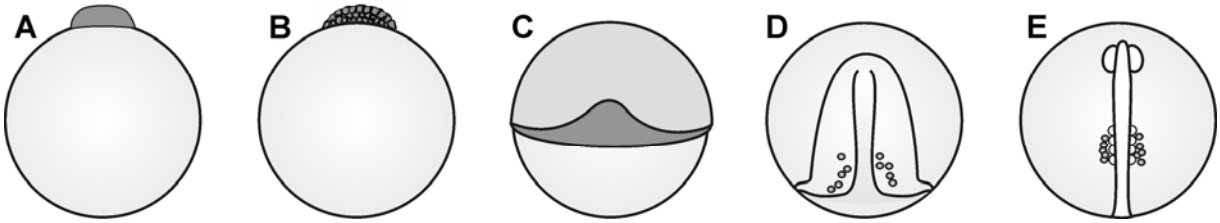


Fig. 8: Schematic illustration of *olvas* gene expression and PGC migration in medaka. (A) one-cell stage; **(B)** late blastula (stage 11); **(C)** early gastrulation stage (stage 13); **(D)** late gastrulation stage (stage 16); **(E)** four-somite stage (stage 20). *Olvas* expressing cells or regions are indicated in dark grey colour.

In medaka, *sdf-1a* is expressed at the onset of gastrulation, and *cxcr4* is maternal deposited. During embryonic development, *sdf-1a* is expressed in the marginal zone with progressive restriction of the expression to the dorsal region of this marginal zone. Knockdown of Cxcr4 in medaka revealed that PGC migration is dependent on Sdf-1/Cxcr4 interaction (Kurokawa et al. 2006). Interestingly, in medaka, in addition to *sdf-1a*, a second putative ligand for Cxcr4 has been described as *sdf-1* (Kurokawa et al. 2006; Yasuoka et al. 2004). I have analyzed their phylogenetic relationship to other Sdf-1 proteins of several vertebrates (Fig. 9A). Medaka Sdf-1a grouped into the Sdf-1b branch and Sdf-1 into the Sdf-1a branch, respectively. Therefore, I redefined the nomenclature of medaka Sdf-1a to Sdf-1b and Sdf-1 to Sdf-1a. Additionally, I compared the synteny of both *sdf-1a* and *sdf-1b* of medaka and zebrafish. *Sdf-1a* in medaka and zebrafish are located on linkage group LG15 and LG13, respectively, whereas *sdf-1b* in medaka was mapped to LG1 and zebrafish *sdf-1b* was located on LG22 (Fig. 9B). The LG 1 and 15 of medaka, and LG 13 and 22 of zebrafish showed strong synteny to each other, and to human chromosome 10 on which the human *SDF-1* is located (Fig. 9B).

This is strong support for the hypothesis that the *sdf-1* gene duplicates in teleost fish arose from the teleost specific WGD (Kasahara et al. 2007; Meyer and Schartl 1999; Meyer and Van de Peer 2005; Postlethwait et al. 2004). Consequently, *sdf-1* gene duplicates are clearly co-orthologs.

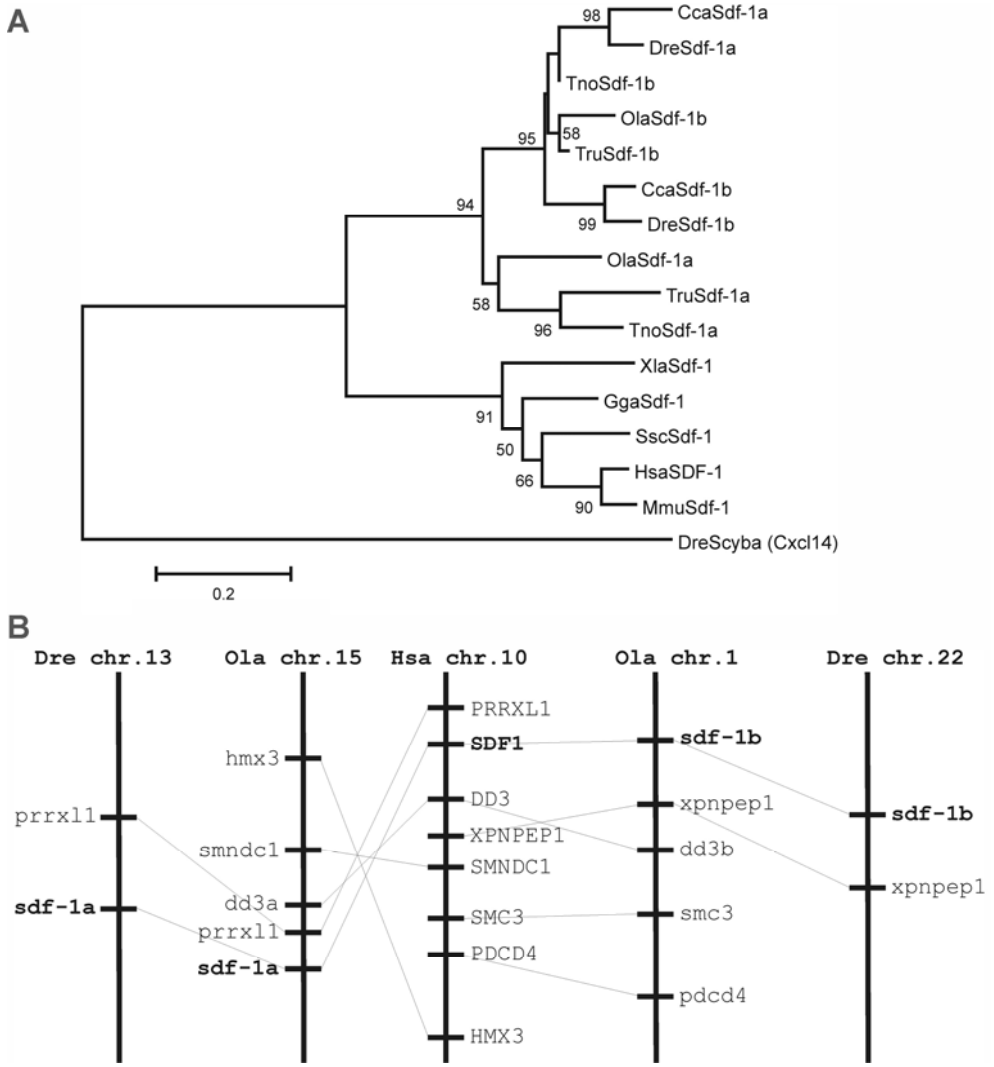


Fig. 9: Phylogenetic and synteny analyses of *sdf-1a* and *sdf-1b*. (A) Phylogenetic tree of Sdf-1 proteins. The branch lengths are drawn to scale to the evolutionary distance based on Poison-corrected Neighbour-Joining method, and the numbers are the percentage of bootstrap values supporting each node from 1000 replicas. Bootstrap values lower than 50 were not included. DreScyba (Cxcl14) was used as outgroup. Scale bar, 0.2 substitutions per site. Cca, *Cyprinus carpio* (common carp), Sdf-1a (AJ627274), Sdf-1b (AJ536027); Dre, *Danio rerio* (zebrafish), Sdf-1a (NM_178307), Sdf-1b (NM_198068), Scyba (AF279919); Gga, *Gallus gallus* (chicken) Sdf-1 (NM_204510); Hsa, *Homo sapiens* (human) SDF-1 (NM_000609); Mmu, *Mus musculus* (mouse) Sdf-1 (NM_021704) ; Ola, *Oryzias latipes* (medaka), Sdf-1a (DQ859774), Sdf-1b (AB214908); Ssc, *Sus scrofa* (pig) Sdf-1 (NM_001009580); Tno, *Tetraodon nigroviridis* (spotted green pufferfish), Sdf-1a (GSTENG00030635001) , Sdf-1b (Chr. 18) ; Tru, *Takifugu rubripes* (Japanese pufferfish) , Sdf-1a (SINFRUG00000163618), Sdf-1b (SINFRUG00000163442) ; Xla, *Xenopus laevis* (African clawed frog) Sdf-1 (NM_001015764). **(B)** Conserved synteny in the *Sdf-1* regions of human, medaka and zebrafish. *Sdf-1* genes are highlighted in bold letters. Lines between the compared chromosomes connect positions of orthologous gene pairs in the two species.

To elucidate their particular influence on PGC migration we have analyzed the expression pattern of both *sdf-1* co-orthologs. In contrast to *cxcr4*, which is present as maternal transcripts, neither *sdf-1a* nor *sdf-1b* was maternally deposited. Both *sdf-1* co-orthologs start to be expressed at stage 10, the mid-blastula transition (Kurokawa et al. 2006, Herpin in prep). Interestingly, quantitative real-time PCR (qPCR) have shown that *sdf-1a* was much higher expressed during early phases of embryonic development (up to stage 16) and *sdf-1b* expression increased around stage 24. In detail, I have analyzed the embryonic expression pattern of *sdf-1a* (Fig. 10) and compared the obtained results with the *sdf-1b* expression pattern (Kurokawa et al. 2006). The spatiotemporal expression analysis revealed that *sdf-1a* (Fig. 10A, B) and *sdf-1b* were coexpressed at the dorsal marginal zone (stage 15). At stage 18 *sdf-1a* mRNA were expressed in the LPM (Fig. 10C), as it has been described for *sdf-1b* (Kurokawa et al. 2006). At later stages, when PGC are migrating posteriorly in the LPM, *sdf-1a* transcripts were detectable in newly formed somites (Fig. 10D, E). In contrast, *sdf-1b* expression is restricted to the posterior LPM (Fig. 10E) (Kurokawa et al. 2006). Hence, Sdf-1a and Sdf-1b during early development appear to be involved in PGC guidance whereas *sdf-1a* is much higher expressed. Later in development Sdf-1b function seems to be the major cue for proper PGC migration to the gonad primordium.

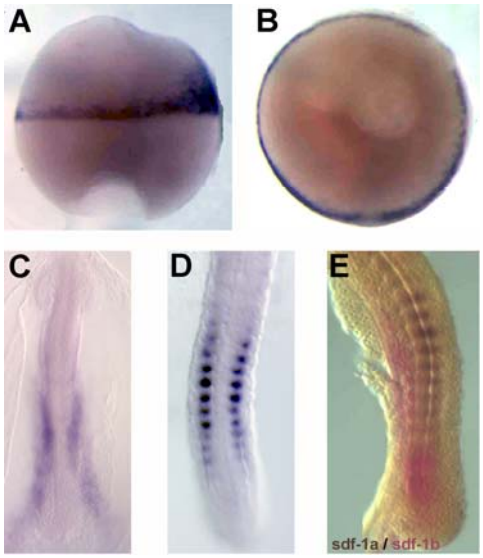


Fig. 10: Medaka Sdf-1a gene expression during embryonic development. (A, B) During mid-gastrulation (stage 15), *sdf-1a* expression is localized in the marginal zone. **(B)** Top view of stage 15 embryo. **(C)** *Sdf-1a* expression became gradually restricted to the LPM (stage 19). **(D)** At stage 24, *sdf-1a* expression is restricted to the posterior somites. **(E)** Non-overlapping expression of *sdf-1a* and *sdf-1b* at stage 24 (lateral view). *Sdf-1b* mRNA expression is localized in the LPM and pre-somitic mesoderm. Experiment in **E** was performed by Cordula Neuner.

Functional analysis of *sdf-1a* and *sdf-1b* by an antisense Morpholino (MO) approach has shown that Sdf-1a is required for the early PGC migration towards the peripheral margin and

Sdf-1b is more involved in later steps of PGC migration (Kurokawa et al. 2006, Herpin in prep). This suggests a function of Sdf-1a/Cxcr4 in the early migration phase and the posterior migration of PGC is dependent on the activity of Sdf-1b/Cxcr4.

Based on our comparison of expression patterns and functional analyses of *sdf-1* co-orthologs we conclude that after the WGD a subfunctionalization of the *sdf-1* co-orthologs has occurred so that in medaka Sdf-1a is involved in early (phase I) and Sdf-1b in later steps of PGC migration (phase III) (Fig. 11). Therefore it would be interesting to analyze *sdf-1* co-orthologs in different teleost species, including zebrafish, to search for lineage specific subfunctionalization which has been already described for several other duplicated genes in teleost (Altschmied et al. 2002; Cresko et al. 2003; Klüver et al. 2005; Postlethwait et al. 2004).

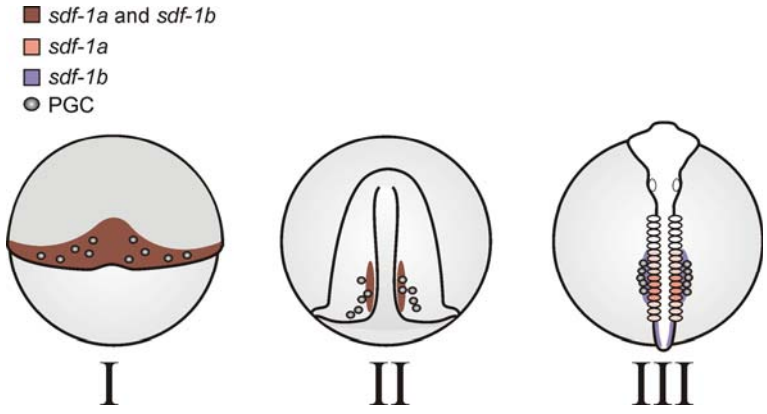


Fig. 11: Phases of *sdf-1/cxcr4* dependent PGC migration. Phase I shows overlapping expression of *sdf-1a* and *sdf-1b* in the marginal zone whereas PGC migration is more dependent on Sdf-1a/Cxcr4 (stage 15). In phase II the movement towards the midline along with convergent movement of somatic cells and less dependent on Sdf-1a and Sdf-1b activity (stage 18). Phase III describes that the posterior migration of PGC towards the LPM is dependent on Sdf-1b. *Sdf-1b* is expressed is the LPM and *sdf-1a* in the somites (stage 24).

2.1.3. Germ cells in *Oryzias celebensis*

In addition to the medaka, I was interested to analyze genes that are involved in gonad development in the closely related species *Oryzias celebensis*, which has no *dmrt1bY* as a male sex-determining gene. In this species, the sex determination system is still unknown. Comparative studies of *Oryzias* species should provide important insights into evolutionary aspects of sex determination. Around 20 species have been classified to belong to the genus *Oryzias*. This genus is a member of the family Adrianichthyidae (Teleostei: Beloniformes). The fish live in fresh-, brackish-, and seawater from India to Japan and south along the Indo-Australian archipelago across Wallace's line to Timor, Sulawesi, and Luzon (Berra 2001). Takehana et al. (2005) have combined molecular phylogenetic analysis and divided the Oryzias species into three major monophyletic species groups, namely the *latipes*, *javanicus*,

and *celebensis* groups (Fig. 12). The *celebensis* group has been isolated for more than 29 million years (Takehana et al. 2005). The most widely investigated member of the family is the medaka. Interestingly, the development of the gonad in *Oryzias celebensis* has been studied. The gonadal anlage of *O. celebensis* develops only on the right side of the dorsal mesentery (Hamaguchi 1983), which is different to the medaka in which the gonad forms at both sides of the mesentery. During earlier stages of development, PGCs in *Oryzias celebensis* are located on both sides of the embryo. Throughout the migration phase, the number of PGCs is constant. These studies were based on light and electron microscopy and so far, no molecular markers for gonad or germ cell development have been described.

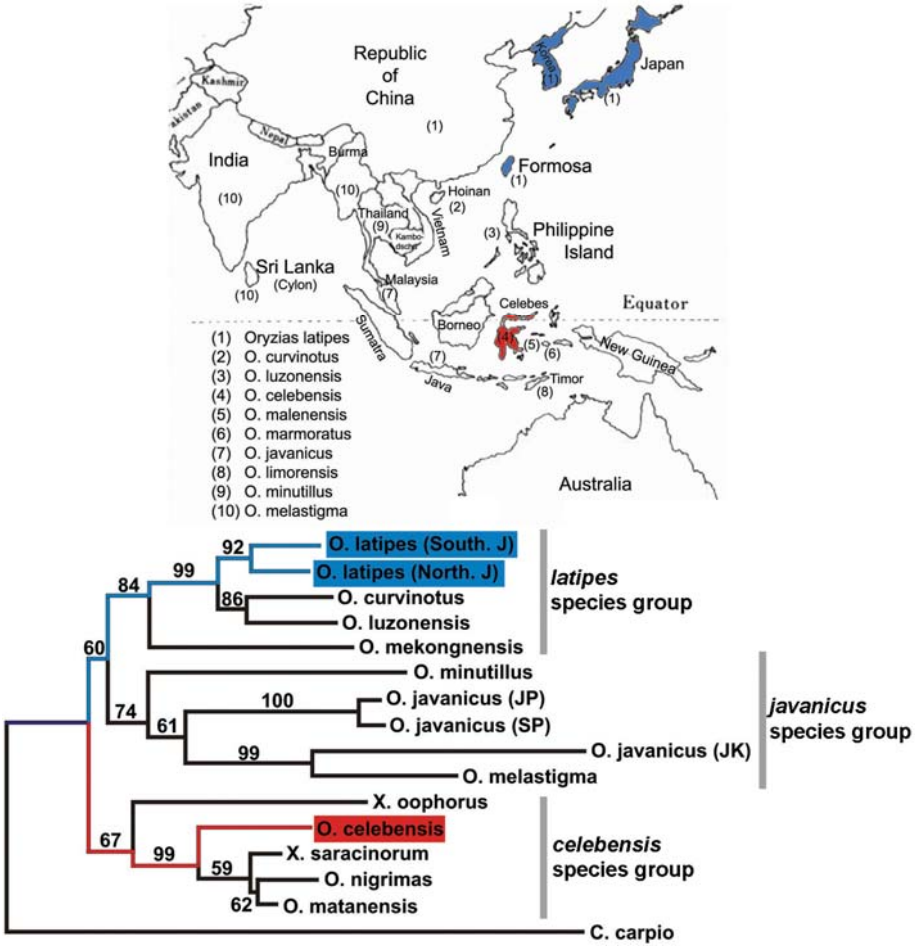


Fig. 12: Geographical distribution and phylogeny of the ricefish species. Distribution of *O. latipes* and *O. celebensis* distribution are highlighted in blue and red, respectively. Phylogenetic tree based on 12s rRNA gene sequences by neighbour-joining method. Numbers are the percentage of bootstrap values supporting each node from 1000 replicas. Modified after Naruse, 1996.

2.1.3.1. Isolation of the vasa homolog and migration of PGCs in *O. celebensis*

Here, I have isolated a partial *vasa* cDNA sequence as a germ cell specific marker in *O. celebensis* (for sequence alignments see Appendix A). I have analyzed the phylogenetic relationship to Vasa proteins and the expression pattern in adult gonads (Fig. 13). The

phylogenetic analysis of different Vasa proteins showed, as expected, that medaka and *Oryzias celebensis* Vasa proteins were closely related (Fig. 13A). The spatial expression pattern of *vasa* in the ovaries of adult fish was restricted to the developing oocytes and the transcripts were uniformly distributed in the cytoplasm, whereas in oocytes of later stages the signals were weaker (Fig. 13B). The testis expression of *vasa* was restricted to the spermatogonia, which are located at the testis periphery (Fig. 13C). The signals became weaker as the spermatogenesis proceeded. *O. celebensis vasa* RNA was exclusively restricted to the germ-line cell lineage. Compared to *olvas* expression in medaka there is no obvious difference in spatial expression (Shinomiya et al. 2000). These results imply that the *O. celebensis vasa* has a similar function as the *olvas* gene in medaka (Shinomiya et al. 2000; Tanaka et al. 2001) and can be used as a germ cell specific marker.

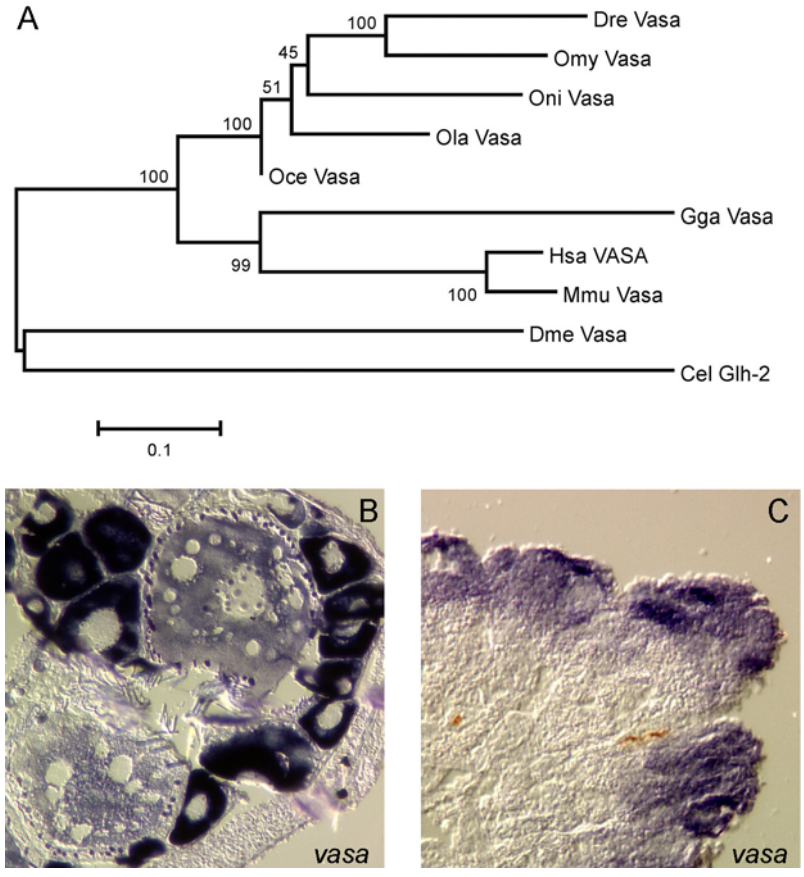


Fig. 13: Comparative analysis and expression pattern of *vasa* in O. celebensis. (A) Phylogenetic tree of Vasa proteins. The branch lengths are drawn to scale to the evolutionary distance based on Poison-corrected Neighbour-Joining method, and the numbers are the percentage of bootstrap values supporting each node from 1000 replicas. Scale bar, 0.1 substitutions per site. **(B)** In adult ovary, *vasa* expression is restricted to oocytes. **(C)** In testis, *vasa* expression is restricted to the spermatogonia. Cel, *Caenorhabditis elegans* (roundworm) Glh-2 (NM_059475); Dme, *Drosophila melanogaster* (Fruit fly) Vasa (NM_165103); Dre, *Danio rerio* (zebrafish) Vasa (AB005147); Gga, *Gallus gallus* (chicken) Vasa (NM_204708); Hsa, *Homo sapiens* (human) Vasa (NM_024415); Mmu, *Mus musculus* (mouse) Vasa (NM_010029); Oce, *Oryzias celebensis* (celebes rice fish); Ola, *Oryzias latipes* (medaka) Vasa (AB063484); Omy, *Oncorhynchus mykiss* (Rainbow trout) Vasa (AB032566); Oni, *Oreochromis niloticus* (Nile tilapia) Vasa (AB032467).

The migration of PGCs during embryogenesis had not been determined through monitoring *vasa* expression in *O. celebensis*. Therefore I used the GFP-zf*nos*3'UTR mRNA which labels PGC in early stages of embryogenesis in different teleost species (Herpin et al. 2007). Microinjection of the GFP-zf*nos*3'UTR mRNA into one- or two-cell stage *O. celebensis* embryos labelled the PGC during embryonic development (Fig. 14). No PGC were detectable in the 16-cell stage. Later during development PGC were seen on both sides of the developing embryo (Fig. 14A, B). At three dpf, PGCs were located alongside the LPM on both sides of the embryo axis (Fig. 14C). This is in line with the observations, which describe that PGCs were located alongside the lateral plate (Hamaguchi 1983). After 6dpf, the GFP positive PGCs had reached the prospective gonad primordium (Fig. 14D, D'). The usage of the GFP-zf*nos*3'UTR mRNA in *O. celebensis* embryos to label PGC was hampered by the strong pigmentation of *O. celebensis*. Nevertheless, PGC can be labelled and the migration to the prospective gonad primordium seem to be like PGC migration which has been described for the medaka (Hamaguchi 1982; Herpin et al. 2007; Kurokawa et al. 2006; Shinomiya et al. 2000; Tanaka et al. 2001). The detailed PGC migration pattern of the different species of the genus *Oryzias* could help to determine differences in the related species.

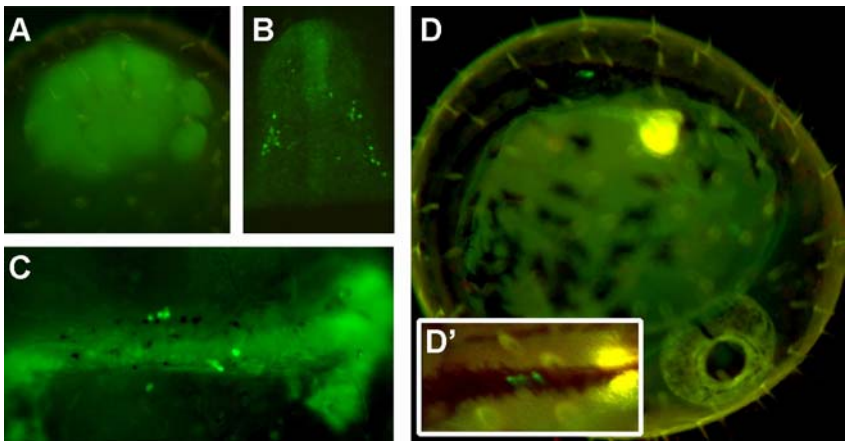


Fig. 14: Time-lapse imaging of PGC migration during early development of GFP-zfnos 3' UTR injected O. celebensis embryo. (A) GFP expression were localized in all cells of the 16-cell stage embryo. **(B)** In early neurula stage, PGCs were localized on both sides of the embryo. **(C)** PGCs migrated towards the midline (3dpf). **(D, D')** PGCs localized in the developing gonad (6dpf).

2.2. Conditional co-regulation of *wt1* genes in medaka ensures PGC maintenance or survival

In mammals, the Wilms' tumor suppressor gene, Wt1, encodes a transcription factor critical for development of the urogenital system and is important for the formation of the indifferent gonad. In mice, Wt1 is required for the survival and proliferation of cells in the genital ridge. In $Wt1^{-/-}$ mice the genital ridge fails to thicken and gonads fail to form during embryonic development (Kreidberg et al. 1993). In the medaka I have isolated two functional wt1 genes

named *wt1a* and *wt1b*, which are present in the splice isoforms + KTS and -KTS (Klüver et al., submitted). Additionally, I have identified an alternative isoform of *wt1a* that lacks exon 4. This alternative splice isoform has been described in turtle (*Trachemys scripta*) but the function is unknown (Spotila and Hall 1998). Phylogenetic analyses of *wt1* genes in vertebrates have indicated that, additionally to the medaka, the zebrafish, the two pufferfishes, and stickleback have two *wt1* genes. Conversely, only one *wt1* gene was identified in species of the tetrapod lineages. Medaka *wt1a* and *wt1b* have been located to linkage groups 6 and 3, respectively. Zebrafish *wt1a* is located on LG 25, *wt1b* was mapped to LG 18, and the human *WT1* gene is located on chromosome 11. Interestingly, the LG 3 and 6 of medaka, and LG 18 and 25 of zebrafish show strong synteny to each other, and to human chromosome 11 (Kasahara et al. 2007). This suggests that the *wt1* gene duplicates in teleost fish arose from the teleosts specific WGD. Thus, *wt1a* and *wt1b* are co-orthologs. However, in teleost fishes the *wt1* function has been studied so far only in the context of pronephros development (Bollig et al. 2006; Hsu et al. 2003; Serluca and Fishman 2001)and it has not been described whether *wt1* genes are involved in gonad development. Therefore, I have analyzed the role of *wt1* co-orthologs during gonad development in medaka (Klüver et al., submitted).

In medaka both *wt1a* and *wt1b* transcripts are maternally deposited. Directly after midblastula transition (MBT), *wt1a* is expressed and *wt1b* was not expressed. In embryos of stage 19-21, *wt1a* expression is located in the LPM. This expression pattern is overlapping with *sdf-1a* expression and PGCs were found to migrate through the *wt1a* positive LPM (Fig. 15). This suggests a role in prepatterning the LPM. However, in zebrafish *wt1b* mRNA is maternally deposited and is expressed directly after MBT, whereas *wt1a* expression begins at 6hpf (Bollig et al. 2006). In medaka, *wt1b* was first expressed around stage 25 and overlapping expression of *wt1a* and *wt1b* was detectable in the developing pronephros region. At stage 33 *wt1a* is specifically expressed in somatic cells of the gonadal anlagen (Klüver et al., submitted). At stage 39, after sex determination has already been initiated by the male specific expression of *dmrt1bY* in the somatic cells of the gonadal anlagen (Kobayashi et al. 2004), *wt1a* was expressed in somatic cells of the gonad primordium of both sexes but is not expressed in the germ cells. In adult tissues, *wt1a* and *wt1b* expressions were detected in the heart, kidney, spleen, testis and ovary, whereas *wt1b* expression was found to be much stronger expressed in the spleen. *Wt1a* expression showed elevated expression in the testis. Interestingly, *wt1a* transcripts were detected in the somatic cells surrounding spermatogonia of the peripheral region in adult testis. Here *wt1b* expression was restricted to the somatic cells of the testis periphery. In gills, liver, and muscle only *wt1b* is expressed. However, in

zebrafish only *wt1a* is expressed in the liver, whereas in medaka *wt1b* is expressed (Bollig et al. 2006). Thus, *wt1* expression differences between the two teleosts are obvious and suggest that *wt1* genes in teleosts followed a lineage specific subfunctionalization (Postlethwait et al. 2004).

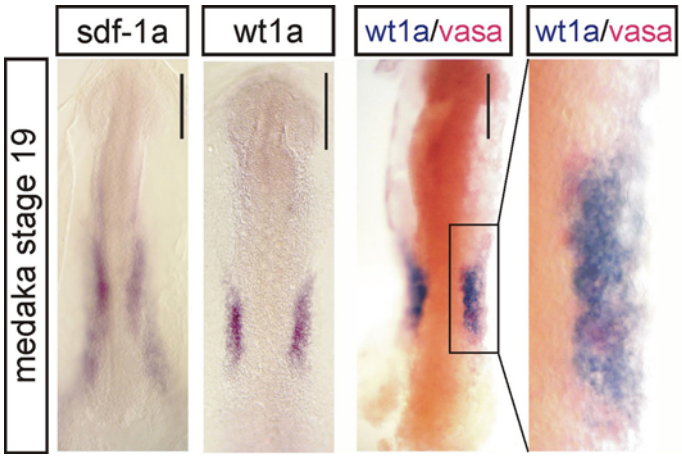


Fig. 15: Prepatterning the lateral plate mesoderm in medaka at stage 19. Overlapping expression of *sdf-1a* and *wt1a* in the LPM. PGCs migrating through the LPM are indicated by *vasa* expression (red staining). Scale bars, 100µm.

Functional analyses revealed redundant and non-redundant functions, and an unexpected regulatory back-up circuit of the wt1 co-orthologs. Remarkably, in the *wt1a* morpholino (MOwt1a) injected embryos I observed a drastic increase of *wt1b* expression. This I verified by whole-mount *in situ* hybridization (WISH) and real-time PCR analyses. Wt1a morphants showed *wt1b* expression in the somatic cells of the gonad primordium and I could observe this expression until hatching (9dpf). In the single and double knockdown experiments, which I have analyzed by *ftz-f1* (*sf1*) expression, the development of the pronephric field was impaired in size. Similar results have been obtained in the zebrafish. The reduction of *Wt1a* levels results in defects in interrenal and pronephros development (Hsu et al. 2003). In mammals the $Wt1^{-/-}$ mice lack kidney, gonad, and adrenal glands (Kreidberg et al. 1993).

This indicates that medaka *wt1* genes are functionally divergent in pronephros development and mammalian and fish *wt1* genes have similar functions during kidney/pronephros development.

In the medaka, neither MOwt1a nor MOwt1b injections had affected the somatic cells of the gonad primordium. As marker gene for somatic cells of the gonad primordium *sox9b* was used (Klüver et al. 2005; Nakamoto et al. 2005; Nakamura et al. 2007; Nakamura et al. 2006). *Sox9b* expression in the somatic cells of the gonadal anlagen was not influenced in either single knockdown of Wt1a and Wt1b or Wt1a and Wt1b double knockdown experiments. However, we obtained strong reduction of *vasa* expressing PGCs in MOwt1a/b injected

embryos around stage 33-36, whereas in the single knockdown of either Wt1a or Wt1b this was not the case. Further, the specification and migration of PGCs in all knockdown experiments were not disturbed. Several studies in mice have implicated a function of Wt1 in germ cell maintenance and survival (Gao et al. 2006; Natoli et al. 2004; Rao et al. 2006). This suggests a conserved role in germ cell survival or maintenance of *wt1* genes during evolution. Interestingly, only in the MOwt1a/b injected embryos we observed the decrease of PGC numbers and together with the increased *wt1b* expression in wt1a morphants, this suggests redundant functions of *wt1a* and *wt1b* in PGC maintenance or survival. Additionally, the up-regulation of *wt1b* in MOwt1a injected embryos revealed an unexpected regulatory back-up circuit of the *wt1* co-orthologs (Fig. 16). This regulatory back-up circuit might have been evolved after the teleost duplication event, whereby all ways of a negative feed back loop could exist (Fig. 16B). During the process of evolution, one gene copy might have lost the regulation, whereas the other gene copy is still able to fulfil the function (Fig. 16C). However, whether the ancestral *wt1* gene showed autoregulation or not is not clear (Fig. 16A). In mammals, it has been shown that WT1 is a powerful direct repressor of its own expression, by binding to its own promoter (Malik et al. 1994; Rupprecht et al. 1994). This shows its critical role as a transcriptional regulatory protein during development. The establishment of this regulatory cross talk during *wt1* co-ortholog evolution is suggested to have offered an immediate fitness advantage due to its direct influence on medaka fertility.

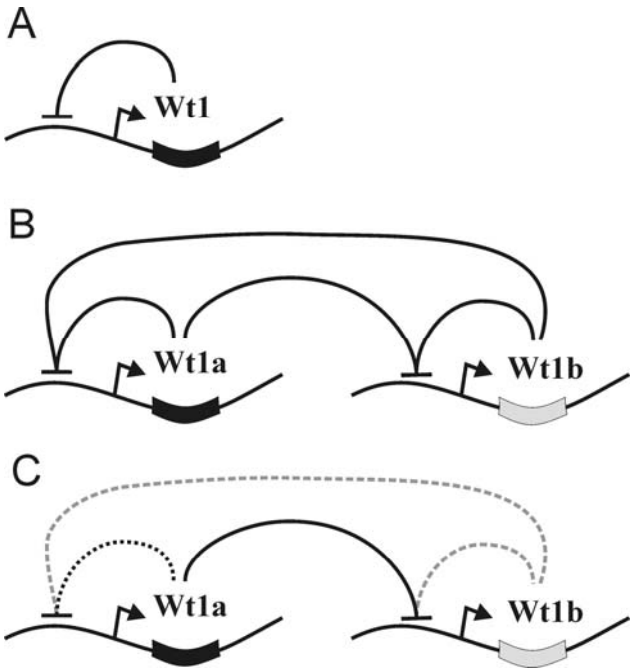


Fig. 16: Regulatory back-up circuit of duplicated *wt1* genes. (A) Auto-regulation of the ancient *wt1* gene. **(B)** Possible interactions of *wt1* genes directly after teleosts specific WGD. Repressive action of Wt1a/Wt1b on *wt1b*/*wt1a* and *wt1a*/*wt1b* expression (black lines). **(C)** Observed (black line), possible (black dashed lines) and probably lost (grey dashed lines) regulatory feedback loops of *wt1* genes in medaka.

2.3. Duplicated *wt1* genes in *Oryzias celebensis*

To find out whether *wt1* genes have similar roles during embryonic development in *O. celebensis*, I have isolated the *wt1* genes in *O. celebensis* and analyzed their expression pattern (for sequence alignments see Appendix A). By using primers of medaka, I could amplify partial sequences of both *wt1* genes. Phylogenetic analysis with the deduced amino acid sequences showed that *O. celebensis* has *wt1a* and *wt1b* co-orthologs like other teleost fishes and both were more related to their medaka orthologs (Fig. 17A). In adult tissues, expression of *wt1a* and *wt1b* was detected in the heart, kidney, liver, spleen, testis and ovary, whereas *wt1a* and *wt1b* expression was more prominent in the testis and in the kidney, respectively (Fig. 17B). In comparison to medaka *wt1* genes, the expression in adult organs is similar and showed differences in the muscle expression. *Wt1b* in medaka was expressed in muscles, whereas in *O. celebensis* neither *wt1a* nor *wt1b* was expressed in muscles. During embryogenesis *wt1a* transcripts were located in the LPM and later became restricted to the pronephric field (Fig. 17C, D), like medaka *wt1a* expression (Klüver et al., submitted).

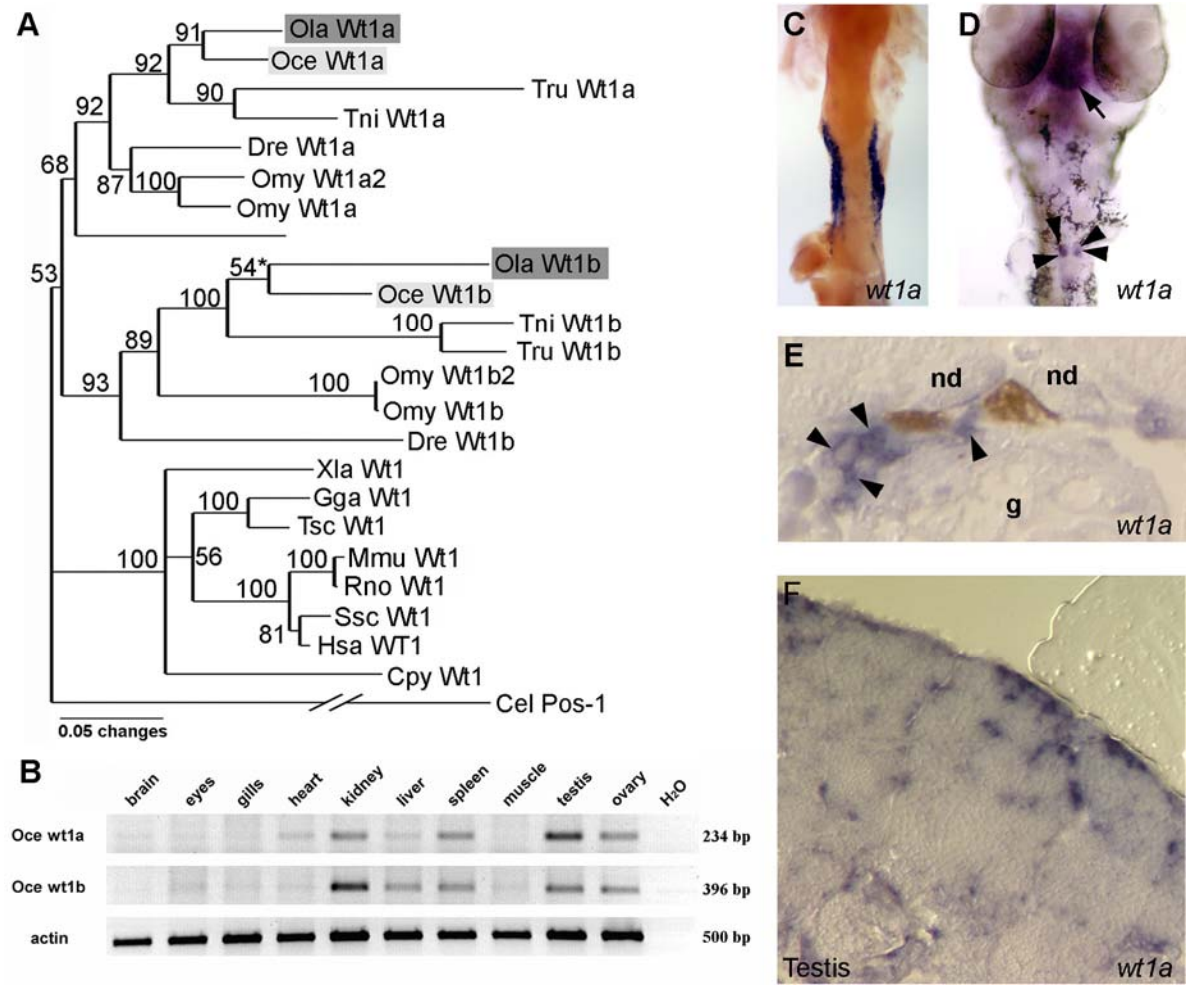


Fig. 17: Identification and expression of duplicated *wt1* genes in *O. celebensis*. (A) Phylogenetic tree of Wt1 proteins by neighbour-joining method. The branch lengths are drawn to scale to the evolutionary distance and the numbers are the percentage of bootstrap values supporting each node from 1000 replicas. Scale bar, 0.05 substitutions per site. Asterisks indicate partial sequence of OceWt1b has been included – that resulted in lower bootstrap value. **(B)** Expression of *O. celebensis wt1a* and *wt1b* in adult tissues. The following primer combinations were used: *wt1a*F2 5´-TGACCAACTGCATGGACACT-3´ and *wt1a*R2 5´-TTGTGCAGCTGTCTGAAGGT-3´, Wt1bF3 5´-TGCAGCAAGAGATACTTTAAGCTC-3´ and *wt1b*R2 5´-CTTTCTTTAAATGGCAGGTTGC-3´, thus amplifying fragments of 234 or 396 bp, respectively. A 500 bp fragment of the constitutively expressed actin gene was amplified with *act*F1 5´-GTAGGTGATGAAGCCCAGAGC-3´ and *act*R1 5´-AGGGAGCTCGTAGCTCTTCTC-3´ primers to quantify transcript amounts. PCR conditions were 25 cycles of 96°C for 30 s, 51°C for 30 s and 72°C for 30 s. **(C)** *Wt1a* is expressed in the LPM at the two somites stage. **(D)** Positive signals of *wt1a* transcript in the heart (arrow) and in the pronephros (arrowheads). **(E)** *Wt1a* expression in the somatic cells of the gonad primordium at hatching day (arrowheads). **(F)** *Wt1a* RNA is localized in the somatic cells of the adult testis. g, gut; nd, nephric duct.

However, *wt1b* expression analysis during embryogenesis was not successful. Therefore, further analysis should help to verify species-specific expression differences in the genus *Oryzias*. During gonad development of *Oryzias celebensis*, in hatched embryos *wt1a* expression was restricted to the somatic cells of the gonad (Fig. 17E). Interestingly, in all investigated embryos the gonad primordium was located only on one side of the dorsal mesentery, which is different to the medaka. This is in line with the observation that the gonadal anlage of *O. celebensis* develops only on the right side of the dorsal mesentery (Hamaguchi 1983). *Wt1a* expression was restricted to the somatic cells of the peripheral region in adult testis (Fig. 17F), as observed in medaka (Klüver et al., submitted). Altogether, this suggests a conserved role of *wt1a* genes in medaka and *O. celebensis* during gonad and pronephros development. However, functional analysis should give detailed information about *wt1* gene function during embryonic development of *O. celebensis.*

2.4. Lineage specific subfunctionalization of *sox9* co-orthologs

Sox9 encodes a transcription factor required for cartilage formation and testis determination in mammals and is the most conserved upstream gene that is involved in sex determination and differentiation among vertebrates (Kent et al. 1996; Morais da Silva et al. 1996; Oreal et al. 1998; Rodriguez-Mari et al. 2005; Smith et al. 1999). However, the expression pattern varies between the species, suggesting that there is functional plasticity in vertebrates.

I have identified *sox9a* and *sox9b* genes in medaka. Phylogenetic analysis revealed that both medaka *sox9* genes are co-orthologs of the single tetrapod *sox9* gene. Medaka *sox9a* and *sox9b* were mapped to LG19 and LG 8, respectively. In comparison, the zebrafish sox9 genes are located on LG 12 (*sox9a*) and LG 3 (*sox9b*) and the *Sox9* bearing chromosome 17 in

human. All the linkage groups show strong conserved synteny to each other (Fig. 18). This provides evidences that sox9 co-orthologs in teleosts species arose through the ancient teleosts specific WGD ~ 350 million years ago (Amores et al. 1998; Kasahara et al. 2007; Meyer and Schartl 1999; Meyer and Van de Peer 2005). To analyze lineage specific differences I have investigated the expression pattern of both genes and compared the results with the zebrafish. In adult tissues medaka *sox9a* was expressed in eyes, brain, gills, testis and ovary, whereas the expression in brain and ovary was more prominent. *Sox9b* showed an expression in the spleen and heart. Further, *sox9b* had overlapping expression with *sox9a* in the eyes, brain, gills and testis. The testis expression of *sox9b* was much stronger than *sox9a*. Therefore, we determined *sox9b* as the testis specific *sox9* gene in the medaka (Klüver et al. 2005). However, zebrafish *sox9a* was expressed in the testis and *sox9b* was ovary specifically expressed. Compared to the medaka we also observed differences in kidney, muscle, and heart expression. These results indicate lineage specific differences in tissue specific expression. The change of tissue specific expression between *sox9a* and *sox9b* in different teleosts are indications for a lineage specific subfunctionalization of regulatory elements that have evolved differentially after the lineages have diverged (Postlethwait et al. 2004).

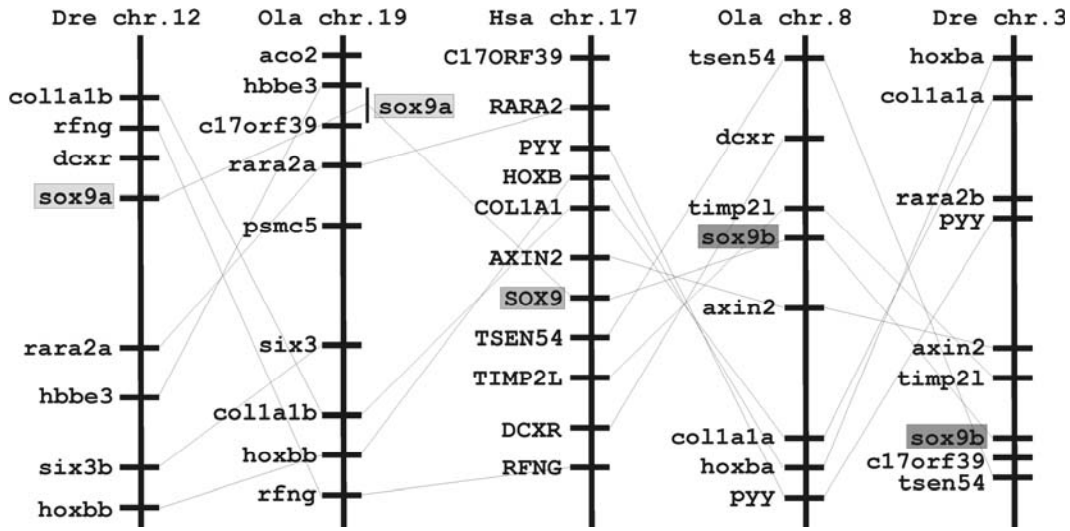


Fig. 18: Conserved syntenies in regions surrounding the *sox9* genes. A comparison of the sox9 bearing chromosomes between human, medaka and zebrafish show that the neighbouring genes are conserved at the same chromosomes. *Sox9* genes are highlighted in grey boxes. Sox9a in Medaka is localized between hbbe3 and c17orf39. Lines between the compared chromosomes connect positions of orthologous gene pairs in the two species.

In addition, I determined differences of expression pattern during embryogenesis (Klüver et al. 2005). The spatiotemporal expression of *sox9a* in medaka during early development was mainly restricted to craniofacial cartilaginous structures and the pectoral fins in later stages. In contrast *sox9b* was expressed in different tissues during embryonic development including

craniofacial cartilaginous structures, neural crest cells, central nervous system, eyes, otic placode, pectoral fins, heart and somites (Klüver et al. 2005). In comparison to stickleback (*Gasterostreus aculeatus*) and zebrafish *sox9a* and *sox9b* expression, the *sox9b* expression patterns during early embryogenesis in all three teleosts species are generally similar. However, the somite specific expression in zebrafish and stickleback is restricted to *sox9a* and no heart expression of *sox9b* has been described (Chiang et al. 2001; Cresko et al. 2003; Yan et al. 2005). Embryonic *sox9a* expression patterns are different in the three species. In stickleback and zebrafish *sox9a* is expressed in the central nervous system (CNS), whereas in medaka *sox9a* is not expressed in the CNS (Chiang et al. 2001; Cresko et al. 2003; Yan et al. 2005). This indicates that the spatiotemporal regulation of *sox9* co-orthologs has changed after the divergence of the corresponding lineages. Although teleost *sox9* co-orthologs display lineage specific expression patterns, their combined expression pattern are similar to that of *sox9* in tetrapods. However, the expression of *sox9* genes in the ovary is either fish specific or is an ancestral gene function that has been lost in the tetrapod lineage. These results support the hypothesis that some subfunctions are shared across lineages (Postlethwait et al. 2004), whereas lineage specific subfunction and partitioning have been acquired during the teleost radiation.

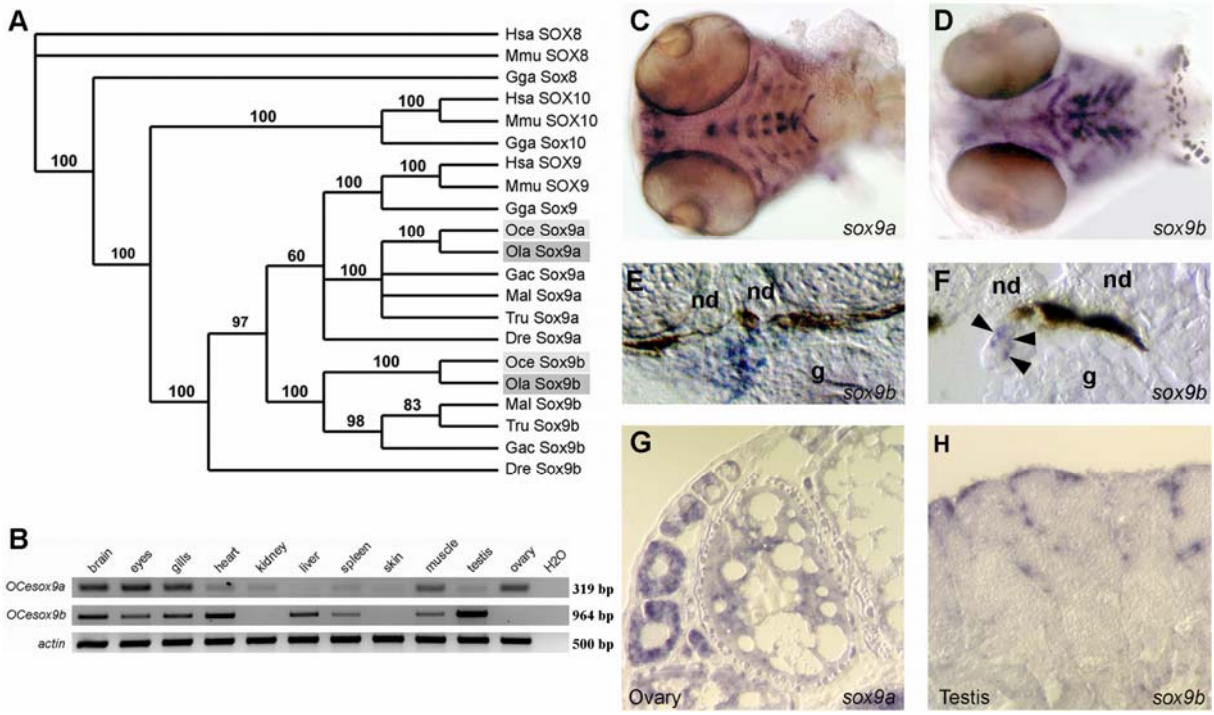


Fig. 19: Identification and expression of duplicated *sox9* genes in *O. celebensis*. (A) Phylogenetic tree of Sox E group proteins of vertebrates constructed by the neighbour-joining method. Numbers on each branch are the bootstrap values in thousand runs. Sox9a and Sox9b of medaka and *O. celebensis* are shaded in dark grey and light grey, respectively. **(B)** Expression of *O. celebensis* *sox9a* and *sox9b* in adult tissues. The following primer combinations were used: OceSox9aF4 5´-GACTCTCCCGCCTCCAGC-3´ and OceSox9aR01 5´-GATGACTGGGGCAGTATTCC-3´,

OceSox9bF1b; 5´-GTCCGACAACCATCTCATCA and OceSox9r1; 5´-CGGGCTCAGCTGCTCCGTCTT , thus amplifying fragments of 319 or 964 bp, respectively. A 500 bp fragment of the constitutively expressed actin gene was amplified with *act*F1 5´-GTAGGTGATGAAGCCCAGAGC-3´ and *act*R1 5´-AGGGAGCTCGTAGCTCTTCTC-3´ primers to quantify transcript amounts. PCR conditions were 30 cycles of 96°C for 30 s, 52°C for 30 s and 72°C for 40 s. **(C)** *Sox9a* expression in the craniofacial cartilage (~stage 33). **(D)** *Sox9b* expression in the craniofacial cartilage (~stage 33). **(E, F)** Expression of *sox9b* is restricted to the somatic cells of the gonad primordium. **(G)** *Sox9a* expression in the adult ovary. **(H)** *Sox9b* RNA is localized in the somatic cells of the adult testis. g, gut; nd, nephric duct.

Additionally, I have isolated *sox9a* and *sox9b* genes in *O. celebensis* (for sequence alignments see Appendix A). Phylogenetic analysis showed that they are orthologs of the *sox9a* and *sox9b* in other teleosts (Fig. 19A). The *sox9a* and *sox9b* expression pattern in adult tissues were nearly identical to the medaka (see above) (Klüver et al. 2005), with the exception that *O. celebensis sox9* genes showed overlapping expression in the muscle and *sox9b* was expressed in the liver (Fig. 19B). During embryogenesis, *sox9a* and *sox9b* transcripts were clearly detectable in the craniofacial cartilage (Fig. 19C, D). Unexpectedly, *sox9b* was expressed in somatic cells surrounding germ cells in the gonad primordium around stage 32 and hatched embryos (Fig. 19E, F). Also here I detected the developing gonad anlagen only on one side of the dorsal mesentery which has been already described earlier (Hamaguchi 1983). In adult gonads, *sox9a* transcripts were located in the cytoplasm of developing oocytes, whereas *sox9b* was expressed in somatic cells of the peripheral region in adult testis. In summary, the *O. celebensis sox9* gene expression clearly shows high similarity to the closely related medaka and is in line with the lineage specific subfunctionalization hypothesis.

2.5. Medaka *sox9b* is involved in gonad development and sex differentiation

In the first expression analysis of medaka, I have not detected a specific *sox9b* expression in the gonad primordium. This might be due to too short incubation times and/or probe quality. Therefore I have analyzed the expression pattern of *sox9b* during gonad development in medaka in detail and could show that *sox9b* was expressed during early phases of gonad development in somatic cells of the gonadal anlagen of both sexes (Fig. 20A), which is identical to the *sox9b* expression in *O. celebensis*. In fact, mammalian *Sox9* is expressed in the bipotential gonad of both sexes and soon after the onset of *Sry* expression, *Sox9* expression is upregulated in the male gonad and downregulated in the female gonad (Kent et al. 1996; Morais da Silva et al. 1996). In medaka the male sex determining gene *dmrt1bY* is expressed specifically in the somatic cells of developing the male gonads at stage 36 (Kobayashi et al.

2004). However, *sox9b* is expressed at stage 30-39 in the developing gonads of both sexes (Fig. 20A, B). Later during development, around 30dph, *sox9b* expression becomes testis specific (Fig. 20C, D). Thereafter, *sox9b* expression continued in somatic cells of the male gonads and I detected *sox9b* transcripts in the somatic cells surrounding spermatogonia of the peripheral region in adult testis, which seem to be Sertoli cells (Fig. 20E, E'). In zebrafish, *sox9a* is expressed in the undifferentiated gonad. Later during sex differentiation *sox9a* becomes testis specific and *sox9b* is expressed in the ovary (Rodriguez-Mari et al. 2005). This shows the lineage specific subfunctionalization of *sox9* regulatory elements that have evolved differently in the medaka and zebrafish between the *sox9* co-orthologs. The male-specific maintenance of *sox9b* expression in medaka corresponds well to the timing of the formation of the acinus (a globular structure that is the seminiferous tubule precursor). This suggests that *sox9b* is more involved in sex differentiation rather than in sex determination. Nakamura et al (2006) have shown that somatic gonadal precursors become established before the formation of the gonad primordium and that later distinct cell populations are involved in formation of the gonadal mesoderm. Interestingly, several genes have been described, which mark these cells, including *sox9b*, *ftz-f1* (*sf1*) and *sdf-1b* (Nakamura et al. 2006). The early function of *sox9b* during formation of the bipotential gonad is yet not clear.

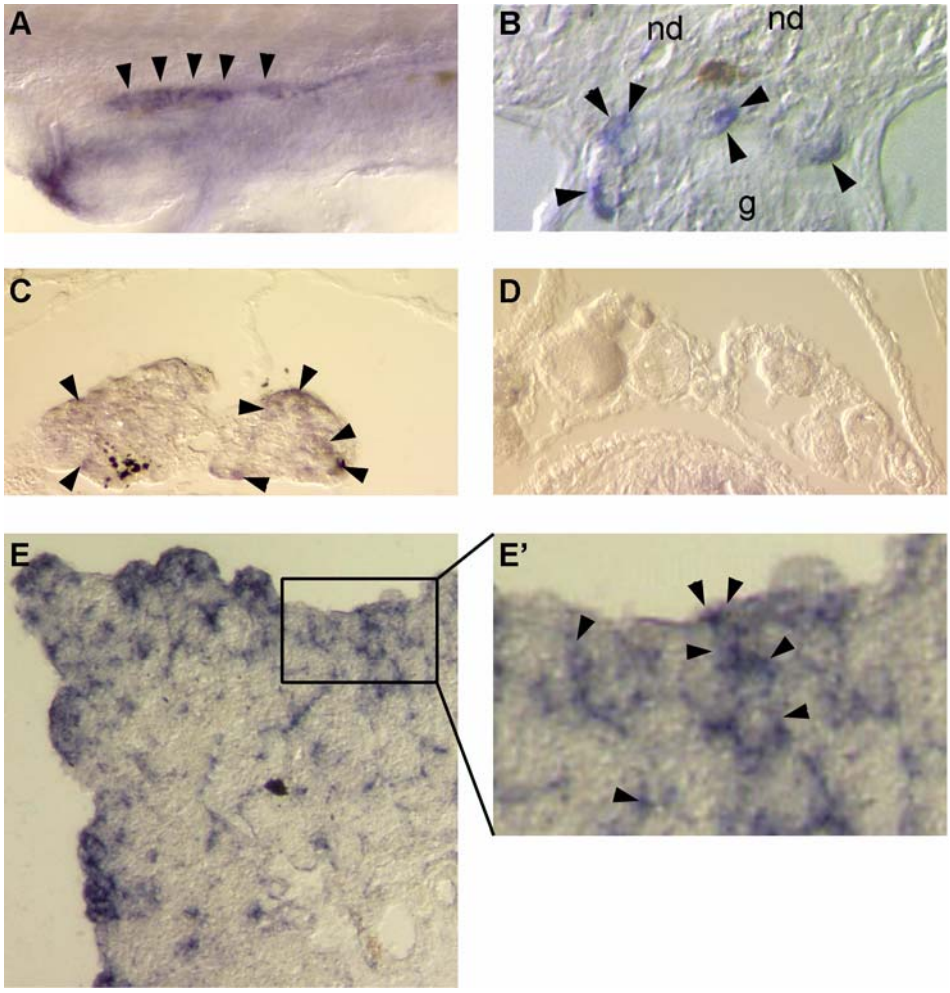


Fig. 20: Medaka *sox9b* expression during sex determination/differentiation. (A) *Sox9b* is expressed in the developing gonads of both sexes. **(B)** S*ox9b* expression is restricted to the somatic cells of the gonad primordium in both sexes. **(C)** *Sox9b* expression persists in the developing testis at 30dph. **(D)** *Sox9b* is not expressed in the developing ovary at 30dph. **(E, E')** *Sox9b* RNA is localized in the somatic cells of the adult testis. Arrowheads indicate *sox9b* expression. g, gut; nd, nephric duct.

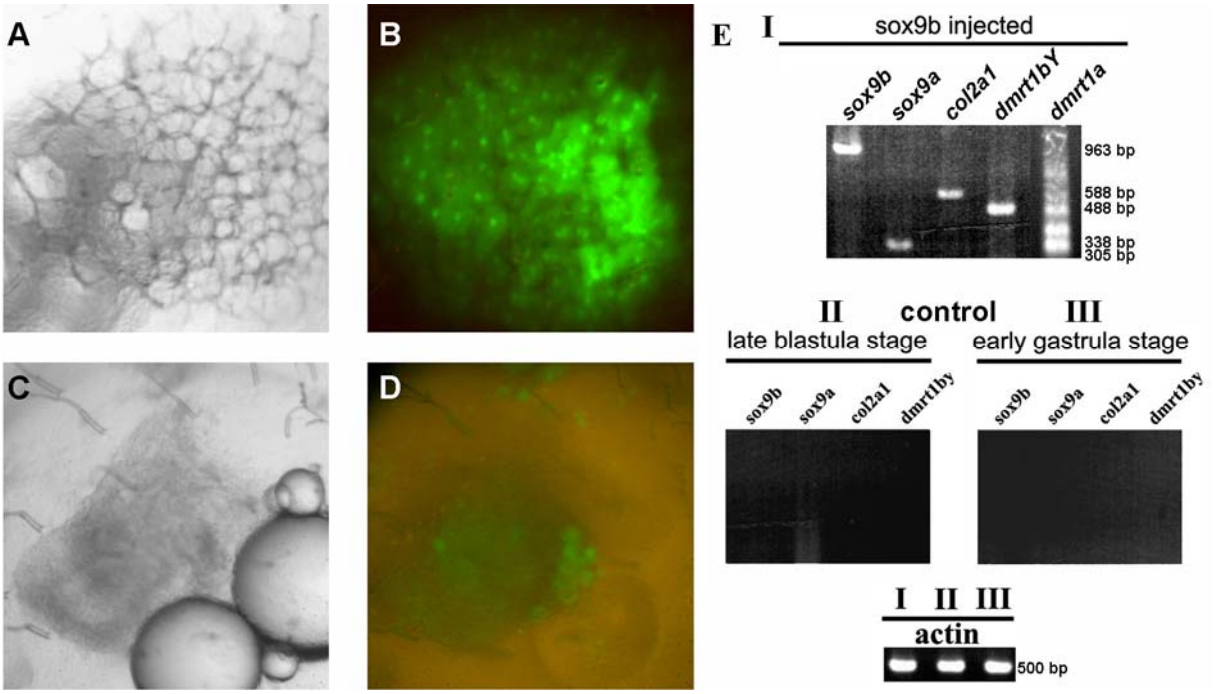


Fig. 21: Overexpression of *sox9b* results in developmental arrest and induction of *dmrt1* expression. (A, B) Sox9b-GFP injected embryo at stage 8. Sox9b-GFP protein is localized in the nucleus **(B)**. **(C, D)** sox9b-GFP injected embryo 20h post injection. **(E)** Expression of *sox9a*, *sox9b*, *col2a1*, *dmrt1a*, and *dmrt1bY* in injected (I) and non-injected embryos directly after MBT (II) and late gastrula stage – 21hpf (III). The following primer combinations were used: *dmrt1a*: DMTm 5'-TCCGGCTCCACAGCGGTC-3' and DMTn 5'-CAGCACAGAGG-GTTGGGGG-3' amplified a fragment of 338 bp. *Dmrt1bY*: DMYa 5'-GGCCGGGTCCCCG-GGTG-3' and DMYc 5'-CTGGTACTGCTGGTAGTTGTG-3' amplified a fragment of 488 bp. *Sox9a*: OlaSox9a3UTR-F3 5'-ATCCGTGCCTTTCCTTTTCT-3' and OlaSox9a3UTR-R2 5'-CCTTTGACCTCGGAAACAAA-3' amplified a fragment of 305 bp. *Sox9b*: Sox9b-F1 5'-GT-CCGACAACCATCTCATCA-3' Sox9-r1 5'-CGGGCTCAGCTGCTCCGTCTT-3' amplified a fragment of 963 bp. *Col2a1a*: Olacol2AUP 5'-GGGCCTATGTCAACTCCAAA-3' and Olacol2ADOWN 5'-GCTGTGTCATCCTGAGTGGA-3' amplified a fragment of 588 bp. PCR conditions were 96°C for 30 s, 51-53°C for 30 s and 72°C for 1 min for 30 cycles.

In mammals, Sox9 function during sex determination and ectopic expression of sox9 in XX gonads is sufficient to induce testis formation in mice, indicating that it can substitute for the sex-determining gene Sry (Vidal et al. 2001). Therefore, I ectopically expressed *sox9b* during early embryogenesis by injection of *sox9b* mRNA or *sox9b*-GFP mRNA (20-30pg) into the one- or two-cell stage of medaka embryos. During early stages of development in the *sox9b*-GFP mRNA injected embryos, the Sox9-GFP protein was localization within the nucleus (Fig. 21A, B). Inside the HMG-box of Sox9b two nuclear localization signals (NLS) at the amino acid positions 105-121 and 176-179 can be identified which are highly conserved up to mammals (Gasca et al. 2002). Unfortunately, all injected embryos arrested in development around 20 hours post injection (Fig. 21C, D). I analyzed gene expression of *dmrt1a*, *dmrt1bY*, *amh*, *col2a1* and *sox9a* in 10-15h old *sox9b* RNA injected embryos just before the

developmental arrest. All genes were found to be expressed. Remarkably, *dmrt1a* expression was dramatically increased. This is in strong contrast to uninjected embryos, which did not show expression of any of these genes (Fig. 21E). Whether this is a specific effect of *sox9b* RNA injection or not has to be analyzed in more detail in the future. These results together with the fact that the autosomal *dmrt1* expression usually starts only around 20dph (Kobayashi et al. 2004; Nanda et al. 2002) can carefully be interpreted that *sox9b* might influence male specific *dmrt1a* expression during sex differentiation. However, further investigation of the transcriptional regulation of the genes would help to decipher the genetic cascade.

2.6. The role of Anti-Müllerian hormone and Anti-Müllerian hormone receptor type II in medaka gonad development

The anti-Müllerian hormone (Amh) function during gonad development has been studied extensively in mammals, but is less understood in other vertebrates. In mammals, Sox9 activates expression of *Amh*, which initiates the regression of the Müllerian ducts and inhibits estrogen action by repressing the expression of aromatase (*cyp19a1*), the enzyme that converts androgens to estrogens. Amh functions primarily through the anti-Müllerian hormone receptor type II (*amhrII*), which is a serine/threonine kinase receptor type II of the TGF-β-receptor superfamily (Josso et al. 2001; Teixeira et al. 2001). I have identified the medaka *amh* and *amhrII* ortholog, which represents the first characterization of *amhrII* in nonmammalian vertebrates (Klüver et al. 2007). However, teleosts fish do not posses a Müllerian duct. The genital ducts are derived from the coelomic epithelium and do not originate from the renal duct system as it is the case in mammals (Suzuki and Shibata 2004). Therefore, the medaka provides the possibility to study the ancestral roles of Amh and AmhrII. The phylogenetic relationship between the medaka Amh and other related members of the TGF-beta superfamily revealed that I have indeed identified the medaka Amh ortholog. The same was true for the AmhrII, which was more related to AmhrII proteins from other vertebrates than to other receptors of the TGF-β superfamily (Klüver et al. 2007). Interestingly, I did not find any duplicated copies of both *amh* and *amhrII*. Also in zebrafish, stickleback and the pufferfishes both *amh* and *amhrII* were present as single copy genes. This suggests that after the teleosts specific WGD one of the duplicated copies might have degenerated. In general, it has been assumed that for at least 20% of the human genes, teleosts has two co-orthologs (Postlethwait et al. 2004). In medaka, *amh* and *amhrII* expression are restricted to the gonads. In adult testis, *amh* and *amhrII* transcripts were localized in the somatic cells surrounding the germ cells and spermatogonia and showed overlapping

expression in the granulosa cell layer of adult ovaries (Klüver et al. 2007). The same *amh* expression pattern has been described in zebrafish and mammals (Lasala et al. 2004; Rodriguez-Mari et al. 2005). The *amhrII* expression pattern is in agreement with mammalian *amhrII* expression in somatic cells like Sertoli cells, Leydig cells and granulosa cells (Baarends et al. 1995a; Baarends et al. 1995b; Josso and Clemente 2003; Teixeira et al. 1996; Teixeira et al. 1999).

During the early and juvenile stages of medaka gonad development, *amh* was identically expressed in the somatic cells of both sexes. This is similar to *sox9b* and *wt1a* expression. These genes were not sexually dimorphic expressed in the somatic cells surrounding germ cells of the developing gonads (Fig. 22). On the contrary, a sexual dimorphic expression of *amh* during gonad development has been described in the zebrafish, Japanese flounder, and mammals (de Santa Barbara et al. 2000; Rodriguez-Mari et al. 2005; Yoshinaga et al. 2004).

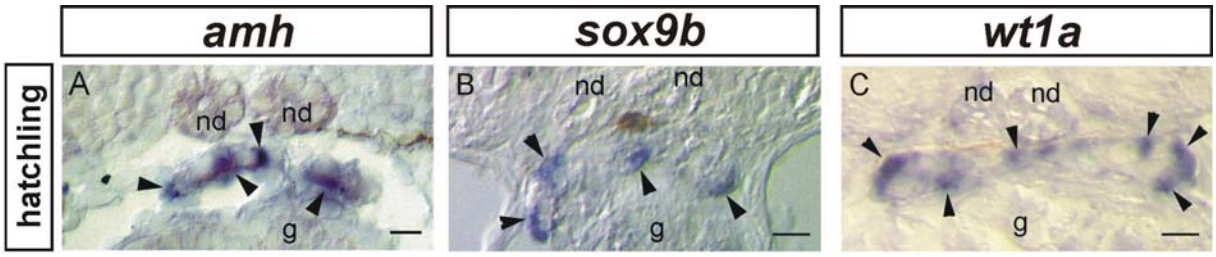


Fig. 22: Overlapping expression of *amh*, *sox9b* and *wt1a* during medaka gonad development. *Amh*, *sox9b* and *wt1a* are expressed in the somatic cells of the developing gonad in both sexes at hatching stage. Arrowheads indicate gene expression. g, gut; nd, nephric duct.

Further, Sox9, Wt-1 and Sf-1 in mammals are involved in the transcriptional activation of *Amh* during testis differentiation (De Santa Barbara et al. 1998; Giuili et al. 1997; Watanabe et al. 2000). However, we have predicted several binding sites in the putative promoter of the medaka *amh* gene (Fig. 23). There are candidate-binding sites for Sox9b, Wt1 and Ftz-F1 (Sf1), which might be involved in the transcriptional regulation of *amh* in medaka. Whether the regulation of *amh* in medaka is dependent on one of these genes or not has to be investigated by a functional promoter analysis.

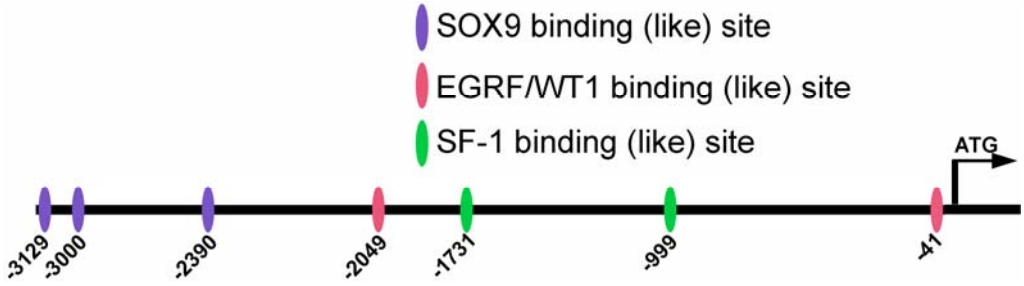


Fig. 23: Putative transcription factor binding sites in 5'-flanking regions of medaka *amh*. Predicted binding sites of Sox9, Ftz-F1 (Sf-1) and Egrf/Wt1 are depicted in the 3,2kb 5' upstream sequence of the *amh* gene.

In somatic cells of the female gonads, *cyp19a1* expression was detected around 7-11dph and *amh* was expressed. This suggests that *amh* and *amhrII* expression has no influence on *aromatase* expression, contrary to what has been shown in higher vertebrates (di Clemente et al. 1992; Nishikimi et al. 2000). In a recent study in medaka an *amhrII* gene mutation has been identified in the *hotei* (*hot*) mutant (Morinaga et al. 2007). The *hot* mutation is a critical amino acid substitution (Y390C) in the *amhrII* gene and the phenotype shows abnormalities only in the *hot*-homozygous animals that includes excessive proliferation of germ cell in both sexes during the onset of germ cell proliferation around hatching stage. Around one-half of the *hot*-homozygous XY fish undergo sex reversal. Moreover, phenotypically male *hot*-homozygotes show initiation of premature meiosis and in *hot*-homozygous females follicular development is arrested at an early stage. This is connected with the expression of *aromatase* in the somatic cells around the oocytes of the gonad, which are detectable in XX *hot*-homozygous and XY *hot*-homozygous larvae that shows ovary-type gonads (Morinaga et al. 2007). So far, it is unclear how the hot mutant amhrII allele affects the downstream signalling of Amh/AmhrII. It can be a gain or a loss of function of AmhrII. Therefore, loss of function experiments of Amh should provide additional insights in the regulation of germ cells. Nevertheless, the *hot* mutant is the first link of Amh/AmhrII function in medaka to germ cell regulation, which has been described in several vertebrates. In mammals, the maturation of germ cells is correlated with a decrease of *Amh* expression in Sertoli cells. In tubules in which germ cells have initiated meiosis, Sertoli cells cease to produce Amh earlier than Sertoli cells with immature germ cells (Al-Attar et al. 1997). Interestingly, in the Japanese eel, the initiation of spermatogenesis and of spermatogonial proliferation correlated with the decrease of *amh* expression. This suggests that Amh is involved in suppressing spermatogenesis (Miura et al. 2002). We detected, in contrast, that the medaka *amh* expression persisted in Sertoli cells during maturation of the neighbouring germ cells (Klüver et al. 2007).

For comparison, I have isolated partial sequences of the *amh* gene in *O. celebensis* (for sequence alignments see Appendix A). The deduced amino acid sequences showed strong evolutionary relation to the medaka Amh, as expected (Fig. 24A). The *amh* expression in *O. celebensis* was detected at 5dpf-2dph and was restricted to the gonad in adult tissues (Fig. 24B, C). The testis specific *amh* expression was determined in the somatic cells (Fig. 24D), which is similar to *amh* in medaka (Klüver et al. 2007). Thus, *amh* is conserved in vertebrates and is involved during the process of gonad formation and maintenance. However, in teleost fish the function is so far not clear. It is likely that Amh is involved in gonad formation and germ cell regulation. Further investigations have to be done to specify the position of Amh

and AmhrII in the gene cascade of sex differentiation and to identify the upstream and downstream interaction partners.

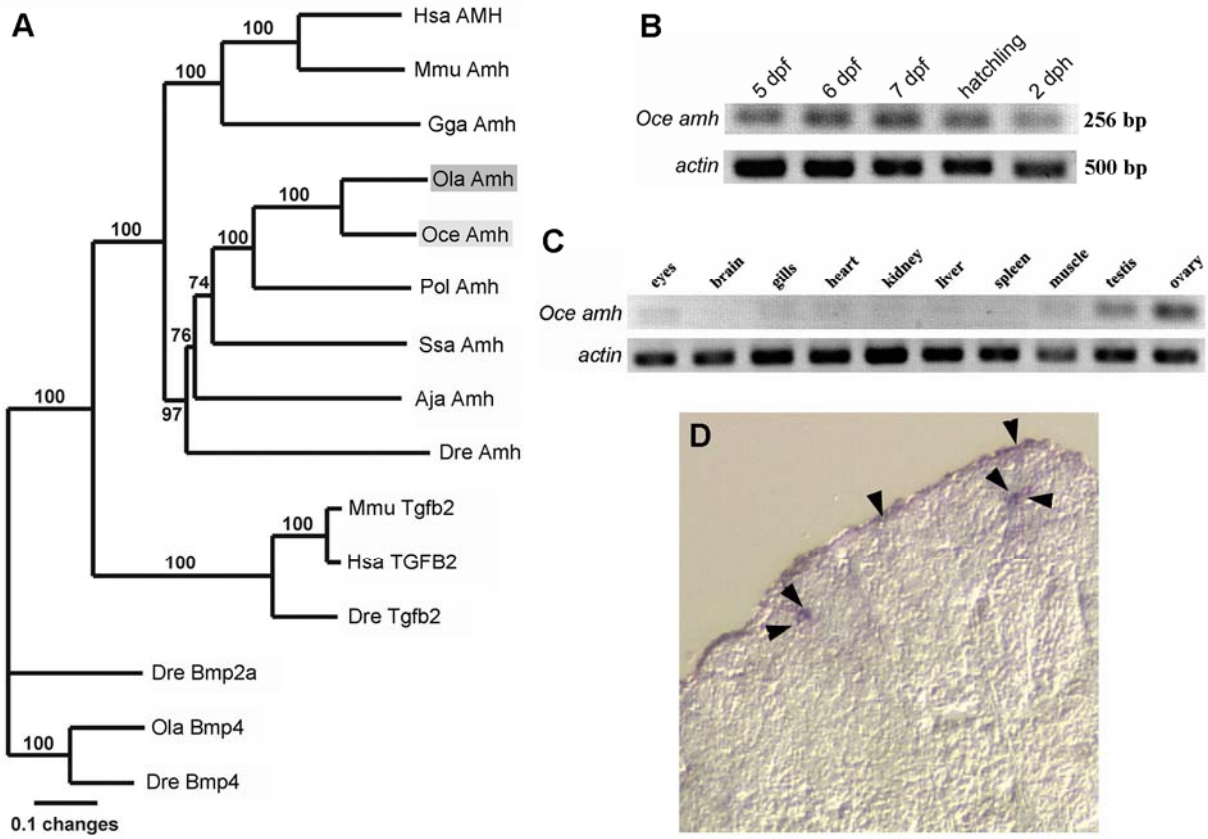


Fig. 24: Identification and expression of *amh* in *O. celebensis*. (A) Protein sequence relationships between the Amh family and other protein families belonging to the TGF-beta superfamily are shown in a phylogenetic tree. Numbers on each branch are the bootstrap values in thousand runs. Amh medaka and *O. celebensis* are shaded in dark grey and light grey, respectively. **(B)** Temporal *amh* expression during embryogenesis. The following primer combinations were used: OCe-AMH-F0 5´-ACAGCAGCCTCACCTTGTTT-3´ and OCe-AMH-R0 5´-GACCTCCAGGTCTCCAACAG-3´ amplify a 256 bp PCR product. A 500 bp fragment of the constitutively expressed actin gene was amplified with *act*F1 5´-GTAGGTGATGAAGCCCAGAGC-3´ and *act*R1 5´-AGGGAGCTCGTAGCTCTTCTC-3´ primers to quantify transcript amounts. PCR conditions were 30 cycles of 96°C for 30 s, 51°C for 30 s and 72°C for 30 s. **(C)** *Amh* expression in adult tissues. **(D)** *Amh* mRNA is localized in the somatic cells of the adult testis (arrowheads).

2.7. *Dmrt1* function in sex determination and/or sex differentiation in different teleosts

The doublesex and mab-3-related transcriptionfactors (*dmrts*) are a vertebrate gene family which encodes transcription factors with a characteristic DNA-binding motif, the DM domain (Hodgkin 2002; Volff et al. 2003b). DM domain proteins are widespread across the animal kingdom and are involved in sexual development. Functional and genetic evidences from different vertebrates support an important role of *dmrt1* in male sexual development. In mammals, Dmrt1 is essential for postnatal testis differentiation (Raymond et al. 2000). In addition, the human DMRT1, DMRT2 and DMRT3 are mapped to a region on chromosome 9

that is implicated in XY sex reversal. In the medaka the Y specific copy *dmrt1bY/dmy* is the male sex determining gene, which emerged approximately 10 mya only in the *latipes* species group of the genus *Oryzias*, including medaka and *Oryzias curvinotus* (Kobayashi et al. 2004; Kondo et al. 2006; Nanda et al. 2002; Takehana et al. 2007; Takehana et al. 2005). Aside from *dmrt1*, up to seven additional *dmrt* genes are present in mouse and human, and four to five in teleost fish (Ottolenghi et al. 2002; Volff et al. 2003b). We have recently analyzed the expression of *dmrt1*, *dmrt2a*, *dmrt4*, and *dmrt5* in the platyfish *Xiphophorus maculatus* (Veith et al. 2006). In particular, I have analyzed the *dmrt1* expression in the testis of *Xiphophorus maculatus* (Fig. 25). Fishes from the genus *Xiphophorus* are viviparous freshwater fishes living in the Atlantic drainages of waters in Belize, Guatemala, Honduras and Mexico (Morizot et al. 1991). However, the master sex-determining gene has yet not been identified. Non of the isolated *dmrt* genes in *Xiphophorus maculatus* localized on the X or Y chromosomes (Veith et al. 2003; Veith et al. 2006). The expression pattern analysis during embryogenesis showed that neither *dmrt1*, which was not expressed until 9.6d (stage 19), nor *dmrt2a*, *dmrt4* or *dmrt5* were expressed in the gonad primordium (Veith et al. 2006). Embryonic expression was detected in the somites (*dmrt2a*), branchial arches (*dmrt2a* and *dmrt4*), and in restricted regions of the CNS in the head (*dmrt4* and *dmrt5*). In adult tissues *dmrt1* was expressed only in the male gonads, whereas *dmrt2a* and *dmrt4* were expressed in gills, and *dmrt5* expression was detected in the brain and eyes (Veith et al. 2006). Interestingly, the *dmrt1* transcripts were detected in spermatogonia and Sertoli cells of the adult testis (Fig. 25B, C). This is in contrast to *dmrt1* expression of various fish species. The medaka, as already described, expresses *dmrt1a* and *dmrt1bY* in Sertoli cells (Kobayashi et al. 2004; Matsuda et al. 2002; Nanda et al. 2002). This has been also described for the Fugu *dmrt1* expression (Yamaguchi et al. 2006). However, the *dmrt1* expression in zebrafish and the marine orange-spotted grouper (*Epinephelus coioides*) is restricted to the germ cells, including spermatogonia, spermatocytes, and spematides (Guo et al. 2005; Xia et al. 2007). *Dmrt1* expression has divergent expression domains in the testis of various fish species. Hence, *dmrt1* has different cellular functions in either Sertoli cells or germ cells in the fish lineages.

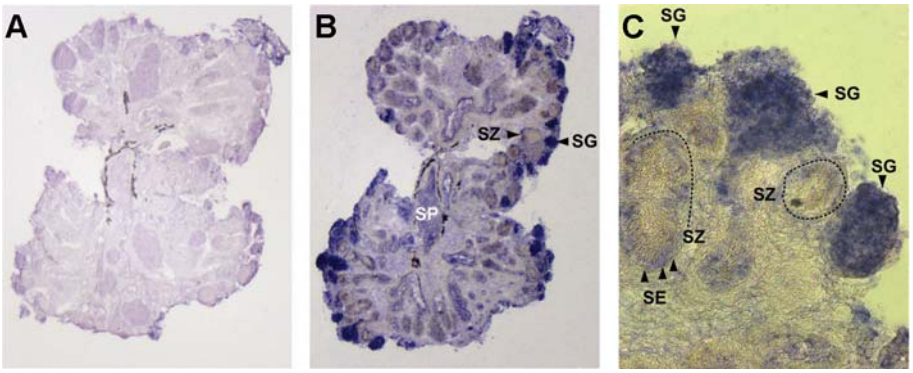


Fig. 25: *In situ* hybridization analysis of *dmrt1* expression in platyfish adult testis. (A) sense control. **(B+C)** antisense probe. SE, Sertoli cells; SG, spermatogonia; SP, free sperm; SZ, spermatozeugmata. (Adapted from Veith *et al*., 2006).

In *O. celebensis*, a *dmrt1* has been isolated (for sequence alignments see Appendix A), but no *dmrt1bY* ortholog is present in the genome (Kondo et al. 2003). The isolated Dmrt1 is more related to the Dmrt1a than to the Dmrt1bY of medaka (Fig. 26A). However, no expression analysis of *dmrt1* in *O. celebensis* has been conducted. Therefore, I have analyzed the *dmrt1* expression of *O. celebensis* during gonad development. In contrast to the medaka, no sex specific markers for *O. celebensis* are available. Nevertheless, I performed single embryo expression analysis. In several embryos, I observed no *dmrt1* expression at 5dpf and an increased expression in about half of the embryos at 6dpf (Fig. 26B). At 7dpf and hatchlings, the expression was still detectable in a subset of embryos (Fig. 26B). In adult gonads of *O. celebensis*, *dmrt1* is expressed in the somatic cell of the testis and in the cytoplasm of developing oocytes (Fig 26C, D). Expression of *dmrt1* in the ovary has been described for mammals and also in different fish species (Guo et al. 2005; Liu et al. 2007; Pask et al. 2003; Xia et al. 2007).

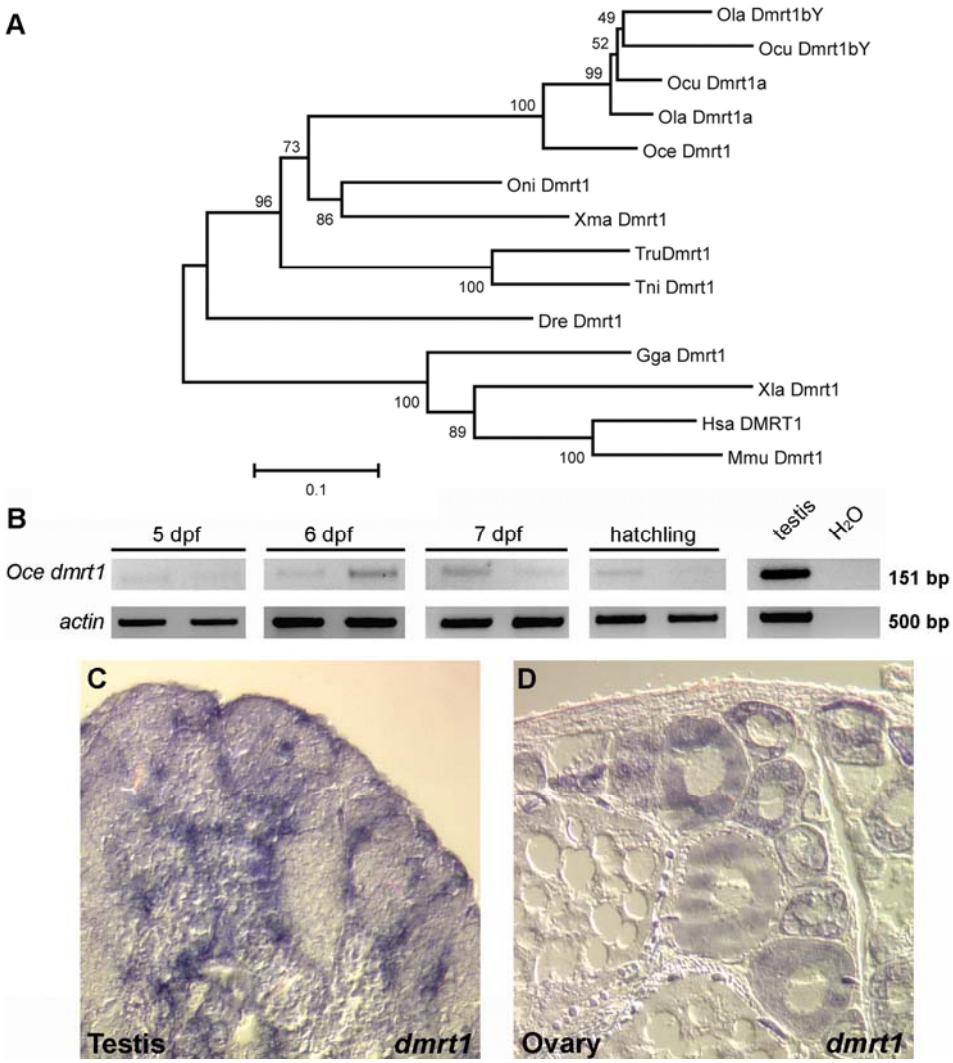


Fig. 26: *O. celebensis dmrt1* expression during development and in adult gonads. (A) Phylogenetic tree of known Dmrt1-related sequences from vertebrates constructed by the neighbour-joining method. Numbers indicate Bootstrap values in thousand replicates. **(B)** *Dmrt1* expression in different stages of development, determined by single embryo RT-PCR. The following primer combinations were used: OceDMRT1F0B 5´-CGCCAAATGCAAGCTGAT-3´ OceDmrt1-R01 5´-GGCTCCAGCTTCATTCTTCA-3´ amplify a 151 bp PCR product. A 500 bp fragment of the constitutively expressed actin gene was amplified with *act*F1 5´-GTAGGTGATGAAGCCCAGAGC-3´and *act*R1 5´-AGGGAGCTCGTAGCTCTTCTC-3´ primers to quantify transcript amounts. PCR conditions were 25 cycles of 96°C for 30 s, 53°C for 30 s and 72°C for 25s. **(C)** *Dmrt1* expression in adult testis is detected in somatic cells. **(D)** *Dmrt1* mRNA in adult ovary is localized in the cytoplasm of developing oocytes.

For comparison, I have investigated the expression of *dmrt1bY*/*dmrt1a* in hatchling and testis and *dmrt1a* in the ovary of medaka (Fig 27). In several hatched embryos (~ 9dpf), *dmrt1bY* is expressed in somatic cells of the developing gonad (Fig. 27A). This is in line with the described *dmrt1bY* expression in male hatchlings (Kobayashi et al. 2004; Matsuda et al. 2002). In medaka *dmrt1a* and *dmrt1bY* expression was restricted to somatic cells of the testis, which seem to be Sertoli cells (Fig. 27B). The expression is similar to *O. celebensis dmrt1* expression (see above). Conversely, transcripts of medaka *dmrt1a* in the ovary were localized

in the granulosa cells (Fig. 27C), whereas in *O. celebensis dmrt1* was expressed in the cytoplasm of developing oocytes. Interestingly, in teleost fish like the zebrafish *dmrt1* expression in the adult ovary is restricted to the germ cells. Others fishes do not express *dmrt1* in the ovary, as we have described for *X. maculatus* (Guo et al. 2005; Veith et al. 2006). This shows that *dmrt1* expression has divergent expression domains in the ovary and testis, as described above. In fish, *dmrt1* mRNA is expressed only in Sertoli cells, or only in germ cells, or in both (Guo et al. 2005; Kobayashi et al. 2004; Veith et al. 2006; Xia et al. 2007). In addition in mouse, *dmrt1* is expressed in pre-meiotic germ cells and in Sertoli cells, whereas in humans DMRT1 is restricted to germ cells in adult testis (Kim et al. 2007; Looijenga et al. 2006). Although it is not known how common combined Sertoli and germ cell expression of Dmrt1 may be among vertebrates.

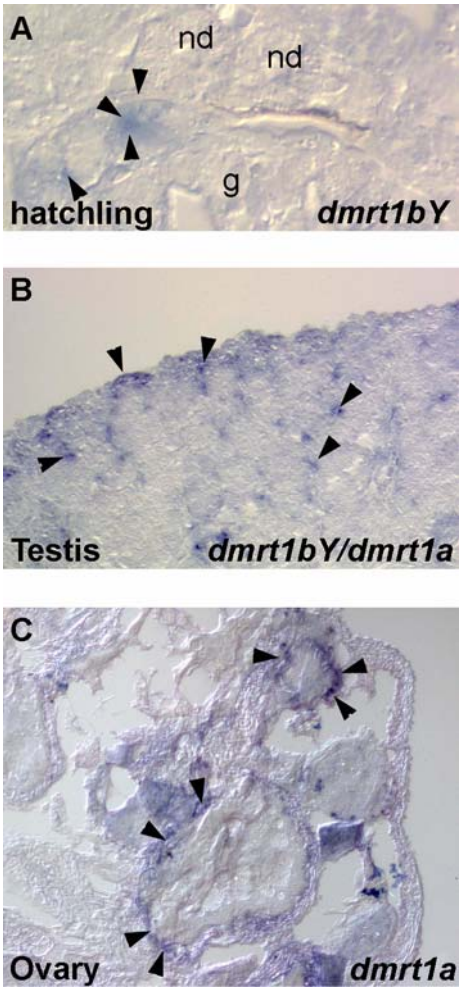


Fig. 27: Medaka *dmrt1bY* and *dmrt1a* expression in hatchling and adult gonads. (A) *Dmrt1bY* mRNA is localized in somatic cells surrounding germ cells in hatched XY embryos (arrowheads). **(B)** Overlapping expression of *dmrt1bY* and *dmrt1a* in the somatic cells of the testis periphery (arrowheads). **(C)** *Dmrt1a* is expressed in the granulosa cells, which surround the developing oocytes (arrowheads). g, gut; nd, nephric duct.

However, the embryonic *dmrt1* expression in *O. celebensis* is similar to the expression pattern for *dmrt1bY* in medaka and is in contrast to the autosomal *dmrt1a*, which is expressed very late, around 20dph (Kobayashi et al. 2004; Nanda et al. 2002). In medaka *dmrt1bY* is involved in repressing the germ cell proliferation in XY embryos, which have been shown in *dmrt1bY* mutants and by loss-of- and gain-of-function studies (Matsuda et al. 2007; Paul-Prasanth et al. 2006). Interestingly, in *O. celebensis* after hatching, two types of gonads can be distinguished, one contained germ cells in meiotic prophase as well as those in the interphase, and the other had only germ cells in the interphase (Hamaguchi 1983). Observations of gonads at later stages revealed that the former is the presumptive ovary and the latter is the presumptive testis. In the presumptive ovary a larger number of germ cells is detectable than in the presumptive testis. Thereafter, the proliferation of germ cells proceeds in the females, whereas in the presumptive testis, the number of germ cells hardly increased until 20dph (Hamaguchi 1983). This suggests that *dmrt1* in *O. celebensis* maybe involved in the regulation of germ cell proliferation. Furthermore, between *dmrt1bY* and *dmrt1a* in medaka subfunctionalization might have taken place and the function of *dmrt1* in *O. celebensis* reflects the more ancestral situation, in which *dmrt1* is already expressed in the undifferentiated gonad and regulates germ cell proliferation. However, detailed analysis in *O. celebensis* has to be undertaken to clarify the localization of *dmrt1* expression during gonad development and the sex determining mechanism (XX-XY or ZW-ZZ mechanism) to provide more evidences for the sex determination and differentiation processes in the genus *Oryzias*.

3. Conclusions and Perspectives

The gonad, including germ cells and associated supporting somatic cells, in fish is similar to other vertebrates. In this thesis, I have studied gonad development in the medaka (*Oryzias latipes*) and in the closely related species *Oryzias celebensis*, which has no *dmrt1bY* as male sex-determining gene. In medaka, it seems that PGC specification is dependent on the inheritance of maternally provided cytoplasmatic determinants (Herpin et al. 2007). In zebrafish (*Danio rerio*), the germ plasm contains *vasa* and *nanos* mRNAs. The germ plasm is localized in distal parts of the first two cleavage planes (Knaut et al. 2000; Koprunner et al. 2001). I have shown that during the early phases of embryonic development *nanos* mRNA is evenly distributed, like *olvas* mRNA. This suggests differences in PGC specification in zebrafish and medaka. If the Nanos and Olvas proteins in medaka are components of the germ plasm like structures has to be analysed in further protein localization studies.

As in many other eukaryotes, the PGCs in fish embryos are specified at some distances from the prospective gonad region and have to migrate towards the gonad (Herpin et al. 2007; Kurokawa et al. 2006; Raz 2002). During the migration phase in medaka, I analyzed the expression pattern of *sdf-1a*. In early phases of embryonic development, I could show that *sdf-1a* and *sdf-1b* mRNA were accumulating in regions where PGCs are found. Later during development, *sdf-1a* is expressed in newly formed somites and *sdf-1b* in the region of the developing gonad. This, together with functional analysis, suggests that Sdf-1a action is primarily involved in the early phases of PGC migration and Sdf-1b is the guidance cue for PGCs to colonize the gonad.

While germ cell specific genes have been described in medaka (Shinomiya et al. 2000) no germ cell marker was known for *O. celebensis*. I have isolated the *vasa* gene homolog in *O. celebensis* and the expression analysis showed a specific transcription in the spermatogonia and developing oocytes in males and females, respectively. This expression-based analysis suggests that *vasa* in *O. celebensis* has a similar function as the *olvas* gene in medaka (Shinomiya et al. 2000; Tanaka et al. 2001) and is suitable as a germ cell specific marker.

Additionally, I have analyzed the PGC migration by injection of GFP-z*fnos*3'UTR mRNA. The results indicate that the PGC migration is similar to the PGC migration in medaka (Kurokawa et al. 2006; Shinomiya et al. 2000; Tanaka et al. 2001).

Many genes that are important in gonadal sex differentiation in mammals, such as *Amh*, *Sox9*, *Wt1*, and *Dmrt1*, are conserved in other vertebrates and show gonad-specific expression during sexual differentiation (Morrish and Sinclair 2002; Wilhelm et al. 2007). Here, I have shown that in the medaka and in *O. celebensis wt1a* and *sox9b* are expressed during early

gonad development of both sexes. This suggests that these genes are involved in the development of the gonad primordium. Additionally, I have analyzed the expression pattern of *amh* and *amhrII*. Both genes are not sexually dimorphic expressed in somatic cells of the developing gonad (Klüver et al. 2007). This suggests that *amh* and *amhrII* are involved in gonad development and have specific functions in the adult gonad. Recently, it was shown that a point mutation in the kinase domain of *amhrII* leads to a dramatic increase in germ cell proliferation (Morinaga et al. 2007). This suggests that Amh/AmhrII signaling is involved in the regulation of germ cell proliferation. Whether AmhrII is the receptor for Amh or not has to be investigated in further protein interaction analyses.

My results provided new insights into the gonad development of the medaka (Fig. 28) and first molecular findings in gonad development of *O. celebensis*. It also could be shown that *sox9b*, *amh* and *amhrII* have influences on gonad development of both sexes more than the sex determination of the male pathway, like it has been described in mammals (Morrish and Sinclair 2002; Wilhelm et al. 2007). However, later during sex differentiation *sox9b* expression became testis specific. Additionally, I have shown that the overexpression of *sox9b* resulted in ectopic expression of *dmrt1a*. Whether this is a specific effect of *sox9b* RNA injection has to be analyzed in more detail in future.

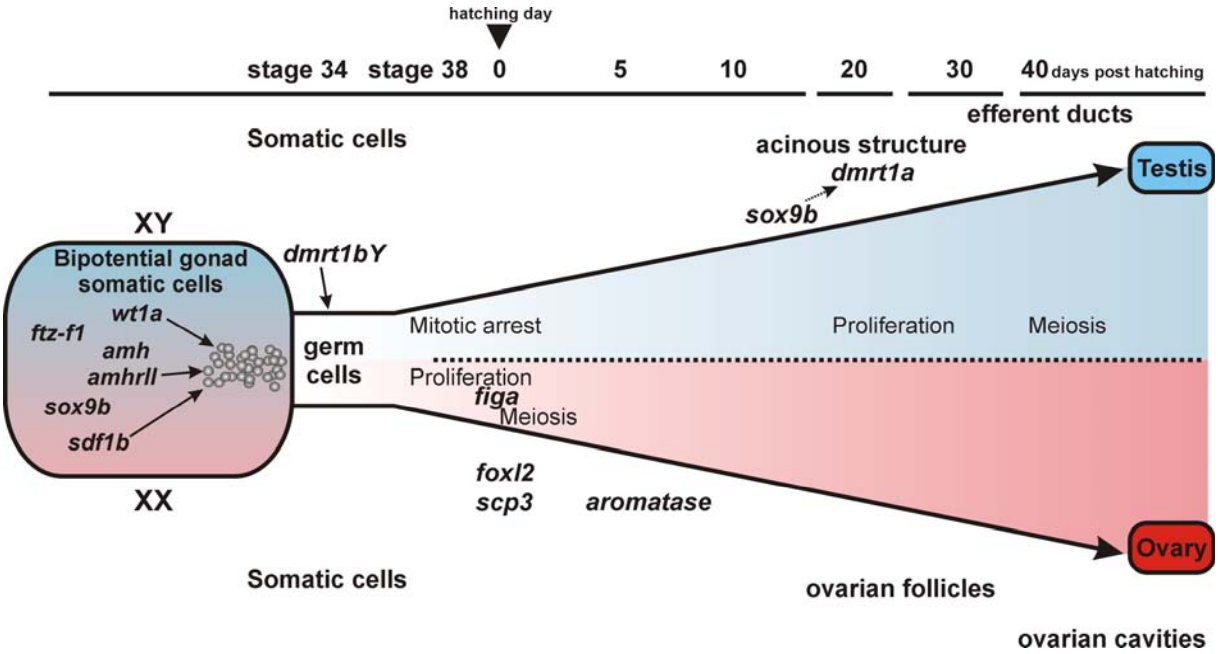


Fig. 28: Updated schematic illustration of sexual development in medaka. In XX and XY undifferentiated gonads *amh/amhrII*, *ftz-f1* (*sf1*), *sdf-1b*, *sox9b*, and *wt1a* are expressed in somatic cells. Amh/AmhrII action is involved in germ cell proliferation. Wt1a is involved in germ cells maintenance and/or survival. Sdf-1b is involved in later steps of PGC migration. Sox9b function in the undifferentiated gonad is so far unclear. During later sex differentiation, *sox9b* expression became male specific and might be involved in activation of *dmrt1a*. PGCs (grey circles). Arrows indicate direct influence on PGCs. Arrow with dashed line display suggested function. Modified after Matsuda, 2005.

During my thesis, I could show by functional analyses of *wt1* genes in the medaka that a conditional co-regulation of *wt1* genes ensures PGC survival and/or maintenance. My data suggests that Wt1a represses *wt1b* expression during early development (Klüver et al., submitted). Therefore, promoter analysis should be done to clarify details of the cross talk between the *wt1* co-orthologs. Wt1b can compensate the knockdown of Wt1a and ensures PGC maintenance and/or survival during gonad development, but not pronephros development. The function is here divergent. The regulatory cross talk between *wt1* co-orthologs in medaka might result in an advantage in fitness due to its direct influence on medaka fertility. Whether this conditional co-regulation of *wt1* co-orthologs is specific for teleost fish species or has developed in a lineage-specific manner in the medaka has to be determined.

The genetic mechanisms triggering sex determination appear to be diverse in nonmammalian vertebrates, especially in fish. The analyzed expression pattern of *dmrt1* in *O. celebensis* during development shows already an expression at 6dpf in about half of the embryos. This is similar to the *dmrt1bY* expression in medaka (Kobayashi et al. 2004). These results together with the assumed inhibitory function of *dmrt1bY* in PGC proliferation (Kobayashi et al. 2004; Matsuda et al. 2007), suggests that *dmrt1* in *O. celebensis* can be involved in the regulation of germ cell proliferation. Furthermore, between *dmrt1bY* and *dmrt1a* in medaka a subfunctionalization might have taken place. The function of *dmrt1* in *O. celebensis* reflects the more ancestral situation, in which *dmrt1* is already expressed in undifferentiated gonad and regulates germ cell proliferation. Whether the expression of *dmrt1* is male specific in *O. celebensis* is currently under investigation. Further studies of the *dmrt1* expression during gonad development and functional analysis in *O. celebensis* should provide supportive information.

Whether the duplication of the autosomal *dmrt1* in medaka that generated the male sex-determining gene *dmrt1bY* has led to a change of the sex-determining genetic cascade or not has to be investigated. Therefore, additional analyses of gene expression patterns and their functional analysis in medaka and closely related species of the genus *Oryzias* should help to decipher the genetic network of sex determination.

4. References

Aida, T. 1921. On the Inheritance of Color in a Fresh-Water Fish, APLOCHEILUS LATIPES Temmick and Schlegel, with Special Reference to Sex-Linked Inheritance. *Genetics* **6:** 554-573.

Al-Attar, L., K. Noel, M. Dutertre, C. Belville, M.G. Forest, P.S. Burgoyne, N. Josso, and R. Rey. 1997. Hormonal and cellular regulation of Sertoli cell anti-Mullerian hormone production in the postnatal mouse. *J Clin Invest* **100:** 1335-1343.

Altschmied, J., J. Delfgaauw, B. Wilde, J. Duschl, L. Bouneau, J.N. Volff, and M. Schartl. 2002. Subfunctionalization of duplicate mitf genes associated with differential degeneration of alternative exons in fish. *Genetics* **161:** 259-267.

Amores, A., A. Force, Y.L. Yan, L. Joly, C. Amemiya, A. Fritz, R.K. Ho, J. Langeland, V. Prince, Y.L. Wang, M. Westerfield, M. Ekker, and J.H. Postlethwait. 1998. Zebrafish hox clusters and vertebrate genome evolution. *Science* **282:** 1711-1714.

Ara, T., Y. Nakamura, T. Egawa, T. Sugiyama, K. Abe, T. Kishimoto, Y. Matsui, and T. Nagasawa. 2003. Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1). *Proc Natl Acad Sci U S A* **100:** 5319-5323.

Arango, N.A., R. Lovell-Badge, and R.R. Behringer. 1999. Targeted mutagenesis of the endogenous mouse Mis gene promoter: in vivo definition of genetic pathways of vertebrate sexual development. *Cell* **99:** 409-419.

Ason, B., D.K. Darnell, B. Wittbrodt, E. Berezikov, W.P. Kloosterman, J. Wittbrodt, P.B. Antin, and R.H. Plasterk. 2006. Differences in vertebrate microRNA expression. *Proc Natl Acad Sci U S A* **103:** 14385-14389.

Baarends, W.M., J.W. Hoogerbrugge, M. Post, J.A. Visser, D.G. De Rooij, M. Parvinen, A.P. Themmen, and J.A. Grootegoed. 1995a. Anti-mullerian hormone and anti-mullerian hormone type II receptor messenger ribonucleic acid expression during postnatal testis development and in the adult testis of the rat. *Endocrinology* **136:** 5614-5622.

Baarends, W.M., J.T. Uilenbroek, P. Kramer, J.W. Hoogerbrugge, E.C. van Leeuwen, A.P. Themmen, and J.A. Grootegoed. 1995b. Anti-mullerian hormone and anti-mullerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. *Endocrinology* **136:** 4951-4962.

Barbaux, S., P. Niaudet, M.C. Gubler, J.P. Grunfeld, F. Jaubert, F. Kuttenn, C.N. Fekete, N. Souleyreau-Therville, E. Thibaud, M. Fellous, and K. McElreavey. 1997. Donor splice-site mutations in WT1 are responsible for Frasier syndrome. *Nat Genet* **17:** 467-470.

Behringer, R.R., M.J. Finegold, and R.L. Cate. 1994. Mullerian-inhibiting substance function during mammalian sexual development. *Cell* **79:** 415-425.

Berra, T.M. 2001. *Freshwater fish distribution*. Academic Press, San Diego, Calif.

Bollig, F., R. Mehringer, B. Perner, C. Hartung, M. Schafer, M. Schartl, J.N. Volff, C. Winkler, and C. Englert. 2006. Identification and comparative expression analysis of a second wt1 gene in zebrafish. *Dev Dyn* **235:** 554-561.

Bullejos, M. and P. Koopman. 2005. Delayed Sry and Sox9 expression in developing mouse gonads underlies B6-Y(DOM) sex reversal. *Dev Biol* **278:** 473-481.

Charlesworth, B. 2002. The evolution of chromosomal sex determination. *Novartis Found Symp* **244:** 207-219; discussion 220-204, 253-207.

Chiang, E.F., C.I. Pai, M. Wyatt, Y.L. Yan, J. Postlethwait, and B. Chung. 2001. Two sox9 genes on duplicated zebrafish chromosomes: expression of similar transcription activators in distinct sites. *Dev Biol* **231:** 149-163.

Cresko, W.A., Y.L. Yan, D.A. Baltrus, A. Amores, A. Singer, A. Rodriguez-Mari, and J.H. Postlethwait. 2003. Genome duplication, subfunction partitioning, and lineage divergence: Sox9 in stickleback and zebrafish. *Dev Dyn* **228:** 480-489.

De Santa Barbara, P., N. Bonneaud, B. Boizet, M. Desclozeaux, B. Moniot, P. Sudbeck, G. Scherer, F. Poulat, and P. Berta. 1998. Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Mullerian hormone gene. *Mol Cell Biol* **18:** 6653-6665.

de Santa Barbara, P., B. Moniot, F. Poulat, and P. Berta. 2000. Expression and subcellular localization of SF-1, SOX9, WT1, and AMH proteins during early human testicular development. *Dev Dyn* **217:** 293-298.

Devlin, R.H. and Y. Nagahama. 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* **208:** 191-364.

di Clemente, N., S. Ghaffari, R.B. Pepinsky, C. Pieau, N. Josso, R.L. Cate, and B. Vigier. 1992. A quantitative and interspecific test for biological activity of anti-mullerian hormone: the fetal ovary aromatase assay. *Development* **114:** 721-727.

Doitsidou, M., M. Reichman-Fried, J. Stebler, M. Koprunner, J. Dorries, D. Meyer, C.V. Esguerra, T. Leung, and E. Raz. 2002. Guidance of primordial germ cell migration by the chemokine SDF-1. *Cell* **111:** 647-659.

Eicher, E. 1982. Primary sex determining genes in mice. In *Prospects for Sexing Mammalian Sperm* (ed. A.R.a.S.G. Boulder). Colorado Associated Univ. Press.

Force, A., M. Lynch, F.B. Pickett, A. Amores, Y.L. Yan, and J. Postlethwait. 1999. Preservation of duplicate genes by complementary, degenerative mutations. *Genetics* **151:** 1531-1545.

Fredga, K. 1983. Aberrant sex chromosome mechanisms in mammals. Evolutionary aspects. *Differentiation* **23 Suppl:** S23-30.

Gao, F., S. Maiti, N. Alam, Z. Zhang, J.M. Deng, R.R. Behringer, C. Lecureuil, F. Guillou, and V. Huff. 2006. The Wilms tumor gene, Wt1, is required for Sox9 expression and maintenance of tubular architecture in the developing testis. *Proc Natl Acad Sci U S A* **103:** 11987-11992.

Gasca, S., J. Canizares, P. De Santa Barbara, C. Mejean, F. Poulat, P. Berta, and B. Boizet-Bonhoure. 2002. A nuclear export signal within the high mobility group domain regulates the nucleocytoplasmic translocation of SOX9 during sexual determination. *Proc Natl Acad Sci U S A* **99:** 11199-11204.

Gessler, M., A. Poustka, W. Cavenee, R.L. Neve, S.H. Orkin, and G.A. Bruns. 1990. Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. *Nature* **343:** 774-778.

Gilbert, S.F. 2006. *Developmental Biology*. Sunderland: Sinauer Associates, Inc.

Giuili, G., W.H. Shen, and H.A. Ingraham. 1997. The nuclear receptor SF-1 mediates sexually dimorphic expression of Mullerian Inhibiting Substance, in vivo. *Development* **124:** 1799-1807.

Gubbay, J., P. Koopman, J. Collignon, P. Burgoyne, and R. Lovell-Badge. 1990. Normal structure and expression of Zfy genes in XY female mice mutant in Tdy. *Development* **109:** 647-653.

Guo, Y., H. Cheng, X. Huang, S. Gao, H. Yu, and R. Zhou. 2005. Gene structure, multiple alternative splicing, and expression in gonads of zebrafish Dmrt1. *Biochem Biophys Res Commun* **330:** 950-957.

Haber, D.A., R.L. Sohn, A.J. Buckler, J. Pelletier, K.M. Call, and D.E. Housman. 1991. Alternative splicing and genomic structure of the Wilms tumor gene WT1. *Proc Natl Acad Sci U S A* **88:** 9618-9622.

Hamaguchi, S. 1982. A light- and electron-microscopic study on the migration of primordial germ cells in the teleost, Oryzias latipes. *Cell and Tissue Research* **227:** 139-151.

Hamaguchi, S. 1983. Asymmetrical Development of the Gonads in the Embryos and Fry of the Fish, *Oryzias celebensis*. *Development, Growth & Differentiation* **25:** 553–561.

Hammes, A., J.K. Guo, G. Lutsch, J.R. Leheste, D. Landrock, U. Ziegler, M.C. Gubler, and A. Schedl. 2001. Two splice variants of the Wilms' tumor 1 gene have distinct functions during sex determination and nephron formation. *Cell* **106:** 319-329.

Harley, V.R., D.I. Jackson, P.J. Hextall, J.R. Hawkins, G.D. Berkovitz, S. Sockanathan, R. Lovell-Badge, and P.N. Goodfellow. 1992. DNA binding activity of recombinant SRY from normal males and XY females. *Science* **255:** 453-456.

Hay, B., L. Ackerman, S. Barbel, L.Y. Jan, and Y.N. Jan. 1988. Identification of a component of Drosophila polar granules. *Development* **103:** 625-640.

Hayashi, K., S.M. de Sousa Lopes, and M.A. Surani. 2007. Germ cell specification in mice. *Science* **316:** 394-396.

He, X. and J. Zhang. 2005. Rapid subfunctionalization accompanied by prolonged and substantial neofunctionalization in duplicate gene evolution. *Genetics* **169:** 1157-1164.

Healy, C., D. Uwanogho, and P.T. Sharpe. 1999. Regulation and role of Sox9 in cartilage formation. *Dev Dyn* **215:** 69-78.

Herpin, A., S. Rohr, D. Riedel, N. Klüver, E. Raz, and M. Schartl. 2007. Specification of primordial germ cells in medaka (Oryzias latipes). *BMC Dev Biol* **7:** 3.

Hodgkin, J. 2002. The remarkable ubiquity of DM domain factors as regulators of sexual phenotype: ancestry or aptitude? *Genes Dev* **16:** 2322-2326.

Houston, D.W. and M.L. King. 2000. Germ plasm and molecular determinants of germ cell fate. *Curr Top Dev Biol* **50:** 155-181.

Hsu, H.J., G. Lin, and B.C. Chung. 2003. Parallel early development of zebrafish interrenal glands and pronephros: differential control by wt1 and ff1b. *Development* **130:** 2107-2116.

Jager, R.J., V.R. Harley, R.A. Pfeiffer, P.N. Goodfellow, and G. Scherer. 1992. A familial mutation in the testis-determining gene SRY shared by both sexes. *Hum Genet* **90:** 350-355.

Josso, N. and N. Clemente. 2003. Transduction pathway of anti-Mullerian hormone, a sex-specific member of the TGF-beta family. *Trends Endocrinol Metab* **14:** 91-97.

Josso, N., N. di Clemente, and L. Gouedard. 2001. Anti-Mullerian hormone and its receptors. *Mol Cell Endocrinol* **179:** 25-32.

Kallman, K. 1984. A new look at sex determination in poeciliid fishes. In *Evolutionary Genetics of Fishes* (ed. T. BJ), pp. 95–171. Plenum Publishing,, New York.

Kasahara, M., K. Naruse, S. Sasaki, Y. Nakatani, W. Qu, B. Ahsan, T. Yamada, Y. Nagayasu, K. Doi, Y. Kasai, T. Jindo, D. Kobayashi, A. Shimada, A. Toyoda, Y. Kuroki, A. Fujiyama, T. Sasaki, A. Shimizu, S. Asakawa, N. Shimizu, S. Hashimoto, J. Yang, Y. Lee, K. Matsushima, S. Sugano, M. Sakaizumi, T. Narita, K. Ohishi, S. Haga, F. Ohta, H. Nomoto, K. Nogata, T. Morishita, T. Endo, I.T. Shin, H. Takeda, S. Morishita, and Y. Kohara. 2007. The medaka draft genome and insights into vertebrate genome evolution. *Nature* **447:** 714-719.

Kent, J., A.M. Coriat, P.T. Sharpe, N.D. Hastie, and V. van Heyningen. 1995. The evolution of WT1 sequence and expression pattern in the vertebrates. *Oncogene* **11:** 1781-1792.

Kent, J., S.C. Wheatley, J.E. Andrews, A.H. Sinclair, and P. Koopman. 1996. A male-specific role for SOX9 in vertebrate sex determination. *Development* **122:** 2813-2822.

Kim, S., V.J. Bardwell, and D. Zarkower. 2007. Cell type-autonomous and non-autonomous requirements for Dmrt1 in postnatal testis differentiation. *Dev Biol* **307:** 314-327.

Klamt, B., A. Koziell, F. Poulat, P. Wieacker, P. Scambler, P. Berta, and M. Gessler. 1998. Frasier syndrome is caused by defective alternative splicing of WT1 leading to an altered ratio of WT1 +/-KTS splice isoforms. *Hum Mol Genet* **7:** 709-714.

Klüver, N., A. Herpin, I. Braasch, J. Drießle, and M. Schartl. Regulatory Back-up Circuit of Medaka Wt1 Co-Orthologs Ensures PGC Maintenance. *in preparation*.

Klüver, N., M. Kondo, A. Herpin, H. Mitani, and M. Schartl. 2005. Divergent expression patterns of Sox9 duplicates in teleosts indicate a lineage specific subfunctionalization. *Dev Genes Evol* **215:** 297-305.

Klüver, N., F. Pfennig, I. Pala, K. Storch, M. Schlieder, A. Froschauer, H.O. Gutzeit, and M. Schartl. 2007. Differential expression of anti-Mullerian hormone (amh) and anti-Mullerian hormone receptor type II (amhrII) in the teleost medaka. *Dev Dyn* **236:** 271-281.

Knaut, H., F. Pelegri, K. Bohmann, H. Schwarz, and C. Nusslein-Volhard. 2000. Zebrafish vasa RNA but not its protein is a component of the germ plasm and segregates asymmetrically before germline specification. *J Cell Biol* **149:** 875-888.

Knaut, H., C. Werz, R. Geisler, and C. Nusslein-Volhard. 2003. A zebrafish homologue of the chemokine receptor Cxcr4 is a germ-cell guidance receptor. *Nature* **421:** 279-282.

Kobayashi, T., M. Matsuda, H. Kajiura-Kobayashi, A. Suzuki, N. Saito, M. Nakamoto, N. Shibata, and Y. Nagahama. 2004. Two DM domain genes, DMY and DMRT1, involved in testicular differentiation and development in the medaka, Oryzias latipes. *Dev Dyn* **231:** 518-526.

Kondo, M., U. Hornung, I. Nanda, S. Imai, T. Sasaki, A. Shimizu, S. Asakawa, H. Hori, M. Schmid, N. Shimizu, and M. Schartl. 2006. Genomic organization of the sex-determining and adjacent regions of the sex chromosomes of medaka. *Genome Res* **16:** 815-826.

Kondo, M., I. Nanda, U. Hornung, S. Asakawa, N. Shimizu, H. Mitani, M. Schmid, A. Shima, and M. Schartl. 2003. Absence of the candidate male sex-determining gene dmrt1b(Y) of medaka from other fish species. *Curr Biol* **13:** 416-420.

Kondo, M., I. Nanda, U. Hornung, M. Schmid, and M. Schartl. 2004. Evolutionary origin of the medaka Y chromosome. *Curr Biol* **14:** 1664-1669.

Koopman, P., A. Munsterberg, B. Capel, N. Vivian, and R. Lovell-Badge. 1990. Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature* **348:** 450-452.

Koprunner, M., C. Thisse, B. Thisse, and E. Raz. 2001. A zebrafish nanos-related gene is essential for the development of primordial germ cells. *Genes Dev* **15:** 2877-2885.

Kreidberg, J.A., H. Sariola, J.M. Loring, M. Maeda, J. Pelletier, D. Housman, and R. Jaenisch. 1993. WT-1 is required for early kidney development. *Cell* **74:** 679-691.

Kurokawa, H., Y. Aoki, S. Nakamura, Y. Ebe, D. Kobayashi, and M. Tanaka. 2006. Time-lapse analysis reveals different modes of primordial germ cell migration in the medaka Oryzias latipes. *Dev Growth Differ* **48:** 209-221.

Lasala, C., D. Carre-Eusebe, J.Y. Picard, and R. Rey. 2004. Subcellular and molecular mechanisms regulating anti-Mullerian hormone gene expression in mammalian and nonmammalian species. *DNA Cell Biol* **23:** 572-585.

Liu, Z., F. Wu, B. Jiao, X. Zhang, C. Hu, B. Huang, L. Zhou, X. Huang, Z. Wang, Y. Zhang, Y. Nagahama, C.H. Cheng, and D. Wang. 2007. Molecular cloning of doublesex and mab-3-related transcription factor 1, forkhead transcription factor gene 2, and two types of cytochrome P450 aromatase in Southern catfish and their possible roles in sex differentiation. *J Endocrinol* **194:** 223-241.

Looijenga, L.H., R. Hersmus, A.J. Gillis, R. Pfundt, H.J. Stoop, R.J. van Gurp, J. Veltman, H.B. Beverloo, E. van Drunen, A.G. van Kessel, R.R. Pera, D.T. Schneider, B. Summersgill, J. Shipley, A. McIntyre, P. van der Spek, E. Schoenmakers, and J.W.

Oosterhuis. 2006. Genomic and expression profiling of human spermatocytic seminomas: primary spermatocyte as tumorigenic precursor and DMRT1 as candidate chromosome 9 gene. *Cancer Res* **66:** 290-302.

Lynch, M. and A. Force. 2000. The probability of duplicate gene preservation by subfunctionalization. *Genetics* **154:** 459-473.

Maack, G. and H. Segner. 2004. Life-stage-dependent sensitivity of zebrafish (Danio rerio) to estrogen exposure. *Comp Biochem Physiol C Toxicol Pharmacol* **139:** 47-55.

Malik, K.T., V. Poirier, S.M. Ivins, and K.W. Brown. 1994. Autoregulation of the human WT1 gene promoter. *FEBS Lett* **349:** 75-78.

Matsuda, M., Y. Nagahama, A. Shinomiya, T. Sato, C. Matsuda, T. Kobayashi, C.E. Morrey, N. Shibata, S. Asakawa, N. Shimizu, H. Hori, S. Hamaguchi, and M. Sakaizumi. 2002. DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature* **417:** 559-563.

Matsuda, M., A. Shinomiya, M. Kinoshita, A. Suzuki, T. Kobayashi, B. Paul-Prasanth, E.L. Lau, S. Hamaguchi, M. Sakaizumi, and Y. Nagahama. 2007. DMY gene induces male development in genetically female (XX) medaka fish. *Proc Natl Acad Sci U S A* **104:** 3865-3870.

Meyer, A. and M. Schartl. 1999. Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. *Curr Opin Cell Biol* **11:** 699-704.

Meyer, A. and Y. Van de Peer. 2005. From 2R to 3R: evidence for a fish-specific genome duplication (FSGD). *Bioessays* **27:** 937-945.

Mishima, Y., A.J. Giraldez, Y. Takeda, T. Fujiwara, H. Sakamoto, A.F. Schier, and K. Inoue. 2006. Differential regulation of germline mRNAs in soma and germ cells by zebrafish miR-430. *Curr Biol* **16:** 2135-2142.

Miura, T., C. Miura, Y. Konda, and K. Yamauchi. 2002. Spermatogenesis-preventing substance in Japanese eel. *Development* **129:** 2689-2697.

Molyneaux, K.A., H. Zinszner, P.S. Kunwar, K. Schaible, J. Stebler, M.J. Sunshine, W. O'Brien, E. Raz, D. Littman, C. Wylie, and R. Lehmann. 2003. The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. *Development* **130:** 4279-4286.

Morais da Silva, S., A. Hacker, V. Harley, P. Goodfellow, A. Swain, and R. Lovell-Badge. 1996. Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. *Nat Genet* **14:** 62-68.

Morinaga, C., D. Saito, S. Nakamura, T. Sasaki, S. Asakawa, N. Shimizu, H. Mitani, M. Furutani-Seiki, M. Tanaka, and H. Kondoh. 2007. The hotei mutation of medaka in the anti-Mullerian hormone receptor causes the dysregulation of germ cell and sexual development. *Proc Natl Acad Sci U S A* **104:** 9691-9696.

Morizot, D.C., S.A. Slaugenhaupt, K.D. Kallman, and A. Chakravarti. 1991. Genetic linkage map of fishes of the genus Xiphophorus (Teleostei: Poeciliidae). *Genetics* **127:** 399-410.

Morrish, B.C. and A.H. Sinclair. 2002. Vertebrate sex determination: many means to an end. *Reproduction* **124:** 447-457.

Muller, H.J.J. 1914. A gene for the fourth chromosome of Drosophila. *Exp. Zool.* **17:** 325–336.

Nagamine, C.M., K. Morohashi, C. Carlisle, and D.K. Chang. 1999. Sex reversal caused by Mus musculus domesticus Y chromosomes linked to variant expression of the testis-determining gene Sry. *Dev Biol* **216:** 182-194.

Nakamoto, M., M. Matsuda, D.S. Wang, Y. Nagahama, and N. Shibata. 2006. Molecular cloning and analysis of gonadal expression of Foxl2 in the medaka, Oryzias latipes. *Biochem Biophys Res Commun* **344:** 353-361.

Nakamoto, M., A. Suzuki, M. Matsuda, Y. Nagahama, and N. Shibata. 2005. Testicular type Sox9 is not involved in sex determination but might be in the development of testicular structures in the medaka, Oryzias latipes. *Biochem Biophys Res Commun* **333:** 729-736.

Nakamura, S., Y. Aoki, D. Saito, Y. Kuroki, A. Fujiyama, K. Naruse, and M. Tanaka. 2007. Sox9b/sox9a2-EGFP transgenic medaka reveals the morphological reorganization of the gonads and a common precursor of both the female and male supporting cells. *Mol Reprod Dev*.

Nakamura, S., D. Kobayashi, Y. Aoki, H. Yokoi, Y. Ebe, J. Wittbrodt, and M. Tanaka. 2006. Identification and lineage tracing of two populations of somatic gonadal precursors in medaka embryos. *Dev Biol* **295:** 678-688.

Nanda, I., M. Kondo, U. Hornung, S. Asakawa, C. Winkler, A. Shimizu, Z. Shan, T. Haaf, N. Shimizu, A. Shima, M. Schmid, and M. Schartl. 2002. A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, Oryzias latipes. *Proc Natl Acad Sci U S A* **99:** 11778-11783.

Nanda, I., Z. Shan, M. Schartl, D.W. Burt, M. Koehler, H. Nothwang, F. Grutzner, I.R. Paton, D. Windsor, I. Dunn, W. Engel, P. Staeheli, S. Mizuno, T. Haaf, and M. Schmid. 1999. 300 million years of conserved synteny between chicken Z and human chromosome 9. *Nat Genet* **21:** 258-259.

Naruse, K., M. Tanaka, K. Mita, A. Shima, J. Postlethwait, and H. Mitani. 2004. A medaka gene map: the trace of ancestral vertebrate proto-chromosomes revealed by comparative gene mapping. *Genome Res* **14:** 820-828.

Natoli, T.A., J.A. Alberta, A. Bortvin, M.E. Taglienti, D.B. Menke, J. Loring, R. Jaenisch, D.C. Page, D.E. Housman, and J.A. Kreidberg. 2004. Wt1 functions in the development of germ cells in addition to somatic cell lineages of the testis. *Dev Biol* **268:** 429-440.

Nef, S., S. Verma-Kurvari, J. Merenmies, J.D. Vassalli, A. Efstratiadis, D. Accili, and L.F. Parada. 2003. Testis determination requires insulin receptor family function in mice. *Nature* **426:** 291-295.

Nelson, J.S. 1994. *Fishes of the world*. J. Wiley, New York.

Nishikimi, H., N. Kansaku, N. Saito, M. Usami, Y. Ohno, and K. Shimada. 2000. Sex differentiation and mRNA expression of P450c17, P450arom and AMH in gonads of the chicken. *Mol Reprod Dev* **55:** 20-30.

Ohno, S. 1967. *Sex Chromosomes and Sex-Linked Genes*. Springer, Berlin.

Ohno, S. 1970. *Evolution by gene duplication*. Springer-Verlag, New York.

Oreal, E., C. Pieau, M.G. Mattei, N. Josso, J.Y. Picard, D. Carre-Eusebe, and S. Magre. 1998. Early expression of AMH in chicken embryonic gonads precedes testicular SOX9 expression. *Dev Dyn* **212:** 522-532.

Otake, H., A. Shinomiya, M. Matsuda, S. Hamaguchi, and M. Sakaizumi. 2006. Wild-derived XY sex-reversal mutants in the Medaka, Oryzias latipes. *Genetics* **173:** 2083-2090.

Ottolenghi, C., M. Fellous, M. Barbieri, and K. McElreavey. 2002. Novel paralogy relations among human chromosomes support a link between the phylogeny of doublesex-related genes and the evolution of sex determination. *Genomics* **79:** 333-343.

Parma, P., O. Radi, V. Vidal, M.C. Chaboissier, E. Dellambra, S. Valentini, L. Guerra, A. Schedl, and G. Camerino. 2006. R-spondin1 is essential in sex determination, skin differentiation and malignancy. *Nat Genet* **38:** 1304-1309.

Pask, A.J., R.R. Behringer, and M.B. Renfree. 2003. Expression of DMRT1 in the mammalian ovary and testis--from marsupials to mice. *Cytogenet Genome Res* **101:** 229-236.

Paul-Prasanth, B., M. Matsuda, E.L. Lau, A. Suzuki, F. Sakai, T. Kobayashi, and Y. Nagahama. 2006. Knock-down of DMY initiates female pathway in the genetic male medaka, Oryzias latipes. *Biochem Biophys Res Commun* **351:** 815-819.

Pontiggia, A., R. Rimini, V.R. Harley, P.N. Goodfellow, R. Lovell-Badge, and M.E. Bianchi. 1994. Sex-reversing mutations affect the architecture of SRY-DNA complexes. *Embo J* **13:** 6115-6124.

Postlethwait, J., A. Amores, W. Cresko, A. Singer, and Y.L. Yan. 2004. Subfunction partitioning, the teleost radiation and the annotation of the human genome. *Trends Genet* **20:** 481-490.

Rao, M.K., J. Pham, J.S. Imam, J.A. MacLean, D. Murali, Y. Furuta, A.P. Sinha-Hikim, and M.F. Wilkinson. 2006. Tissue-specific RNAi reveals that WT1 expression in nurse cells controls germ cell survival and spermatogenesis. *Genes Dev* **20:** 147-152.

Rastogi, S. and D.A. Liberles. 2005. Subfunctionalization of duplicated genes as a transition state to neofunctionalization. *BMC Evol Biol* **5:** 28.

Raymond, C.S., M.W. Murphy, M.G. O'Sullivan, V.J. Bardwell, and D. Zarkower. 2000. Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. *Genes Dev* **14:** 2587-2595.

Raz, E. 2002. Primordial germ cell development in zebrafish. *Semin Cell Dev Biol* **13:** 489-495.

Roberts, L.M., J.A. Visser, and H.A. Ingraham. 2002. Involvement of a matrix metalloproteinase in MIS-induced cell death during urogenital development. *Development* **129:** 1487-1496.

Rodriguez-Mari, A., Y.L. Yan, R.A. Bremiller, C. Wilson, C. Canestro, and J.H. Postlethwait. 2005. Characterization and expression pattern of zebrafish Anti-Mullerian hormone (Amh) relative to sox9a, sox9b, and cyp19a1a, during gonad development. *Gene Expr Patterns* **5:** 655-667.

Rongo, C., H.T. Broihier, L. Moore, M. Van Doren, A. Forbes, and R. Lehmann. 1997. Germ plasm assembly and germ cell migration in Drosophila. *Cold Spring Harb Symp Quant Biol* **62:** 1-11.

Rupprecht, H.D., I.A. Drummond, S.L. Madden, F.J. Rauscher, 3rd, and V.P. Sukhatme. 1994. The Wilms' tumor suppressor gene WT1 is negatively autoregulated. *J Biol Chem* **269:** 6198-6206.

Santos, A.C. and R. Lehmann. 2004a. Germ cell specification and migration in Drosophila and beyond. *Curr Biol* **14:** R578-589.

Santos, A.C. and R. Lehmann. 2004b. Isoprenoids control germ cell migration downstream of HMGCoA reductase. *Dev Cell* **6:** 283-293.

Satoh, N. and N. Egami. 1972. Sex differentiation of germ cells in the teleost, Oryzias latipes, during noraml embryonic development. *J Embryol Exp Morphol* **28:** 385-395.

Schmitt-Ney, M., H. Thiele, P. Kaltwasser, B. Bardoni, M. Cisternino, and G. Scherer. 1995. Two novel SRY missense mutations reducing DNA binding identified in XY females and their mosaic fathers. *Am J Hum Genet* **56:** 862-869.

Schultheis, C., Q. Zhou, A. Froschauer, I. Nanda, Y. Selz, C. Schmidt, S. Matschl, M. Wenning, A.M. Veith, M. Naciri, R. Hanel, I. Braasch, A. Dettai, A. Bohne, C. Ozouf-Costaz, S. Chilmonczyk, B. Segurens, A. Couloux, S. Bernard-Samain, M. Schmid, M. Schartl, and J.N. Volff. 2006. Molecular Analysis of the Sex-Determining Region of the Platyfish *Xiphophorus maculatus*. *Zebrafish* **3:** 299-309.

Schupbach, T. and E. Wieschaus. 1989. Female sterile mutations on the second chromosome of Drosophila melanogaster. I. Maternal effect mutations. *Genetics* **121:** 101-117.

Serluca, F.C. and M.C. Fishman. 2001. Pre-pattern in the pronephric kidney field of zebrafish. *Development* **128:** 2233-2241.

Seydoux, G. and S. Strome. 1999. Launching the germline in Caenorhabditis elegans: regulation of gene expression in early germ cells. *Development* **126:** 3275-3283.

Shinomiya, A., N. Shibata, M. Sakaizumi, and S. Hamaguchi. 2002. Sex reversal of genetic females (XX) induced by the transplantation of XY somatic cells in the medaka, Oryzias latipes. *Int J Dev Biol* **46:** 711-717.

Shinomiya, A., M. Tanaka, T. Kobayashi, Y. Nagahama, and S. Hamaguchi. 2000. The vasa-like gene, olvas, identifies the migration path of primordial germ cells during embryonic body formation stage in the medaka, Oryzias latipes. *Dev Growth Differ* **42:** 317-326.

Slanchev, K., J. Stebler, G. de la Cueva-Mendez, and E. Raz. 2005. Development without germ cells: the role of the germ line in zebrafish sex differentiation. *Proc Natl Acad Sci U S A* **102:** 4074-4079.

Smith, C.A., M.J. Smith, and A.H. Sinclair. 1999. Gene expression during gonadogenesis in the chicken embryo. *Gene* **234:** 395-402.

Spotila, L.D. and S.E. Hall. 1998. Expression of a new RNA-splice isoform of WT1 in developing kidney-gonadal complexes of the turtle, Trachemys scripta. *Comp Biochem Physiol B Biochem Mol Biol* **119:** 761-767.

Stebler, J., D. Spieler, K. Slanchev, K.A. Molyneaux, U. Richter, V. Cojocaru, V. Tarabykin, C. Wylie, M. Kessel, and E. Raz. 2004. Primordial germ cell migration in the chick and mouse embryo: the role of the chemokine SDF-1/CXCL12. *Dev Biol* **272:** 351-361.

Sutou, S., Y. Mitsui, and K. Tsuchiya. 2001. Sex determination without the Y chromosome in two Japanese rodents Tokudaia osimensis osimensis and Tokudaia osimensis spp. *Mamm Genome* **12:** 17-21.

Suzuki, A. and N. Shibata. 2004. Developmental process of genital ducts in the medaka, Oryzias latipes. *Zoolog Sci* **21:** 397-406.

Takahashi, H. 1977. Juvenile hermaphroditism in the zebrafish, *Brachydanio rerio*. *Bull. Fac. Fish., Hokkaido***:** 57-65.

Takehana, Y., D. Demiyah, K. Naruse, S. Hamaguchi, and M. Sakaizumi. 2007. Evolution of different Y chromosomes in two medaka species, Oryzias dancena and O. latipes. *Genetics* **175:** 1335-1340.

Takehana, Y., K. Naruse, and M. Sakaizumi. 2005. Molecular phylogeny of the medaka fishes genus Oryzias (Beloniformes: Adrianichthyidae) based on nuclear and mitochondrial DNA sequences. *Mol Phylogenet Evol* **36:** 417-428.

Tanaka, M., M. Kinoshita, D. Kobayashi, and Y. Nagahama. 2001. Establishment of medaka (Oryzias latipes) transgenic lines with the expression of green fluorescent protein fluorescence exclusively in germ cells: a useful model to monitor germ cells in a live vertebrate. *Proc Natl Acad Sci U S A* **98:** 2544-2549.

Taylor, J.S., I. Braasch, T. Frickey, A. Meyer, and Y. Van de Peer. 2003. Genome duplication, a trait shared by 22000 species of ray-finned fish. *Genome Res* **13:** 382-390.

Taylor, J.S. and J. Raes. 2004. Duplication and divergence: the evolution of new genes and old ideas. *Annu Rev Genet* **38:** 615-643.

Teixeira, J., W.W. He, P.C. Shah, N. Morikawa, M.M. Lee, E.A. Catlin, P.L. Hudson, J. Wing, D.T. Maclaughlin, and P.K. Donahoe. 1996. Developmental expression of a candidate mullerian inhibiting substance type II receptor. *Endocrinology* **137:** 160-165.

Teixeira, J., D.J. Kehas, R. Antun, and P.K. Donahoe. 1999. Transcriptional regulation of the rat Mullerian inhibiting substance type II receptor in rodent Leydig cells. *Proc Natl Acad Sci U S A* **96:** 13831-13838.

Teixeira, J., S. Maheswaran, and P.K. Donahoe. 2001. Mullerian inhibiting substance: an instructive developmental hormone with diagnostic and possible therapeutic applications. *Endocr Rev* **22:** 657-674.

Tevosian, S.G., K.H. Albrecht, J.D. Crispino, Y. Fujiwara, E.M. Eicher, and S.H. Orkin. 2002. Gonadal differentiation, sex determination and normal Sry expression in mice require direct interaction between transcription partners GATA4 and FOG2. *Development* **129:** 4627-4634.

Thorpe, J.L., M. Doitsidou, S.Y. Ho, E. Raz, and S.A. Farber. 2004. Germ cell migration in zebrafish is dependent on HMGCoA reductase activity and prenylation. *Dev Cell* **6:** 295-302.

Tsunekawa, N., M. Naito, Y. Sakai, T. Nishida, and T. Noce. 2000. Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells. *Development* **127:** 2741-2750.

Upadhyay, S., J. Luciani, and Z. L. 1981. The role of the mesonephros in the development of the mouse testis and its excurrent pathways. In *Development and Function of Reproductive Organs* (ed. B.A.a.P. H.). Amsterdam: Excerpta Medica.

Veith, A.M., A. Froschauer, C. Korting, I. Nanda, R. Hanel, M. Schmid, M. Schartl, and J.N. Volff. 2003. Cloning of the dmrt1 gene of Xiphophorus maculatus: dmY/dmrt1Y is not the master sex-determining gene in the platyfish. *Gene* **317:** 59-66.

Veith, A.M., M. Schafer, N. Klüver, C. Schmidt, C. Schultheis, M. Schartl, C. Winkler, and J.N. Volff. 2006. Tissue-specific expression of dmrt genes in embryos and adults of the platyfish Xiphophorus maculatus. *Zebrafish* **3:** 325-337.

Vidal, V.P., M.C. Chaboissier, D.G. de Rooij, and A. Schedl. 2001. Sox9 induces testis development in XX transgenic mice. *Nat Genet* **28:** 216-217.

Volff, J.N., M. Kondo, and M. Schartl. 2003a. Medaka dmY/dmrt1Y is not the universal primary sex-determining gene in fish. *Trends Genet* **19:** 196-199.

Volff, J.N., D. Zarkower, V.J. Bardwell, and M. Schartl. 2003b. Evolutionary dynamics of the DM domain gene family in metazoans. *J Mol Evol* **57 Suppl 1:** S241-249.

Wagner, T., J. Wirth, J. Meyer, B. Zabel, M. Held, J. Zimmer, J. Pasantes, F.D. Bricarelli, J. Keutel, E. Hustert, and et al. 1994. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell* **79:** 1111-1120.

Wang, X.G. and L. Orban. 2007. Anti-Mullerian hormone and 11 beta-hydroxylase show reciprocal expression to that of aromatase in the transforming gonad of zebrafish males. *Dev Dyn* **236:** 1329-1338.

Watanabe, K., T.R. Clarke, A.H. Lane, X. Wang, and P.K. Donahoe. 2000. Endogenous expression of Mullerian inhibiting substance in early postnatal rat sertoli cells requires multiple steroidogenic factor-1 and GATA-4-binding sites. *Proc Natl Acad Sci U S A* **97:** 1624-1629.

Wilhelm, D. 2007. R-spondin1--discovery of the long-missing, mammalian female-determining gene? *Bioessays* **29:** 314-318.

Wilhelm, D. and C. Englert. 2002. The Wilms tumor suppressor WT1 regulates early gonad development by activation of Sf1. *Genes Dev* **16:** 1839-1851.

Wilhelm, D., S. Palmer, and P. Koopman. 2007. Sex determination and gonadal development in mammals. *Physiol Rev* **87:** 1-28.

Wittbrodt J, M.A., Schartl M. 1998. More genes in fish? *BioEssays* **20:** 511-515.

Wittbrodt, J., A. Shima, and M. Schartl. 2002. Medaka--a model organism from the far East. *Nat Rev Genet* **3:** 53-64.

Wrobel, K.H. 2003. The genus Acipenser as a model for vertebrate urogenital development: the mullerian duct. *Anat Embryol (Berl)* **206:** 255-271.

Xia, W., L. Zhou, B. Yao, C.J. Li, and J.F. Gui. 2007. Differential and spermatogenic cell-specific expression of DMRT1 during sex reversal in protogynous hermaphroditic groupers. *Mol Cell Endocrinol* **263:** 156-172.

Yamaguchi, A., K.H. Lee, H. Fujimoto, K. Kadomura, S. Yasumoto, and M. Matsuyama. 2006. Expression of the DMRT gene and its roles in early gonadal development of the Japanese pufferfish Takifugu rubripes. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics. Papers presented at the TODAI International Symposium on Functional Genomics of Pufferfish Recent Advances and Perspective, The University of Tokyo, Yayoi Auditorium, Tokyo, Japan, 3rd -6th November 2004* **1:** 59-68.

Yan, Y.L., J. Willoughby, D. Liu, J.G. Crump, C. Wilson, C.T. Miller, A. Singer, C. Kimmel, M. Westerfield, and J.H. Postlethwait. 2005. A pair of Sox: distinct and overlapping functions of zebrafish sox9 co-orthologs in craniofacial and pectoral fin development. *Development* **132:** 1069-1083.

Yasuoka, A., Y. Hirose, H. Yoda, Y. Aihara, H. Suwa, K. Niwa, T. Sasado, C. Morinaga, T. Deguchi, T. Henrich, N. Iwanami, S. Kunimatsu, K. Abe, H. Kondoh, and M. Furutani-Seiki. 2004. Mutations affecting the formation of posterior lateral line system in Medaka, Oryzias latipes. *Mech Dev* **121:** 729-738.

Yoon, C., K. Kawakami, and N. Hopkins. 1997. Zebrafish vasa homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells. *Development* **124:** 3157-3165.

Yoshinaga, N., E. Shiraishi, T. Yamamoto, T. Iguchi, S. Abe, and T. Kitano. 2004. Sexually dimorphic expression of a teleost homologue of Mullerian inhibiting substance during gonadal sex differentiation in Japanese flounder, Paralichthys olivaceus. *Biochem Biophys Res Commun* **322:** 508-513.

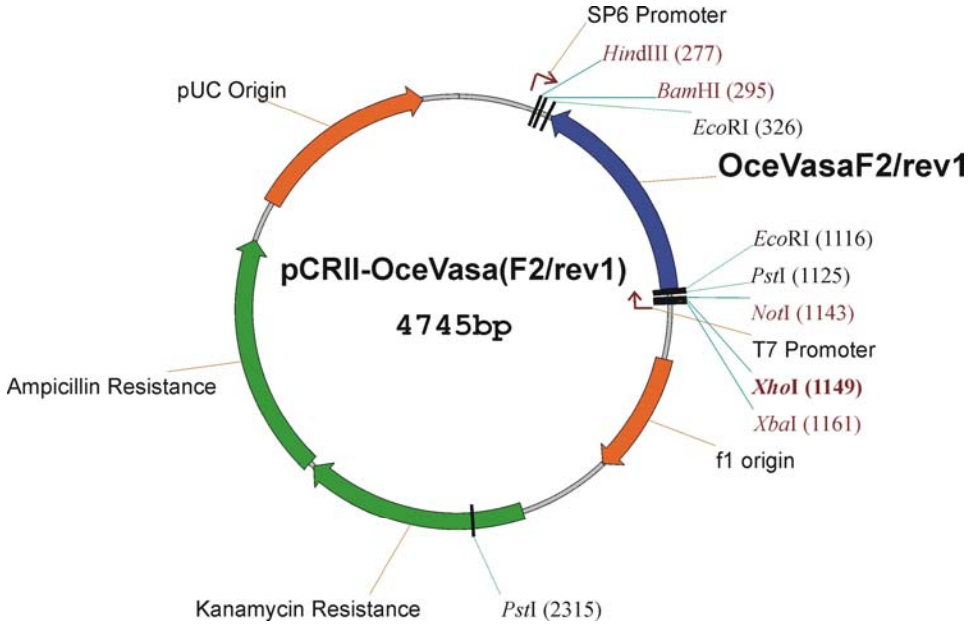
Zarkower, D. 2001. Establishing sexual dimorphism: conservation amidst diversity? *Nat Rev Genet* **2:** 175-185.

Zhao, Q., H. Eberspaecher, V. Lefebvre, and B. De Crombrugghe. 1997. Parallel expression of Sox9 and Col2a1 in cells undergoing chondrogenesis. *Dev Dyn* **209:** 377-386.

Appendix A

A.1. *Oryzias celebensis vasa* (F2/rev1) cloned into pCRII

Primer combination vasaF2;CCAACCAGGGAGCTCATCAA and vasa-rev1;TCCCGATGCGG-TGGAC amplified a 772-bp cDNA fragment.



For antisense DIG-labeled RNA probe synthesis cut *Xho*I and use SP6-RNA-Polymerase (Roche, Mannheim).

A.1.1. *Ocevasa* and *Olvas* nucleotide sequence alignment

```
OcevasaF2/rev1  ------------------------------------------------------------
Olvas           ATGGACGACTGGGAGGAAGAGGAAACCGCACCGTCCTTCGCGCCCGTCAGCAGCACGGAT

OcevasaF2/rev1  ------------------------------------------------------------
Olvas           GCAGCTCCGCAGAGAAGCTCCTGGAACGGAGGCGCCAGGGACTCAGGAAATGACGGAGAC

OcevasaF2/rev1  ------------------------------------------------------------
Olvas           AGCTGGAACCGCTCCAACCGAGGGCGAGGAGGTTCGGCCGGGAGAGGAGGCAGAGGAGGG

OcevasaF2/rev1  ------------------------------------------------------------
Olvas           CGCGGCAGAGGCTTCGGACGGTCTGACCAGGACGAGCTCAATGGAGGCGGCGGAGACTCT

OcevasaF2/rev1  ------------------------------------------------------------
Olvas           GAAAACGGGTTCAGAGGAAGAGGCCGAGGAGGAAGAGGAGGCTTCAGATCAGGTGGGGGG
```

```
Ocevasa_F2/rev1  ------------------------------------------------------------
Olvas            GAGCGAGGAAGAGGAGGAGGTTATCGAGGACGGGATGAAGACGTCTTTGCTGCAGGAGAC

Ocevasa_F2/rev1  ------------------------------------------------------------
Olvas            GGCAGAGGGGCCGAGAACAGCGATGCAGCTGACCCAGAACGGCCCAAAGTGACCTACATC

Ocevasa_F2/rev1  ------------------------------------------------------------
Olvas            CCCCCGAGCCTCCCGGAGGACGAGGACTCCATCTTCTCCCACTACAAGATGGGCATCAAC

Ocevasa_F2/rev1  ------------------------------------------------------------
Olvas            TTCGACAAGTACGACGACATCCTGGTGGACGTCAGCGGGACCAACCTGCCCGCCGCCATC

Ocevasa_F2/rev1  ------------------------------------------------------------
Olvas            ATGACCTTTGAGGAGGCCAAGCTGTGCGAGTCGTTGGAGAACAACATCAGCAGGTCCGGA

Ocevasa_F2/rev1  ------------------------------------------------------------
Olvas            TACGTCAAGCCGACGCCCGTCCAGAAGTACGGCCTCCCCATCATCTCCGCCGGCAGAGAC

Ocevasa_F2/rev1  ------------------------------------------------------------
Olvas            CTGATGGCGTGCGCTCAGACCGGCTCCGGAAAAACTGCGGCGTTCCTGCTGCCCATCCTG

Ocevasa_F2/rev1  ------------------------------------------------------------
Olvas            CAGCAGCTGATGGCAGACGGCGTGGCGGCCAGCCGCTTCAGCGAGATCCAGGAGCCAGAG

                                      vasaF1
Ocevasa_F2/rev1  ---------------CCAACCAGGGAGCTCATCAACCAGATTTACCAGGAAGCTCGGAAG
Olvas            GCCGTGATTGTGGCTCCAACCAGGGAGCTCATCAACCAGATTTACCAGGAGGCCCGGAAG
                             ********************************** ** ******

Ocevasa_F2/rev1  TTTTCCTTTGGGACCTGCGTGCGGCCCGTGGTGGTTTACGGAGGCGTGAACACCGGCTAC
Olvas            TTCTCCTTTGGGACCTGCGTGCGGCCCGTGGTGGTCTACGGAGGCGTGAACACCGGCTAC
                 ** ******************************** ************************

Ocevasa_F2/rev1  CAGATTAGGGAGATCCAGAAAGGCTGCAACGTTCTGTGCGGGACTCCGGGGCGTCTGCTG
Olvas            CAGATGAGGGAGATCGAGAAGGGCTGCAACGTGCTGTGCGGGACTCCGGGACGCCTGCTG
                 ***** ********* **** *********** ***************** ** ******

Ocevasa_F2/rev1  GACATGATCGGCAAAGGAAAGGTGGGGCTGAGTAAAGTTCGCCACTTGGTCCTGGACGAA
Olvas            GACATGATCGGCAGAGGAAAGGTGGGCCTGAGTAAAGTTCGCCACCTGGTCCTGGACGAA
                 ************* ************ ****************** **************

Ocevasa_F2/rev1  GCCGACCGCATGTTGGACATGGGCTTCGAGCCGGACATGCGCCGCCTGGTGGGTTTCCCG
Olvas            GCCGACCGCATGTTGGACATGGGCTTCGAGCCGGACATGCGCCGGCTGGTGGGCTCCCCG
                 ******************************************** ******** * ****

Ocevasa_F2/rev1  GGTATGCCGTCCAAAGAGGAGCGCCAGACTCTGATGTTCAGCGCCACCTTCCCCGAGGAC
Olvas            GGTATGCCGTCCAAAGAGGAGCGGCAGACGCTGATGTTCAGCGCCACCTTCCCCGAGGAC
                 ********************** ***** ******************************
```

```
OcevasaF2/rev1  ATCCAGAGGTTGGCGGCTGACTTCCTGAAGGTGGACTACCTGTTTGTGGCTGTGGGGGTG
Olvas           ATCCAAAGGTTGGCGGCCGACTTCCTGAAGGTGGACTACCTGTTTGTGGCCGTGGGGGTG
                ***** ********** ******************************** *********

OcevasaF2/rev1  TTGGGCGGAGCGTGCGCCGACGTGGAGCAGACCTTCATACAGGTCACCAAATTCAACAAG
Olvas           GTGGGCGGAGCCTGCACCGACGTGGAGCAGACCTTCCTACAGGTCACCAAATTCAACAAG
                 ********** *** ******************** ***********************

OcevasaF2/rev1  AGGGAGAAGCTCCTGGACCTCCTCAGGACCACAGGGTCGGAGCGGACCATGGTGTTCGTG
Olvas           CGGGAGCAGCTCCTGGACCTCCTCAGGACCATCGGGTCTGAGCGGACCATGGTGTTCGTG
                 ***** ************************  ***** ********************
OcevasaF2/rev1  GAGACCAAGAGGCAGGCCGACTTCATCGCCGCGTGCCTGTGCCAGGAGAAGGTTCCCACC
Olvas           GAGACCAAGAGGCAGGCCGACTTCATCGCCGCCTTCCTGTGCCAGGAGAAGGTTCCGACC
                ******************************** * ********************* ***

OcevasaF2/rev1  ACCAGCATTCACGGCGACAGAGAGCAGCGTGAGCGGGAGAAGGCGCTGACGGATTTCCGC
Olvas           ACCAGCATCCACGGCGACAGAGAGCAGCGCGAGCGGGAGAAGGCGCTGGCGGATTTCCGC
                ******** ******************** ****************** ***********

OcevasaF2/rev1  TCAGGCAAGTGTCCCGTCCTGGTGGCCACCTCGGTGGCGTCCCGTGGCCTGGACATCCCA
Olvas           TCAGGCAAGTGTCCCGTCCTGGTGGCCACCTCGGTGGCGTCCCGCGGCCTGGACATCCCT
                ******************************************** **************
                                                                  Vasa-rev1
OcevasaF2/rev1  GACGTTCAACATGTAGTGAACTTTGACCTCCCAAACAACATTGACGACTACGTCCACCGC
Olvas           GACGTCCAGCACGTGGTGAACTTTGACCTGCCCAACACCATTGACGACTACGTCCACCGC
                ***** ** ** ** ************** ** **** **********************

OcevasaF2/rev1  ATCGGGA-------------------------------------------------------
Olvas           ATCGGGAGAACGGGCCGCTGCGGCAACACCGGCAGGGCCGTGTCCTTCTACGACCCCGAC
                *******

OcevasaF2/rev1  ------------------------------------------------------------
Olvas           GTGGACAGTCAGCTGGCCCGCTCGCTGGTCGGCATCCTGGCCAAGGCTCAGCAGGAGGTG

OcevasaF2/rev1  ------------------------------------------------------------
Olvas           CCGTCATGGCTGGAGGAGTCGGCGTTCGGCGCTCACGGTTCCGCCGCCTTCAACCCTTCG

OcevasaF2/rev1  ------------------------------------------------------------
Olvas           GGAAGGACGTTTGCTTCCACAGACTCCAGGAAGGGTGGTTCTTTCCAGGACAGCAGTGTG

OcevasaF2/rev1  ------------------------------------------------------
Olvas           AAGACGCAGCCTGCAGCGCCCCCTGCTGCTGCTGATGAAGATGACTGGGAGTGA
```

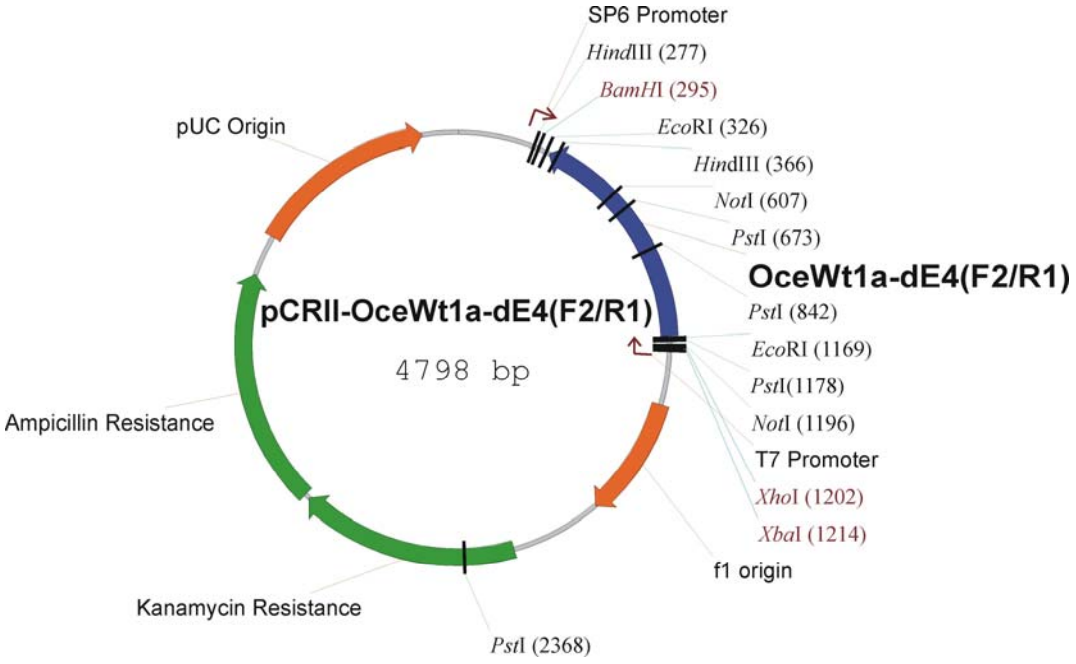
A.1.2. *Oce*Vasa and OlVas amino acid alignment

```
OceVasaF2/rev1  ------------------------------------------------------------
OlVas           MDDWEEEETAPSFAPVSSTDAAPQRSSWNGGARDSGNDGDSWNRSNRGRGGSAGRGGRGG

OceVasaF2/rev1  ------------------------------------------------------------
OlVas           RGRGFGRSDQDELNGGGGDSENGFRGRGRGGRGGFRSGGGERGRGGGYRGRDEDVFAAGD
```

```
OceVasaF2/rev1  ------------------------------------------------------------
OlVas           GRGAENSDAADPERPKVTYIPPSLPEDEDSIFSHYKMGINFDKYDDILVDVSGTNLPAAI

OceVasaF2/rev1  ------------------------------------------------------------
OlVas           MTFEEAKLCESLENNISRSGYVKPTPVQKYGLPIISAGRDLMACAQTGSGKTAAFLLPIL


OceVasaF2/rev1  ------------------------PTRELINQIYQEARKFSFGTCVRPVVVYGGVNTGY
OlVas           QQLMADGVAASRFSEIQEPEAVIVAPTRELINQIYQEARKFSFGTCVRPVVVYGGVNTGY
                                        **********************************

OceVasaF2/rev1  QIREIQKGCNVLCGTPGRLLDMIGKGKVGLSKVRHLVLDEADRMLDMGFEPDMRRLVGFP
OlVas           QMREIEKGCNVLCGTPGRLLDMIGRGKVGLSKVRHLVLDEADRMLDMGFEPDMRRLVGSP
                *:***:******************:******************************** *

OceVasaF2/rev1  GMPSKEERQTLMFSATFPEDIQRLAADFLKVDYLFVAVGVLGGACADVEQTFIQVTKFNK
OlVas           GMPSKEERQTLMFSATFPEDIQRLAADFLKVDYLFVAVGVVGGACTDVEQTFLQVTKFNK
                ****************************************:****:******:*******

OceVasaF2/rev1  REKLLDLLRTTGSERTMVFVETKRQADFIAACLCQEKVPTTSIHGDREQREREKALTDFR
OlVas           REQLLDLLRTIGSERTMVFVETKRQADFIAAFLCQEKVPTTSIHGDREQREREKALADFR
                **:******* ******************** ************************:***

OceVasaF2/rev1  SGKCPVLVATSVASRGLDIPDVQHVVNFDLPNNIDDYVHRIG-----------------
OlVas           SGKCPVLVATSVASRGLDIPDVQHVVNFDLPNTIDDYVHRIGRTGRCGNTGRAVSFYDPD
                *********************************.*********

OceVasaF2/rev1  ------------------------------------------------------------
OlVas           VDSQLARSLVGILAKAQQEVPSWLEESAFGAHGSAAFNPSGRTFASTDSRKGGSFQDSSV


OceVasaF2/rev1  -----------------
OlVas           KTQPAAPPAAADEDDWE
```

A.2. *Oryzias celebensis wt1a*-dE4 (F2/R1) cloned into pCRII

Primer combination Olawt1aF2;TGACCAACTGCATGGACACT and Olawt1aR1;AAACTGG-GATGGCTCAGATG amplified an 825-bp cDNA fragment.



For antisense DIG-labeled RNA probe synthesis cut *Xho*I and use SP6-RNA-Polymerase (Roche, Mannheim).

A.2.1. *Ocewt1a*-dE4 and *Olawt1a*-dE4 nucleotide sequence alignment

```
Ocewt1a-dE4     ------------------------------------------------------------
Olawt1a-dE4     ATGGGTTCAGATGTTCGTGACCTGAACTCCCTGCTGCCCCCAGTCCCGGCAGGCCCCAGC

Ocewt1a-dE4     ------------------------------------------------------------
Olawt1a-dE4     CCCGGCTGCCCAGCCCTGCCTGTCAGTACAGCCCCTCAGTGGGCTCCTGTCCTGGACTTC

Ocewt1a-dE4     ------------------------------------------------------------
Olawt1a-dE4     CACCCTGCTGCCTCACCTTACTCCTCCCTGGCCGCACCGCACTCCTTCATCAAACAGGAA

Ocewt1a-dE4     ------------------------------------------------------------
Olawt1a-dE4     CCCAGTTGGGGGGCCGCCAGTGACCCACACCACCTTGACTCCGACCCCCACTGTGGCCTC

Ocewt1a-dE4     ------------------------------------------------------------
Olawt1a-dE4     AGCGCCTTCACTGTGCACTTCTCGGGACAGTTCACAGGCACCGGAGCTTGCAGGTATGGA
```

```
Ocewt1a-dE4     ------------------------------------------------------------
Olawt1a-dE4     GCAGCATTCGGAGCACCCCCTCCTCCACCCACAGCTCCCAGCCAGCCTCCACCTGTGGCA

Ocewt1a-dE4     -----------------------------------------------TGACCAAC
Olawt1a-dE4     GCACCACACCACCATCAGCCCCCCAGCAGGATGTTCAGTAACCCACCATATCTGACCAAC
                                                               ********

                  Wt1a-F2
Ocewt1a-dE4     TGCATGGACACTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGCAGTCGGGTTCGAT
Olawt1a-dE4     TGCATGGACACTCAGCCGCCGGCTCGCAACCAGACAGGTTACAGCACAGTCGGATTCGAT
                ************                                   ******* ******

Ocewt1a-dE4     GGAGCCGGTAATTACGGCCACACTCCCACGCACCACAGCTCGCAGTTCTCCAGCCACTCC
Olawt1a-dE4     GGAGCCGGTAATTACGGCCACACTCCCACGCACCACAGCTCGCAGTTCTCCGGCCACTCC
                ************************************************* ********

Ocewt1a-dE4     TTCAAGCATGAAGATGCTTTGGCCCAGCAGAACACAATGGGTGAGCAGCAGTATCCTGTG
Olawt1a-dE4     TTCAAACATGAAGACGCTTTGGCTCAGCAGAACACAATGGGTGAGCAGCAGTATCCTGTG
                ***** ******** ******** ***********************************

Ocewt1a-dE4     CCTCCTCCGGTGTATGGATGCCACACACCTTCAGACAGCTGCACGAGCAGCCAGGCTCTG
Olawt1a-dE4     CCTCCTCCGGTGTATGGATGCCACACACCTTCAGACAGCTGCACAAGCAGCCAGGCTCTA
                ******************************************** **************

Ocewt1a-dE4     CTGCTGAGGAACCCCTACAACAGTCATGCAGCTGGCTATGACAGTGACCCCAGCACCCCG
Olawt1a-dE4     CTGCTGAGAAACCCCTACAACAGTCATGCAGCTGGCTATGACAGTGACCCCAGCACCCCG
                ******** ***************************************************

Ocewt1a-dE4     ATGGTGTACAGCTGCAGCACGCAGTACCGCATACACACACACGGAGTCTTCCGAGGCATT
Olawt1a-dE4     ATGGTGTACAGCTGCAGCACCCAGTACCGCATACACACTCACGGAGTCTTCCGAGGCATT
                ******************** ***************** **********************

Ocewt1a-dE4     CAGGATGTGCGACGGGTGCCCAGCATCGCTCCTGCAATAGTTCGGTCAGAAAGCAGTGAG
Olawt1a-dE4     CAGGATGTGCGACGGGTGCCCAGCATTGCTCCTGCAATAGTTCGGTCAGAAAGCAGTGAG
                ************************** *********************************

Ocewt1a-dE4     AAGCGGCCGTTCATGTGCGGCTACCCAGGATGCAACAAGCGCTACTTCAAGCTGTCTCAC
Olawt1a-dE4     AAGCGACCGTTCATGTGCGGCTACCCAGGATGCAACAAGCGCTACTTCAAGCTGTCTCAC
                ***** ******************************************************

Ocewt1a-dE4     CTGCAGATGCACAGCCGCAAACACACAGGTGAGAAACCTTACCAGTGCGAGTTCACGGAC
Olawt1a-dE4     TTGCAGATGCACAGCCGCAAACACACAGGTGAGAAACCTTACCAGTGTGAGTTCACCGAC
                 ********************************************* ******** ***

Ocewt1a-dE4     TGCGGCCGCCGCTTTTCACGCTCTGACCAGCTCAAAAGACACCAGCGGAGGCACACAGGA
Olawt1a-dE4     TGTGGCCGCCGCTTTTCACGCTCTGACCAGCTCAAAAGACACCAGCGGAGGCACACAGGA
                ** ************************************************************

Ocewt1a-dE4     GTTAAACCGTTCGAATGCGAGACGTGTCAGAGAAAGTTCTCTCGGTCTGACCACCTTAAG
Olawt1a-dE4     GTTAAACCGTTCGAATGCGAGACGTGTCAGAGAAAGTTCTCTCGGTCTGACCACCTTAAG
                ************************************************************

Ocewt1a-dE4     ACACACACACGGACTCATACAG---------GCGAGAAACCGTTTAACTGTCGGTGGCCG
Olawt1a-dE4     ACACACACACGGACTCATACAGGTAAAACAAGCGAGAAACCGTTTAACTGTCGGTGGCCG
                ********************         ****************************

Ocewt1a-dE4     AACTGCCAGAAGAAGTTTGCCCGAAGCGACGAGTTGGCGCGACACCACAACATGCACCAG
Olawt1a-dE4     AACTGCCAGAAGAAGTTCGCCCGAAGCGACGAGTTGGTGCGACACCACAACATGCACCAA
                ***************** ****************** ********************
```

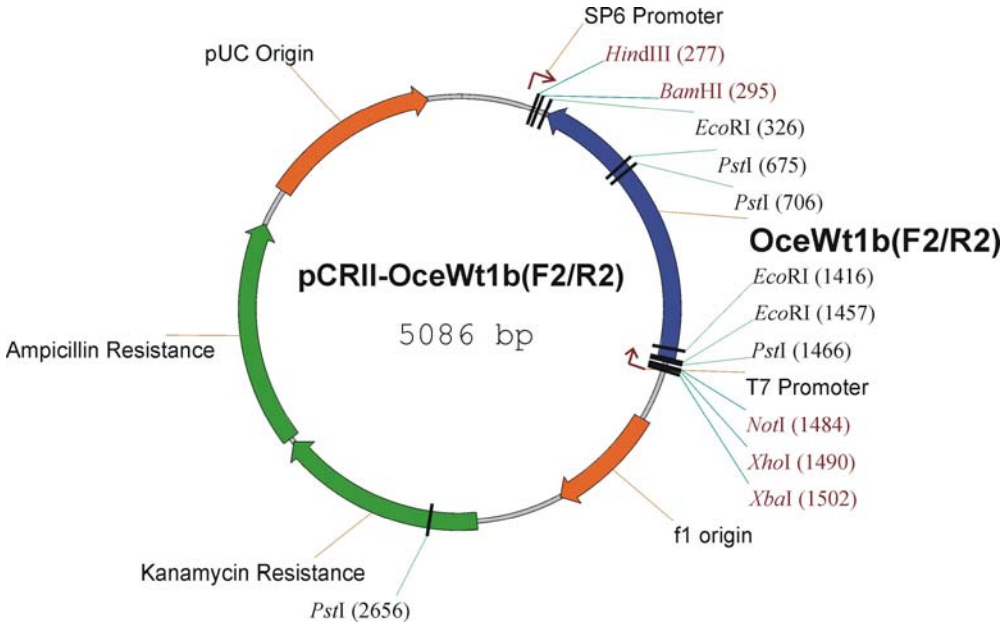
```
                                                  Wt1a-R1
Ocewt1a-dE4     AGGAACCTCACCAAGCTTCAGCTGTCCATCTGAGCCATCCCAGTTT
Olawt1a-dE4     AGGAACCTCACCAAGCTTCAGCTGTCCATCTGAGCCATCCCAGTTT
                *********************************************
```

A.2.2. *Oce*Wt1a-dE4 and *Ola*Wt1a-dE4 amino acid alignment

```
OceWt1a-dE4     ------------------------------------------------------------
OlaWt1a-dE4     MGSDVRDLNSLLPPVPAGPSPGCPALPVSTAPQWAPVLDFHPAASPYSSLAAPHSFIKQE


OceWt1a-dE4     ------------------------------------------------------------
OlaWt1a-dE4     PSWGAASDPHHLDSDPHCGLSAFTVHFSGQFTGTGACRYGAAFGAPPPPPTAPSQPPPVA


OceWt1a-dE4     ------------------TNCMDTXXXXXXXXXXXAVGFDGAGNYGHTPTHHSSQFSSHS
OlaWt1a-dE4     APHHHQPPSRMFSNPPYLTNCMDTQPPARNQTGYSTVGFDGAGNYGHTPTHHSSQFSGHS
                                  ******          :*********************.**

OceWt1a-dE4     FKHEDALAQQNTMGEQQYPVPPPVYGCHTPSDSCTSSQALLLRNPYNSHAAGYDSDPSTP
OlaWt1a-dE4     FKHEDALAQQNTMGEQQYPVPPPVYGCHTPSDSCTSSQALLLRNPYNSHAAGYDSDPSTP
                ************************************************************

OceWt1a-dE4     MVYSCSTQYRIHTHGVFRGIQDVRRVPSIAPAIVRSESSEKRPFMCGYPGCNKRYFKLSH
OlaWt1a-dE4     MVYSCSTQYRIHTHGVFRGIQDVRRVPSIAPAIVRSESSEKRPFMCGYPGCNKRYFKLSH
                ************************************************************

OceWt1a-dE4     LQMHSRKHTGEKPYQCEFTDCGRRFSRSDQLKRHQRRHTGVKPFECETCQRKFSRSDHLK
OlaWt1a-dE4     LQMHSRKHTGEKPYQCEFTDCGRRFSRSDQLKRHQRRHTGVKPFECETCQRKFSRSDHLK
                ************************************************************

OceWt1a-dE4     THTRTHTG---EKPFNCRWPNCQKKFARSDELARHHNMHQRNLTKLQLSI
OlaWt1a-dE4     THTRTHTGKTSEKPFNCRWPNCQKKFARSDELVRHHNMHQRNLTKLQLSI
                ********   *********************.*****************
```

A.3. *Oryzias celebensis wt1b* (F2/R2) cloned into pCRII

Primer combination Olawt1b-F2;CTCCACCCTGGTTCTCCCTA and Olawt1b-R2;CTTTCTTT-AAATGGCAGGTTGC amplified an 1113-bp cDNA fragment.



For antisense DIG-labeled RNA probe synthesis cut *Xho*I and use SP6-RNA-Polymerase (Roche, Mannheim).

A.3.1. *Ocewt1b* and *Olawt1b* nucleotide sequence alignment

```
Ocewt1bF2R2     ------------------------------------------------------------
Olawt1b         ATGTCGGTGGGTTCAGATGTTTGCGATCTCACCCTCCTACCATCAGCACCCCCCATGCCA


Ocewt1bF2R2     ------------------------------------------------------------
Olawt1b         TCCTTACCAGGAGCTGTTGGCAGCTGTGGGATGTCTGTTGGTAGTGGTCAGTGGACCAAT

                                  Wt1bF2
Ocewt1bF2R2     ---------CTCCACCCTGGTTCTCCCTACGGCTCATTGAATTCCCACCACTCCCTAATC
Olawt1b         TTGCTGGACCTCCACCCTGGTTCTCCCTACAGCTCCTTAAACTCCCACCATTCCCTAATC
                         ********************** **** ** ** ******** *********

Ocewt1bF2R2     AAGCAGGAGCCTGGCTGGGGAACCGCTGACCCTATGGAGGACCCTCACTGTGGACTGGGG
Olawt1b         AAGCAGGAGCCTGGCTGGGGAACCGCTGACCCAATGGAGGACCCTCACTGTGGACTGGGA
                ******************************** *************************

Ocewt1bF2R2     GCCTTCACTGTGCACTTCTCAGGTCAGTTTACAGGCTCCGGTCCCTGTCGAGTCAGTGCG
Olawt1b         GCCTTCACTGTGCACTTCTCAGGTCAGTTAACAGGCTCTGGTCCATGTCGAGTCGGTGCA
                **************************** ******** ***** ********* ****
```

```
Ocewt1bF2R2     TTTGGAGAACCAGCAAGCCAACCAAGGATGTTTCCCAATGCAGCTTACATGCCCAGCTGT
Olawt1b         TTTGGAGAACCCGTGAGCCAACCAAGGATGTTTCCCAATGCAGCGTACCTGCCCAGCTGT
                *********** *  ***************************** *** ***********

Ocewt1bF2R2     GTGGACAGCTCCCCTCTGCCCAGGAACCAGGGTTATGGCACACTGGCTTTGGACAGCAAC
Olawt1b         ATGGACAGCTCCCCTCTCCCCAGGAACCAGGGTTACGGCCCACTGGCTTTGGACAGCAAC
                 *************** ***************** *** ********************

Ocewt1bF2R2     CCCAGTTATAGTCACGCTGCATCCCACCACACCCCACAGCTCACCACCCTGTCCTTCAAG
Olawt1b         CCTGGTTATAGTCACTCAGCATCCCACCACAACCCCCAGCTCTCCAGCCTGTCCTTCAAG
                **  *********** * ************ *** ****** *** *************
Ocewt1bF2R2     CATGAGGACACGCTGTCGCCACCAAACAACATAGTTGACCAACAGTATCCAACAGCAAAT
Olawt1b         CATGAGGACACGCTGTCACCAACAAACAACATA---GACCAACAGTATCCAACACTAAAC
                ***************** *** ***********   ******************  ***

Ocewt1bF2R2     CCCATGTTTGGTTGTCACAATCCTTCCGAATCCAATCCCAGCAGCCAAACTCTGCTGCTG
Olawt1b         CCCATGTTCGGTTGTCACAATCCTTCAGAATCCACTCCCAACAGCCAAACTCTGCTGCTG
                ******** ***************** ******* ***** ******************

Ocewt1bF2R2     AGGAACTACAGCAGTGATAATTTGTATCAAATGGCATCTCAGCTGGAGTGTGTCNNNNNN
Olawt1b         AGGAACTACAGCAGTGATAATTTGTACCAAATGGCATCTCAGCTGGAATGTGTCACATGG
                ************************** ******************* ******

Ocewt1bF2R2     NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Olawt1b         AATCAGATGAATACCCTGGCATCCCCCACAAAGACTGGCCAAGCAGCTAGTTTTGACAGC

Ocewt1bF2R2     NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Olawt1b         GACCCCATGCTCGTCAGTGCCCAGTACCACATTCACACACATGGTGTGTTCAGGGGGCTT

Ocewt1bF2R2     NNNNNNNNNNNNNNNGTTTCTGGTATGACTCCTCCAGTTGCAAAGTCCTCAGAAGCCAGT
Olawt1b         CAGGATATAAGAAGGGTTCCTGGTGTGACTCCTCCAGTTGCAAAGTCCTCAGAAGCCAGT
                           *** ***** ***********************************

Ocewt1bF2R2     GAGAGGCGGCCTTTTGTGTGCATCTATCCAGGCTGCAGCAAGAGATACTTCAAGCTCTCA
Olawt1b         GAGAGGCGGCCTTTTGTGTGTGTCTATCCAGGCTGCAGCAAGAGATACTTTAAGCTCTCA
                ********************  **************************** *********

Ocewt1bF2R2     CATCTGCAGATGCACGGGCGGAAACACACAGGAGAGAAGCCATACCAGTGTGATGTCACA
Olawt1b         CATCTGCAGATGCACGGACGGAAACACACAGGAGAAAAGCCATATCAGTGTGATGTCACA
                ***************** ***************** ******** ***************

Ocewt1bF2R2     GAATGTGGTCGCAGATTCTCGCGTTCAGATCAGTTGAAGAGACACCAGCGCAGACACACA
Olawt1b         GAATGTGGTCGCAGATTCTCGCGTTCAGACCAGTTAAAGAGACACCAGCGCAGACACACA
                **************************** ***** ************************

Ocewt1bF2R2     GGAGTGAAGCCTTTTCAGTGTCAGACGTGTCAAAGAAAGTTCTCACGGTCAGATCACCTT
Olawt1b         GGAGTGAAGCCTTTTCAGTGTCAGACGTGTCAAAGAAAGTTCTCACGGTCAGATCACCTT
                ************************************************************

Ocewt1bF2R2     AAGACTCACACTCGGACTCATACAGGTAAAACAAGTGAGAAGCCTTTTACCTGCCGCTGG
Olawt1b         AAGACTCACACTCGGACTCATACAG---------GTGAGAAGCCTTTTACCTGCCGCTGG
                ************************         ************************

Ocewt1bF2R2     TCCAACTGTCAGAAGAAGTTTGCCCGCTCTGACGAGCTGGTGCGCCACCACAGCATGCAC
Olawt1b         TCCAACTGTCAGAAGAAGTTTGCCCGCTCTGACGAGCTGGTGCGCCACCACAGCATGCAC
                ************************************************************
```

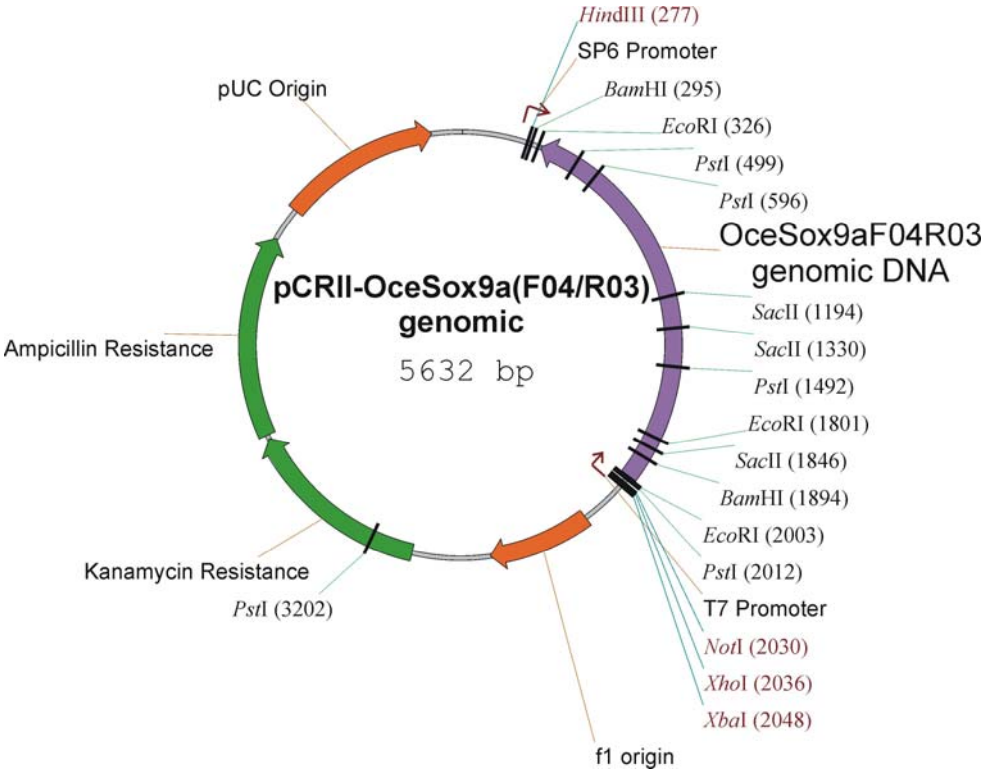
```
                                        Wt1bR2
Ocewt1bF2R2     CAGAGGAACCTGACCAAGCTGCAACCTGCCATTTAAAGAAAG
Olawt1b         CAGAGGAACCTGAGCAAGCTGCAACCTGCCATTTAAAGAAAG
                ************* ****************************
```

A.3.2. *Oce*Wt1b and *Ola*Wt1b amino acid alignment

```
OceWt1b         -------------------------------------------LHPGSPYGSLNSHHSLI
OlaWt1b         MSVGSDVCDLTLLPSAPPMPSLPGAVGSCGMSVGSGQWTNLLDLHPGSPYSSLNSHHSLI
                                                          *******.*********

OceWt1b         KQEPGWGTADPMEDPHCGLGAFTVHFSGQFTGSGPCRVSAFGEPASQPRMFPNAAYMPSC
OlaWt1b         KQEPGWGTADPMEDPHCGLGAFTVHFSGQLTGSGPCRVGAFGEPVSQPRMFPNAAYLPSC
                ****************************:********.*****.***********:***

OceWt1b         VDSSPLPRNQGYGTLALDSNPSYSHAASHHTPQLTTLSFKHEDTLSPPNNIVDQQYPTAN
OlaWt1b         MDSSPLPRNQGYGPLALDSNPGYSHSASHHNPQLSSLSFKHEDTLSPTNNI-DQQYPTLN
                :************.*******.***:****.***::***********.*** ****** *

OceWt1b         PMFGCHNPSESNPSSQTLLLRNYSSDNLYQMASQLECVXXXXXXXXXXXXXXXXXXXXX
OlaWt1b         PMFGCHNPSESTPNSQTLLLRNYSSDNLYQMASQLECVTWNQMNTLASPTKTGQAASFDS
                ***********.*.************************

OceWt1b         XXXXXXXXXXXXXXXXXXXXXXXXVSGMTPPVAKSSEASERRPFVCIYPGCSKRYFKLS
OlaWt1b         DPMLVSAQYHIHTHGVFRGLQDIRRVPGVTPPVAKSSEASERRPFVCVYPGCSKRYFKLS
                                  *.*:******************:************

OceWt1b         HLQMHGRKHTGEKPYQCDVTECGRRFSRSDQLKRHQRRHTGVKPFQCQTCQRKFSRSDHL
OlaWt1b         HLQMHGRKHTGEKPYQCDVTECGRRFSRSDQLKRHQRRHTGVKPFQCQTCQRKFSRSDHL
                ************************************************************

OceWt1b         KTHTRTHTGKTSEKPFTCRWSNCQKKFARSDELVRHHSMHQRNLTKLQPAI
OlaWt1b         KTHTRTHTG---EKPFTCRWSNCQKKFARSDELVRHHSMHQRNLSKLQPAI
                *********   *********************************:******
```

A.4. *Oryzias celebensis sox9a* (F04/R03) cloned into pCRII

Primer combination Sox9F04; TCGCATGAATCTCCTCGAC and Sox9R03; GTCAGCTGCGT-GTAGACCG amplified a 1659-bp genomic DNA fragment.



For antisense DIG-labeled RNA probe synthesis cut *Sac*II and use SP6-RNA-Polymerase (Roche, Mannheim).

A.4.1. *Ocesox9a* and *Olasox9a* nucleotide sequence alignment

```
                 Sox9F04
Ocesox9a         ATGAATCTCCTCGACCCCTACCTGAAGATGACAGAGGAACAAGACAAATGTCTCTCTGAC
Olasox9a         ATGAATCTCCTCGACCCTTACCTGAAGATGACGGAGGAACAAGACAAATGTCTCTCTGAC
                 ***************** ************** ****************************

Ocesox9a         GCCCCGAGCCCGAGCATGTCCGAGGACTCTGCGGGATCCCCGTCCAGCCCGTCCGGGTCG
Olasox9a         GCCCCGAGCCCGAGCATGTCCGAGGACTCCGCGGGATCCCCGGCCAGCCCGTCCGGGTCG
                 ***************************** ************ *****************

Ocesox9a         GGCTCCGACACCGAGAACACCCGGCCGCGGGAAAACGGGCTGATGCGCGCGGACGGGGCT
Olasox9a         GGCTCCGACACCGAGAACACCCGGCCGCGGGAAAACGGCCTGATGCGCGCGGACGGAGCT
                 ************************************ ***************** ***

Ocesox9a         CTGAGCGAATTCAAGAAGGACGAGGACGACAAGTTCCCTGCGTGCATCCGCGAGGCGGTG
Olasox9a         CTGAGCGACTTCAAGAAGGACGAAGACGACAAGTTTCCCGCGTGCATCCGGGAGGCGGTG
                 ******** ************** *********** ** *********** *********

Ocesox9a         TCTCAGGTGCTGAAGGGCTACGACTGGACCCTCGTGCCGATGCCGGTGCGCGTAAACGGA
Olasox9a         TCTCAGGTGCTGAAGGGCTACGACTGGACGCTCGTGCCGATGCCGGTGCGCGTAAACGGA
                 **************************** ******************************
```

```
Ocesox9a        TCTACAAAGAACAAGCCGCACGTGAAGAGGCCGATGAACGCCTTCATGGTGTGGGCGCAG
Olasox9a        TCTACAAAGAACAAGCCGCACGTGAAGAGACCAATGAACGCCTTCATGGTGTGGGCGCAG
                ***************************** ** *************************

Ocesox9a        GCGGCGCGCAGGAAGCTCGCGGATCAGTACCCCCACCTGCACAACGCGGAGCTCAGCAAA
Olasox9a        GCGGCGCGCAGGAAGCTCGCGGATCAGTACCCCCACCTGCACAACGCGGAGCTCAGCAAA
                ************************************************************

Ocesox9a        ACTCTGGGGAAGCTGTGGAGACTCCTGAATGAGGGGGAGAAGCGGCCGTTCGTGGAGGAG
Olasox9a        ACTCTGGGGAAGCTGTGGAGACTCCTCAATGAGGGGGAGAAGCGGCCGTTCGTGGAGGAG
                ************************** ********************************

Ocesox9a        GCGGAGCGGCTCCGCGTGCAGCATAAGAAGGACCACCCGGACTACAAGTACCAGCCGCGG
Olasox9a        GCGGAGCGGCTCCGCGTGCAGCATAAGAAGGACCACCCGGACTACAAGTACCAGCCGCGA
                ************************************************************

Ocesox9a        CGGAGGAAGTCCGTGAAGAGCGGAGGGAGCGAGGCGGAGGAGGGCGGGGAGCACATCTCC
Olasox9a        CGGCGGAAGTCCGTGAAGAGCGGCGGGAGCGAGGCGGAGGACGGCGGGGAGCACATCTCC
                *** ****************** ***************** ******************

Ocesox9a        ACCAACGCCATCTTCAAGGCTCTGCAACAGGCGGACTCTCCCGCCTCCAGCATGGGGGAG
Olasox9a        ACCAACGCCATCTTCAAGGCTCTGCAGCAGGCGGACTCCCCCGCCTCCAGCATGGGGGAG
                ************************** *********** *********************

Ocesox9a        GTGCACTCCCCCGCGGAGCACTCAGGCTCACAGGGACCCCCCACCCCTCCCACCGCCCCA
Olasox9a        GTGCACTCCCCCGCGGAGCACTCAGGCTCACAGGCACCCCCAACCCCTCCCACCACCCCA
                ********************************** ****** ************ *****

Ocesox9a        AAGACTGACAGCTCAGCAAAGGTGGACCTAAAGCGTGAGGGGGGGCTGCGCCCGCTGCCA
Olasox9a        AAGACGGACTGCTCAGCAAAGATGGACCTAAAGCGTGAGGGGGGGCTGCGCCCACTGCCG
                ***** *** *********** ******************************** *****

Ocesox9a        GACGGCGCCCCCGGGCGCCAGCTTAACATCGACTTCCGTGACGTGGACATCAGTGAGCTC
Olasox9a        GACGGCGCCCCCGGACGCCAGCTCAACATCGACTTCCGTGACGTGGACATCGGCGAGCTG
                ************** ******** *************************** * *****

Ocesox9a        AGCAGTGATGTCATCTCCCACATTGAGACGTTTGACGTCAACGAGTTCGACCAGTACCTC
Olasox9a        AGCAGTGACGTCATCTCCCACATTGAGACGTTCGACGTCAACGAGTTCGACCAGTACCTC
                ******** ********************** ****************************

Ocesox9a        CCCCCCAACGGGCATCCGGGCCCCGCCCCTGGGAATACTGCCCCAGTCATCTACAGCGGC
Olasox9a        CCCCCCAACGGGCACCCTGGCGCCGCCCCTGGGAGCACCGCCCCCGTCAGCTACAGCGGC
                ************** ** *** ************  ** ***** **** **********

Ocesox9a        AGCTACAGCATCAGCAACGGGCCCC-CC-CTGAGCCCGCAGGCAGGCGGGGGTCAGGCCT
Olasox9a        AACTACAGCATCAGCGGC--GCCCCGCCTCTGAGCCCGCAGGCAGGCGGGGGGCCGGCCT
                * *************  *  ***** ** ************************ * *****

Ocesox9a        GGATGGCAAAGGGCCACGGTCACCAGCAGCAGCACA-CTCTGACCCCCCTGGGGACCAGC
Olasox9a        GGATGGCTAAGGCCCACAGTCAGCAGCAGCAGCACAGC-CTGACCCCCCTGGGGACCAGC
                ******* **** **** **** ************* * **********************

Ocesox9a        GGGGGCTCAGAGGCCGCTCAGCACAGGACCCAGATTAAGACAGAGCAGCTGAGCCCGAGT
Olasox9a        GGGGGTTCAGAGGCCGCCCTGCGCAGGACCCAGATTAAGACGGAGCAGCTGAGCCCGAGT
                ***** *********** * ** ****************** ******************
```

```
Ocesox9a        CACTACAGCGAGCAGCAGGGCTCCCCCCAGCACGCCGCCTACAGCCCCTTCAACCTGCAG
Olasox9a        CACTACAGCGAGCAGCAGGGCTCCCCCCAGAACGCCCCCTACAGCCCCTTCAACCTGCAG
                ****************************** ***** ***********************

Ocesox9a        CACTACAGCCCCCCCTCCTCCTCGTACCCGCCCATCTCCCGGGCGCAGCACTATGACTAC
Olasox9a        CACTACAGCCCCCCCTCCTCCTCGTACCCGCCCATCTCCCGAGCGCAGCAGTATGATTAC
                **************************************** ******** ***** ***

Ocesox9a        CCCGACACCCAGGGAGGGAGCTTCTACAGCCCTGCAGGGGCGGGGCAGGGCTCTGGTCTG
Olasox9a        CCCGACCCCCAGGGAGGGGGCTTCTACAGCCCTGCAGGGGCGGGGCAGGGTTCTGGGCTG
                ****** ********** ******************************** ***** ***

Ocesox9a        TACTCGACTTTCAGCTACATGGGTAGCCCCAGCCAGAGGCCCATGTACACCCCCATTGCC
Olasox9a        TACTCGACTTTCAGTTACATGAGCAGCCCCAGCCAGAGGCCCATGTACACCCCCATCGCC
                ************** ****** * ********************************* ***

Ocesox9a        GACAACGCTGGGGTGCCGTCCATCCCCCAA---AGCCCGCAGCCCTGGGAACCGGCGCCG
Olasox9a        GACAACGCGGGGGTGCCGTCCATCCCCCAGGGCAGCCCGCAGCACTGGGAGCAGGCGCCG
                ******** ********************    ********** ****** * *******

Ocesox9a        GTCTACACGCAACTGACCCGGCCCTGA
Olasox9a        GTCTACACGCAGCTGACCCGGCCCTGA
                *********** ***************
```

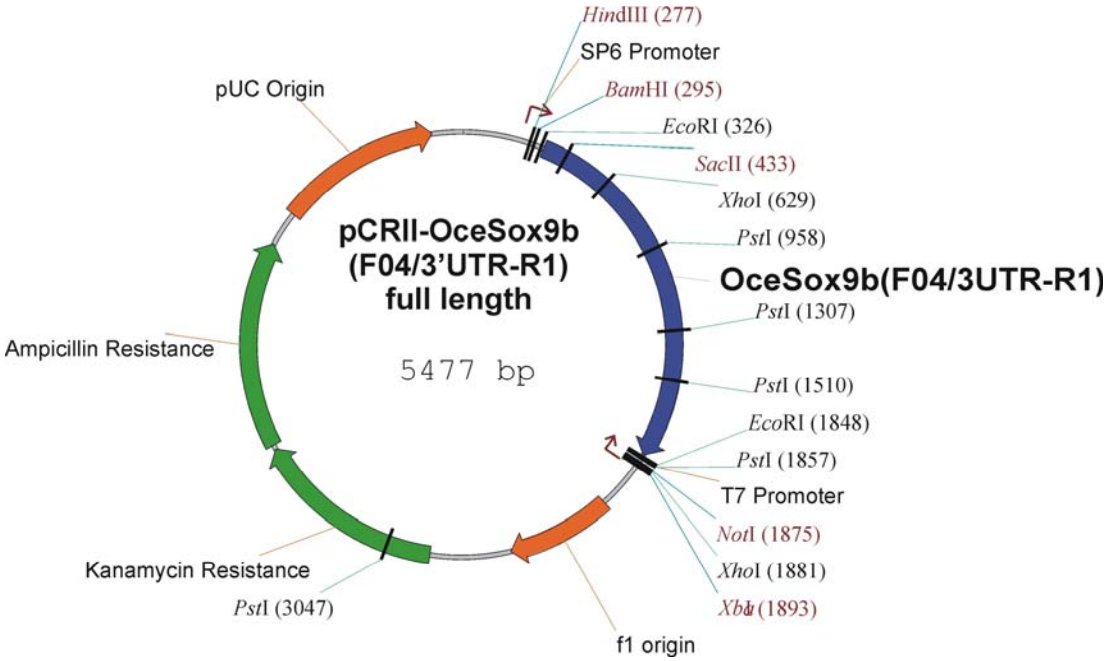
A.4.2. *Oce*Sox9a and *Ola*Sox9a amino acid alignment

```
OceSox9a        MNLLDPYLKMTEEQDKCLSDAPSPSMSEDSAGSPSSPSGSGSDTENTRPRENGLMRADGA
OlaSox9a        MNLLDPYLKMTEEQDKCLSDAPSPSMSEDSAGSPASPSGSGSDTENTRPRENGLMRADGA
                **********************************:**************************
                                                                 N-NLS1
OceSox9a        LSEFKKDEDDKFPACIREAVSQVLKGYDWTLVPMPVRVNGSTKNKPHVKRPMNAFMVWAQ
OlaSox9a        LSDFKKDEDDKFPACIREAVSQVLKGYDWTLVPMPVRVNGSTKNKPHVKRPMNAFMVWAQ
                **:**********************************************************
                 N-NLS2
OceSox9a        AARRKLADQYPHLHNAELSKTLGKLWRLLNEGEKRPFVEEAERLRVQHKKDHPDYKYQPR
OlaSox9a        AARRKLADQYPHLHNAELSKTLGKLWRLLNEGEKRPFVEEAERLRVQHKKDHPDYKYQPR
                ************************************************************
                C-NLS
OceSox9a        RRKSVKSGGSEAEEGGEHISTNAIFKALQQADSPASSMGEVHSPAEHSGSQGPPTPPTAP
OlaSox9a        RRKSVKSGGSEAEDGGEHISTNAIFKALQQADSPASSMGEVHSPAEHSGSQAPPTPPTTP
                *************:**************************************.******:*

OceSox9a        KTDSSAKVDLKREGGLRPLPDGAPGRQLNIDFRDVDISELSSDVISHIETFDVNEFDQYL
OlaSox9a        KTDCSAKMDLKREGGLRPLPDGAPGRQLNIDFRDVDIGELSSDVISHIETFDVNEFDQYL
                ***.***:*****************************.**********************

OceSox9a        PPNGHPGPAPGNTAPVIYSGSYSISNGPPLSPQAGGGQAWMAKGHGHQQQHTLTPLGTSG
OlaSox9a        PPNGHPGAAPGSTAPVSYSGNYSISGAPPLSPQAGGGPAWMAKAHSQQQQHSLTPLGTSG
                *******.***.**** ***.****..********** *****.*.:****:********

OceSox9a        GSEAAQHRTQIKTEQLSPSHYSEQQGSPQHAAYSPFNLQHYSPPSSSYPPISRAQHYDYP
OlaSox9a        GSEAALRRTQIKTEQLSPSHYSEQQGSPQNAPYSPFNLQHYSPPSSSYPPISRAQQYDYP
                ***** :**********************:*.**********************:****
```

```
Oce Sox9a       DTQGGSFYSPAGAGQGSGLYSTFSYMGSPSQRPMYTPIADNAGVPSIPQ-SPQPWEPAPV
Ola Sox9a       DPQGGGFYSPAGAGQGSGLYSTFSYMSSPSQRPMYTPIADNAGVPSIPQGSPQHWEQAPV
                *.***.******************.********************** *** ** ***

Oce Sox9a       YTQLTRP
Ola Sox9a       YTQLTRP
                *******
```

A.5. *Oryzias celebensis sox9b* (F04/3'UTR-R1) cloned into pCRII

Primer combination Sox9F04; TCGCATGAATCTCCTCGAC and OceSox9b-3UTR-R1; GGA-GGGGTGGAGTAAAGACC amplified a 1504-bp cDNA fragment.



For antisense DIG-labeled RNA probe synthesis cut *BamH*I and use T7-RNA-Polymerase (Roche, Mannheim).

A.5.1. *Ocesox9b* and *Olasox9b* nucleotide sequence alignment

```
                    Sox9F04
Ocesox9b        ATGAATCTCCTCGACCCATACCTGAAGATGACAGAAGAACAGGAGAAGTGTCACTCCGAC
Olasox9b        ATGAATCTCCTCGATCCATACCTGAAGATGACAGAAGAACAGGAGAAGTGTCACTCCGAC
                ************** ********************************************

Ocesox9b        GCTCCCAGCCCCAGCATGTCCGAGGACTCCGCGGGCTCGCCATGTCCGTCTGGCTCCGGC
Olasox9b        GCTCCCAGTCCCAGCATGTCTGAGGACTCCGCAGGTTCGCCTTGTCCGTCCGGCTCCGGT
                ******** *********** *********** ** ***** ******** ********

Ocesox9b        TCGGACACCGAAAACACTCGGCCGTCCGACAACCATCTCATCAGGGGTCCGGACTACAAG
Olasox9b        TCGGACACCGAAAACACCCGGCCGTCCGACAACCATCTCATCAGGGGGCCGGACTACAAG
                ***************** ***************************** ************

Ocesox9b        AAAGAGGGCGAAGAGGAGAAGTTCCCCGTGTGCATCAGAGACGCCGTGTCCCAAGTGCTC
Olasox9b        AAAGAGGGTGAAGAAGAGAGGTTCCCCGTGTGCATTAGAGACGCGGTGTCCCAAGTGCTC
                ******** ***** **** *************** ******** ***************

Ocesox9b        AAGGGTTACGACTGGACTCTGGTGCCGATGCCAGTGCGCGTGAACGGCTCGAGTAAAAGC
Olasox9b        AAGGGCTACGACTGGACTCTGGTGCCGATGCCAGTACGCGTCAACGGCTCGAGTAAAAGC
                ***** ***************************** ***** ******************
```

```
Ocesox9b        AAACCTCACGTGAAAAGACCCATGAACGCATTCATGGTCTGGGCTCAAGCAGCACGCAGG
Olasox9b        AAACCTCACGTCAAAAGGCCCATGAATGCATTCATGGTCTGGGCTCAGGCAGCACGGAGG
                *********** ***** ******** ******************** ******** ***

Ocesox9b        AAACTGGCCGATCAATACCCGCATTTGCACAACGCAGAGCTCAGCAAAACTCTTGGCAAA
Olasox9b        AAACTGGCCGACCAATACCCGCATTTGCACAACGCAGAGCTCAGCAAAACTCTTGGAAAA
                *********** ****************************************** ***

Ocesox9b        CTTTGGAGGCTCCTCAATGAGGTGGAGAAGCGGCCGTTTGTGGAGGAGGCTGAGCGACTG
Olasox9b        CTTTGGAGGCTCCTTAATGAGGTGGAGAAGCGGCCGTTTGTGGAGGAGGCTGAGCGACTG
                ************** ********************************************

Ocesox9b        AGGGTGCAGCACAAGAAGGATCACCCCGACTATAAGTATCAGCCCAGACGGAGAAAATCT
Olasox9b        AGGGTCCAGCACAAGAAGGATCATCCCGACTATAAATACCAGCCCAGGCGGAGAAAATCT
                ***** ***************** *********** ** ******** ************

Ocesox9b        GTCAAGAACGGCCAAAGTGAAGCCGAGGACAGTGAGCCGACTCACATCTCTCCAAATGCG
Olasox9b        GTCAAGAACGGTCAAAGCGAAGCGGAGGACAGCGAACCGACTCACATCTCTCCAAATGCG
                *********** ***** ***** ******** ** ************************

Ocesox9b        ATCTTCAAAGCGCTGCAGCAGGTCGACTCTCCTGCGTCCAGCTTGGGCGAGGCGCACTCT
Olasox9b        ATCTTCAAAGCGCTGCAGCAGGCCGACTCTCCTGCGTCCAGCATGGGCGAGGCGCACTCT
                ********************** ******************* *****************

Ocesox9b        CCAGGAGAACATTCAGGTCAGTCACAGGGGCCACCAACACCCCCAACAACCCCCAAGACA
Olasox9b        CCAGGAGAACATTCAGGTCAGTCACAGGGGCCACCAACACCCCCAACAACCCCCAAGACA
                ************************************************************

Ocesox9b        GATCTTCCTACCAGCAAAGCTGACCTGAAGCGAGAGGGGCGCCCTGTACAGGAGGGCACC
Olasox9b        GATCTTCCTTCCAGCAAAGCTGACCTGAAGCGCGAGGGCCGCCCAGTGCAAGAGGGCACC
                ********* ********************** ***** ***** ** ** *********

Ocesox9b        AGCCGCCAGCTCAACATAGACTTTGGCGCTGTGGACATCGGTGAGCTGAGCAGCGACGTC
Olasox9b        AGCCGCCAGCTCAACATAGACTTTGGCACTGTGGACATCGGTGAGCTGAGCAGCGACGTC
                **************************** ********************************

Ocesox9b        ATCTCCAACATTGGCAGCTTCGACGTCGATGAGTTCGATCAGTACCTGCCCCCTCACAGC
Olasox9b        ATCTCCAACATCGGCAGCTTCGACGTCGATGAGTTCGATCAGTACCTGCCCCCTCACAGC
                *********** ************************************************

Ocesox9b        CACGCCGGGATGACCGGCACAGCCCAGACGGGCTACTCCAACAACTACGCTATCAACAGC
Olasox9b        CACGCTGGGATGACCGGCACAGCCCAGACCAGCTACTCCAACAACTACGTTATCAACAGC
                ***** ************************  ****************** **********

Ocesox9b        TCTGCAGTTGGCCAGACAGCCAACGTTGGAGCCCACGCCTGGATGCCCAAGCAGCCGCAT
Olasox9b        TCTGCCGTTGGCCAGACAGCCAATGTTGGAGCCCATGCCTGGATGCCCAAACAGCAGCAT
                ***** ***************** *********** ************** **** ****

Ocesox9b        TCTTTGGCCACCCTGGGTGGGGGTGGAGATCAAAGCCAGCAGGGTCAGCAGAGAACCACT
Olasox9b        TCTTTGGCCACCCTCGGTGGGGGTGGAGATCAAAGCCAACAGGGTCAACAGAGAACCACT
                ************** ********************** ******** ************

Ocesox9b        CAGATCAAGACGGAGCAGCTGAGCCCCAGCCACTACAGCGAGCAGCAGAGCTCCCCGCAG
Olasox9b        CAGATCAAGACGGAGCAGCTGAGCCCCAGCCACTACAGCGAGCAGCAAAGCTCCCCACAG
                ************************************************ ******** ***
```

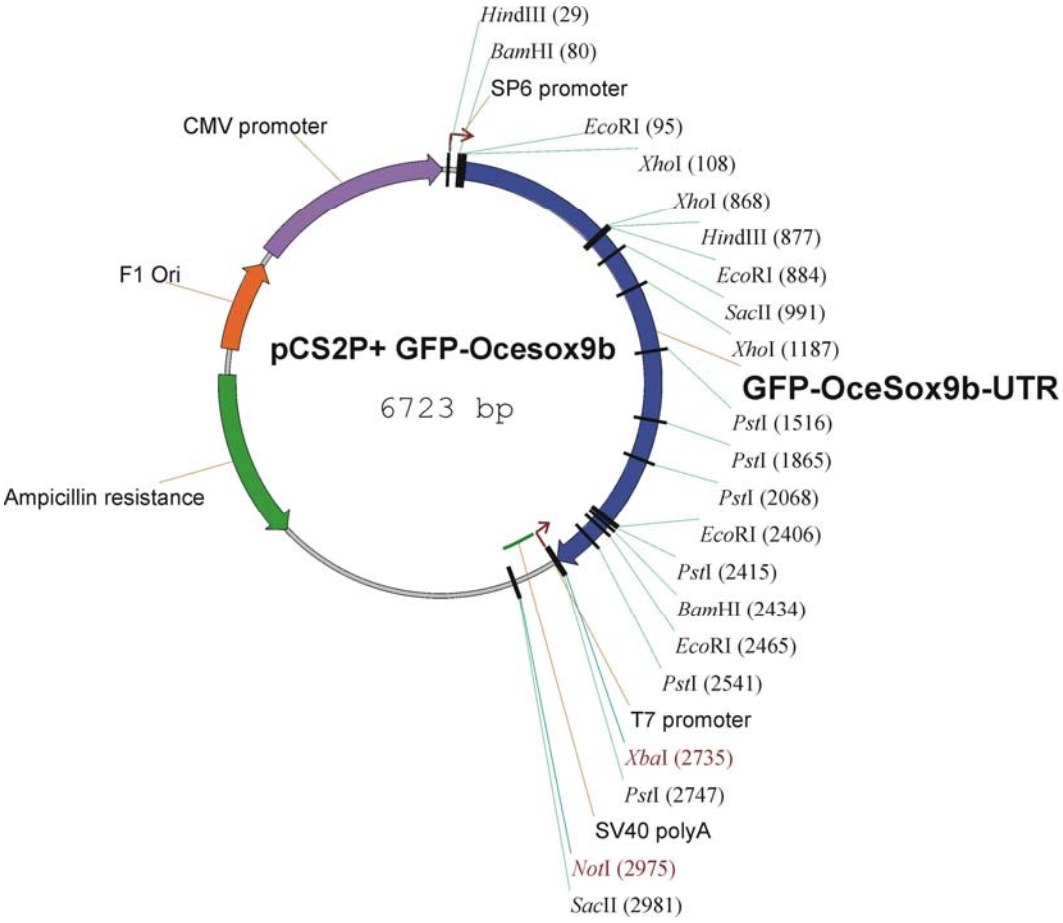
```
Ocesox9b        CACGTCAGCTATGGGTCCTTCAACCTGCAGCACTACAGCACCTCCTCTTACCCCTCCATC
Olasox9b        CACGTCAGCTACGGGTCCTTCAACCTGCAGCACTACAGCACCTCTTCTTACCCCTCCATC
                *********** ******************************** ***************

Ocesox9b        ACAAGAGCACAGTATGACTATTCAGACCACCAAAACAGTGCCAACTCCTACTACAGCCAT
Olasox9b        ACAAGAGCACAGTATGACTATTCAGACCACCAAAATAGTGCCAACTCTTACTACAGCCAT
                ********************************** *********** ************

Ocesox9b        GCAGCTGGTCAGGGCTCCAACATGTACTCCACCTTCAGCTACATGAGCCCCAGCCAGCGG
Olasox9b        GCAGCTGGTCAAGGCTCCAACATGTACTCCACCTTCAGCTACATGAGCCCCAGCCAGAGG
                *********** ********************************************** **

Ocesox9b        CCGATGTACACCCCGATCGCTGACAGTACTGGGGTGCCATCTGTGCCGCAGACCCACAGT
Olasox9b        CCGATGTATACCCCCATCGCTGACAGTACTGGGGTGCCATCTGTGCCACAGACCCACAGT
                ******** ***** ******************************** ************

Ocesox9b        CCTCAGCACTGGGAGCAGCAGCCCATATACACACAACTGTCCAGACCATGA
Olasox9b        CCACAGCACTGGGAGCAGCAGCCCATATACACCCAGCTGTCCAGACCGTGA
                ** ***************************** ** *********** ***
```

A.5.2. *Oce*Sox9b and *Ola*Sox9b amino acid alignment

```
OceSox9b        MNLLDPYLKMTEEQEKCHSDAPSPSMSEDSAGSPCPSGSGSDTENTRPSDNHLIRGPDYK
OlaSox9b        MNLLDPYLKMTEEQEKCHSDAPSPSMSEDSAGSPCPSGSGSDTENTRPSDNHLIRGPDYK
                ************************************************************
                                                          N-NLS1      N-NLS2
OceSox9b        KEGEEEKFPVCIRDAVSQVLKGYDWTLVPMPVRVNGSSKSKPHVKRPMNAFMVWAQAARR
OlaSox9b        KEGEEERFPVCIRDAVSQVLKGYDWTLVPMPVRVNGSSKSKPHVKRPMNAFMVWAQAARR
                ******:*****************************************************
                                                                  C-NLS1
OceSox9b        KLADQYPHLHNAELSKTLGKLWRLLNEVEKRPFVEEAERLRVQHKKDHPDYKYQPRRRKS
OlaSox9b        KLADQYPHLHNAELSKTLGKLWRLLNEVEKRPFVEEAERLRVQHKKDHPDYKYQPRRRKS
                ************************************************************

OceSox9b        VKNGQSEAEDSEPTHISPNAIFKALQQVDSPASSLGEAHSPGEHSGQSQGPPTPPTTPKT
OlaSox9b        VKNGQSEAEDSEPTHISPNAIFKALQQADSPASSMGEAHSPGEHSGQSQGPPTPPTTPKT
                ***************************.******:************************

OceSox9b        DLPTSKADLKREGRPVQEGTSRQLNIDFGAVDIGELSSDVISNIGSFDVDEFDQYLPPHS
OlaSox9b        DLPSSKADLKREGRPVQEGTSRQLNIDFGTVDIGELSSDVISNIGSFDVDEFDQYLPPHS
                ***:*************************:******************************

OceSox9b        HAGMTGTAQTGYSNNYAINSSAVGQTANVGAHAWMPKQPHSLATLGGGGDQSQQGQQRTT
OlaSox9b        HAGMTGTAQTSYSNNYVINSSAVGQTANVGAHAWMPKQQHSLATLGGGGDQSQQGQQRTT
                **********.*****.********************* *********************

OceSox9b        QIKTEQLSPSHYSEQQSSPQHVSYGSFNLQHYSTSSYPSITRAQYDYSDHQNSANSYYSH
OlaSox9b        QIKTEQLSPSHYSEQQSSPQHVSYGSFNLQHYSTSSYPSITRAQYDYSDHQNSANSYYSH
                ************************************************************

OceSox9b        AAGQGSNMYSTFSYMSPSQRPMYTPIADSTGVPSVPQTHSPQHWEQQPIYTQLSRP
OlaSox9b        AAGQGSNMYSTFSYMSPSQRPMYTPIADSTGVPSVPQTHSPQHWEQQPIYTQLSRP
                ***********************************************************
```

A.6. GFP-OceSox9b cloned into pCS2P+

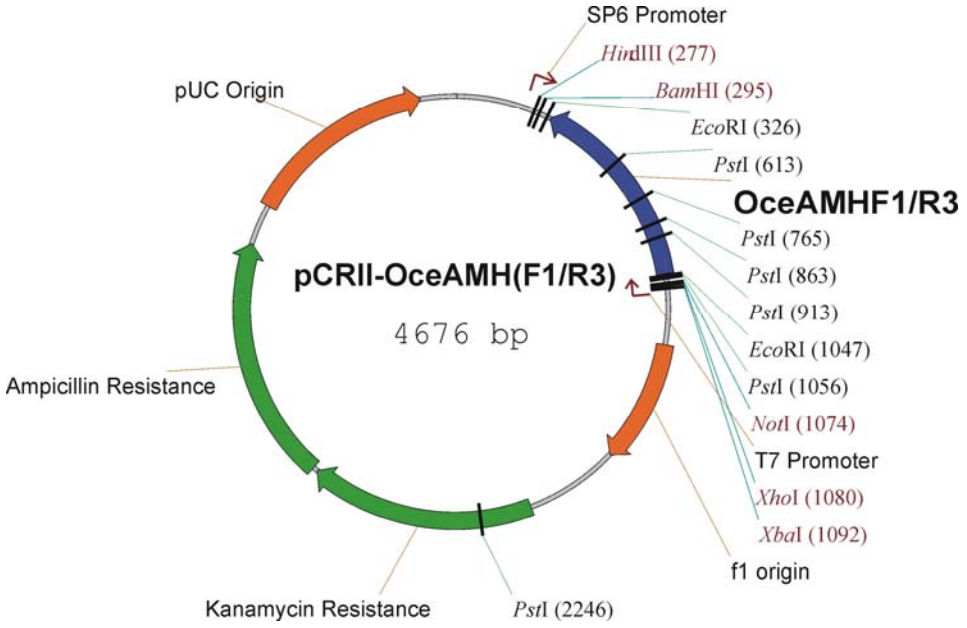
Cloning strategy: *Ocesox9b* (F04/3'UTR-R1) subcloned into pEGFP-C1 (*EcoRI*). pEGFP-C1-OceSox9b with *NheI*/*XbaI* and subcloned into pCS2P+ (*XbaI*).



For 5'-capped mRNA synthesis cut *NotI* and use the SP6-mMessage mMachinekit (Ambion).

A.7. *Oryzias celebensis amh* (F1/R3) cloned into pCRII

Primer combination OlaMIS-F1;ATGTTGCTTGTGCTGCTGTT and OlaAMH-R3;ATGAGGT-CTGCACAGGAACC amplified a 703-bp cDNA fragment.



For antisense DIG-labeled RNA probe synthesis cut *NotI* and use SP6-RNA-Polymerase (Roche, Mannheim).

A.7.1. *Oceamh* and *Olaamh* nucleotide sequence alignment

```
                    OlaMIS-F1
Oceamh          ATGTTGCTTGTGCTGCTGTTNNNNNNNNNNNNNNNCCGGGACTCAGCAGGCCCAGCACCAC
Olaamh          ATGTTGCTTGTGCTGCTGTTCTGGATCGGGCTCGCTGGGACCCAGCAGGTCCAGCACCAC
                ********************               * ***** ******* **********

Oceamh          ATCTCCACAGG------TCAGCGGGTGAAGCCGTGCTTTGAGCACGACGCGGTCGCAGCG
Olaamh          AGCTCCACCGGAACAGATCGGCGGGTGGAGCCGTGCTTTGTGGATGATGCGGTGGCAGCG
                * ****** **      ** ******* ************ * * ** ***** ******

Oceamh          CTGCGTGAAGCCCTGCAGGCTGATGGAGCGCTCTCCAACAGCAGCCTCACCTTGTTTGGA
Olaamh          CTGCGTGAAGCGCTGCAGACTGACGGAGCGCTCTCCAACAGCAGCCTCACCCTGTTTGGA
                *********** ****** **** ************************** ********

Oceamh          ACCTGCAGAGCACCGGATCATGCCACCCGCTCTGCCTTGCTGGTGCTTTCAGAAGAGATG
Olaamh          GCCTGCAGAGCGCCGGATCCTTCCTCCCGCTCCACCTTGTTGGTGCTTTCAGAGGAGAGG
                 ********** ******* * ** *******  ***** ************* **** *

Oceamh          CAGAGAAAGACGGGTGTAGGTCTGGAGGTCCTGCACCCAGCTGCAGCGTTTGCGGCAGTG
Olaamh          CGGAGATGGCCAGATGGAGGTCTGAAGGTCCGGCAACCAGCTGCAGTGTTTGCAACAGAG
                * ****  * * * ** ******* ****** *** ********** ******  *** *

Oceamh          GAGCAAGGGGTCGTCAGCCTGACGATTGACTTCCCATCATCCCCTGCACTGGAGCTAAAC
Olaamh          GAGCGAGGGGTCGTCACCCTGACCTTTGACCTCCCAGCATCCTCTTTGCTGGAGATGAAC
                **** ********** ******  ***** ***** ***** **   ****** * ***
```

```
Oceamh          ---CCGGTGTTGCTGCTGGCCTTTGAAAGCCCCCCCACTGTTGGAGACCTGGAGGTCACG
Olaamh          TTGCTGCTGCTGCTGCTGGCCTTTGAAAGCCCCGCCGCTGCTGGAGACCTGGAGGTCACG
                   * * ** ********************** ** *** ******************

Oceamh          TTCAGCAGCCGCTTTCTGCAGCCGCACTCACAGTCTGCTTGTATCTCAGAAGGAACTCTC
Olaamh          TTCAGCAGCCGCTTTCTGCTGCCACACTCACAGTCTGCTTGTATCTCAGAAGGAACCCTC
                ****************** *** ******************************** ***

Oceamh          TACGTCCTCCTGAAGGGCACATCTTCCAGCAGCAGCTCTCAGGACATATGGAGGATCTCC
Olaamh          TATGTTCTCCTGATGGGCAGATCTTCCAGCAGCAGCTCTCAGCACAGATGGACAATCAGC
                ** ** ******* ***** ********************** *** *****  ***  *

Oceamh          GTTGAAGCGAAATCCACTGAAATGAACCAAAACCTGATCGACGCCATCGTTGGTGGGAAA
Olaamh          GCTGAAGCAAAATCACCAAGAATGAACCAAAACCTGATAGACGCCATCATTGGTGGGAAA
                * ****** *****  *   ****************** ********* ***********

Oceamh          TCTGGAGGTCTGCTTGGCGTGACACCACTTCTNNNNNNNNNNNNNNNNNNNNNNNNNNN
Olaamh          CCTGGAGGTCCGGTTGGCGTGACGCCACTTCTGCTTTTCACAGGAGAGGAAGGAATCAAT
                 ********* * ********** ********

                                                        OlaAMH-R3
Oceamh          NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGTTCCTGTGCAGACCTCAT--------
Olaamh          GGCAGCCGTCCAGCTGCCTTTGGTTCCTCCGGGGTTCCTGTGCAGACCTCATTCGTCTGT
                                          ********************

Oceamh          ------------------------------------------------------------
Olaamh          GAACTGAGGAGGTTCCTGGAGGGCGTCCTGCCTCAGGACCAGGACAAACAGCCACTGCTC

Oceamh          ------------------------------------------------------------
Olaamh          CAGCTGGACTCCCTCCAGGCCCTGCCCCCCCTTCCCCTGGGCTTGTCCTCCAGTGAGACC

Oceamh          ------------------------------------------------------------
Olaamh          CTGCTGGCGGCGCTTATTAATTCTTCTGCTCCAACTATCTTCTACTTCAACAACTGGGGC

Oceamh          ------------------------------------------------------------
Olaamh          TCCAAGTCTCAGGGCCTCCGGGGACAGCTGATCCTGTCTCCTGCGCTGTTGGTGGAGCTC

Oceamh          ------------------------------------------------------------
Olaamh          AGGCTCAGGCTGGAGCAGAGCGAGATGCTCATTCTGGATCTGGCAGAGCAGGAAACGGTT

Oceamh          ------------------------------------------------------------
Olaamh          TCTCCCAGAACCACGGCCCGACTGGAAAGGCTGAAGGACCTCAGTGCTTCTCAGCAGATG

Oceamh          ------------------------------------------------------------
Olaamh          GAAGCAGCGGAGAGTCACTTCTGTGCCTTTCTTCTGCTGAAGGCGCTGAAGACGGTGGCG

Oceamh          ------------------------------------------------------------
Olaamh          CATGCGTACGACATGACCAGGCGACTGCGGAGCACCAGGGCGGGTCCCGACAGCCCGCCC
```

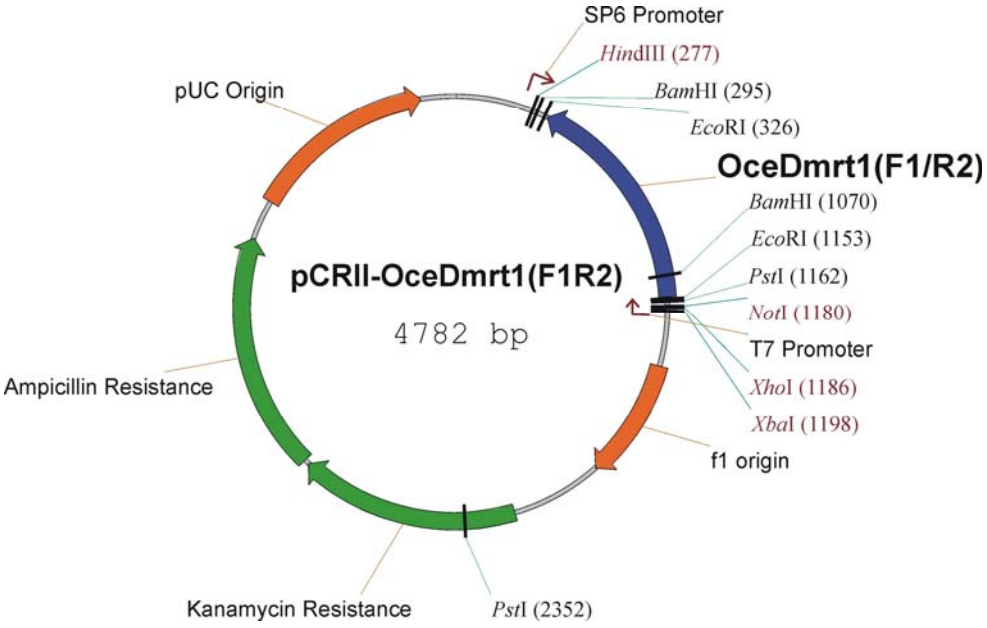
```
Oceamh          ------------------------------------------------------------
Olaamh          AAGGGCAACATCTGTGGGCTGAGGAGCCTCACCGTGTCCTTTGAGAAGCTTCTTCTGGGT

Oceamh          ------------------------------------------------------------
Olaamh          CCGCAGAGCGCCAACATCAACAACTGCCGCGGGGTGTGCTCCTTCCCTCTGATCAACGGC

Oceamh          ------------------------------------------------------------
Olaamh          AACAACCACGCCATCCTGCTCACCTCGCACACGGAGAGCGTGGCGGAAGAGCGCGCGCCC

Oceamh          ------------------------------------------------------------
Olaamh          TGCTGCGTGCCCGTGGCCTATGACCCCCTGGAGGTCTTGGACTGGAATGATGAAGGCTCC

Oceamh          --------------------------------------------------
Olaamh          TTCCTGTCCATCAAGCCGGACATGATCGCGCGGGAGTGTGGCTGCCGCTGA
```

A.7.2. *Oce*Amh and *Ola*Amh amino acid alignment

```
OceAmh          MLLVLL------GTQQAQHHISTG--QRVKPCFEHDAVAALREALQADGALSNSSLTLFG
OlaAmh          MLLVLLFWIGLAGTQQVQHHSSTGTDRRVEPCFVDDAVAALREALQTDGALSNSSLTLFG
                ******      ****.*** ***  :**:*** .***********:*************

OceAmh          TCRAPDHATRSALLVLSEEMQRKTGVGLEVLHPAAAFAAVEQGVVSLTIDFPSSPALELN
OlaAmh          ACRAPDPSSRSTLLVLSEERRRWPDGGLKVRQPAAVFATEERGVVTLTFDLPASSLLEMN
                :***** ::**:******* :* .. **:* :***.**: *:***:**:*:*:*. **:*

OceAmh          PVLLL-AFESPPTVGDLEVTFSSRFLQPHSQSACISEGTLYVLLKGTSSSSSSQDIWRIS
OlaAmh          LLLLLLAFESPAAAGDLEVTFSSRFLLPHSQSACISEGTLYVLLMGRSSSSSSQHRWTIS
                 :*** *****.:.************ ***************** * *******. * **

OceAmh          VEAKSTEMNQNLIDAIVGGKSGGLLGVTPL-----------------------------
OlaAmh          AEAKSPRMNQNLIDAIIGGKPGGPVGVTPLLLFTGEEGINGSRPAAFGSSGVPVQTSFVC
                .****..*********:***.** :*****

OceAmh          ------------------------------------------------------------
OlaAmh          ELRRFLEGVLPQDQDKQPLLQLDSLQALPPLPLGLSSSETLLAALINSSAPTIFYFNNWG

OceAmh          ------------------------------------------------------------
OlaAmh          SKSQGLRGQLILSPALLVELRLRLEQSEMLILDLAEQETVSPRTTARLERLKDLSASQQM

OceAmh          ------------------------------------------------------------
OlaAmh          EAAESHFCAFLLLKALKTVAHAYDMTRRLRSTRAGPDSPPKGNICGLRSLTVSFEKLLLG

OceAmh          ------------------------------------------------------------
OlaAmh          PQSANINNCRGVCSFPLINGNNHAILLTSHTESVAEERAPCCVPVAYDPLEVLDWNDEGS

OceAmh          ----------------
OlaAmh          FLSIKPDMIARECGCR
```

A.8. *Oryzias celebensis dmrt1* (F1/R2) cloned into pCRII

Primer combination OceDMRT1F1;CTGAGATCTCGGGCCATCAT and OceDMRT1R2;CTC-TCAAACTCCGCCTTGAC amplified an 809-bp cDNA fragment.



For antisense DIG-labeled RNA probe synthesis cut *NotI* and use SP6-RNA-Polymerase (Roche, Mannheim).

A.8.1. *Ocedmrt1*, *Oladmrt1a* and *Oladmrt1bY* nucleotide sequence alignment

```
Ocedmrt1        ATGAGCAGGGAGGGGCAGTGCAGACCGGTGCCGGAGGGCCCCGGTTCCGGTTCGGATCCG
Oladmrt1bY      ATGAGCAAGGAGAAGCAGTGCAG------GCCG---------------GGT---------
Oladmrt1a       ATGAGCAAGGAGAAGCAGGGCAGGCCGGTTCCGGAGGG-CCCGGCTC-----CGGGTCCA
                ******* ****  **** ****       ***

Ocedmrt1        CAGAGGTCCCCCCGGATGCCCAAATGCTCCCGGTGCCGGAACCACGGCTTCGTGTCTCCG
Oladmrt1bY      ---------CCCCGGGTGCCCAAGTGCTCCCGCTGCCGGAACCACGGCTTGAAGACCCCG
Oladmrt1a       CAGCGGTCCCCCCGGATGCCCAAGTGCTCCCGCTGCCGGAACCACGGCTTCGTGTCTCCG
                         ****** ******* ******** *****************   * * ***

Ocedmrt1        CTGAAAGGCCACAAGCGCTTCTGCCGCTGGAAGGACTGCCGCTGCGCCAAATGCAAGCTG
Oladmrt1bY      CTGAAAGGCCACAAGCGCTTCTGCCGCTGGAAAGACTGCCAGTGCTTCAAATGCGAGCAG
Oladmrt1a       CTGAAAGGCCACAAGCGCTTCTGCCGCTGGAAGGACTGCCGCTGCGCCAAATGCAAGCTG
                ********************************* *******  ***  ******* *** *

Ocedmrt1        ATCGCGGAGCGCCAGAGAGTCATGGCGGCGCAGGTTGCCCTGAGAAGGCAGCAGGCTCAA
Oladmrt1bY      ATCATGGTCCGCCAGAGAGTCATGGCGGCGCAGGTCGCCGATAGGAGGCAGCAGGCTCAA
Oladmrt1a       ATTGCGGAGGGCCAGAGAGTCATGGCGGCGCAGGTCGCCTTGAGGAGGCAGCAGGCTCAA
                **   **   ************************* ***   ** ***************

Ocedmrt1        GAGGAGGAGCTTGGGATTTGTAGTCCAGAAACTTCGTCTGGCCCTGAAGTGATGATGAAG
Oladmrt1bY      GAGGAGGAGCTTGGGATTTGTAGTCCAGAGGCCTCGTCAGGCCCTGAAGTGGTGGTGAAG
Oladmrt1a       GAGGAGGAGCTTGGGATTTGTAGTCCAGAGGCCTCGTCCGGCCCTGAAGTGACGGTGAAG
                ****************************  * ***** ************  * *****
```

```
Ocedmrt1        AATGAAGCTGGAGCCGACTGCCTGTTCTCCATGGAGGGACGATCCGTAACACCAGCCGTC
OladmrtlbY      AATGAAGCCGGAGCCGACTGTCTCTTCTCCGTGGAGGGACGATCCGGCACGCTAGCCATC
Oladmrt1a       AATGAAACTGGAGCCGACTGTCTCTTCTCCATGGAGGGGCGATCCGGCACGCCAGGCGTC
                ****** * ********** ** ****** ******* *******  ** * ** * **

Ocedmrt1        ACCCCCA---CC---CCTTTGTCTGGCTCAGGGAGCTGCTCGGCTTCGTCGTCCAGCCCA
OladmrtlbY      CCCCCCAGCCCCAATCCTCTGTCTGCCGCAGGGAGCTACTCTGCTTCATCGTCCAGCCCA
Oladmrt1a       CCCCCCAAC------CCTCTGTCTGCCGCAGGGAGCTGCTCGGCGTCATCGTCCAGCCCA
                 ******        *** ****** * ********* *** ** ** ************

Ocedmrt1        TTGGCTGCGGCCCGGGTTCACGCCGAGGAACCGTCTGACCTGCTGCTGGAAACTTCTTAC
OladmrtlbY      TCGGCTGCGGCCCGTGTTTACGGCGAGGAAGCGTCTGACTGCCTGCTGGAAACCTCCTAC
Oladmrt1a       TCGGCTGCGGCCCGTGTTTACGGCGAGGAAGCGTCTGACCTGCTGCTGGAAACCTCCTAC
                * ************ *** *** ******* ********   ************ ** ***

Ocedmrt1        TACAATTTTTACCAGCCTTCAAGATATTCCTCTTACTATGGAAACCTGTACAACTACCAG
OladmrtlbY      TACAACTTCTACCAGCCTTCCAGATATTCCTCCTATTATGGAAACCTGCACAACTACCAG
Oladmrt1a       TACAACTTCTACCAGCCTTCCAGATACTCCTCCTACTATGGAAACCTG------TACCAG
                ***** ** ********** ***** ***** ** ************      ******

Ocedmrt1        CAGTACCAG---ATGCCCCACACTGACAGCCGCCTGTCCGGCCACAGCATGTCCTCTCAG
OladmrtlbY      CAGTACCAGCAGATGCCCCCCTCCGACGGCCGCCTGTCCGGCCACAGCATGCCCTCTCAG
Oladmrt1a       CAGTAC---CAGATGCCCCCCTCCGACGGCCGCCTGTCCGGCCACAGCATGCCCTCTCAG
                ******      ******* * * *** ********************** ********

Ocedmrt1        TACCGCATGCACTCCTTCTACCCCGGGACCCCTTACTTTCCCCAGGGACTGGGCTCGTCG
OladmrtlbY      TACCGCATGCACTCCTTCTACCCCGGGACCACCTACCTGCCCCAGGGCCTGGGCTCGCCG
Oladmrt1a       TACCGCATGCACTCCTTCTACCCCGGGACCGCCTACCTGCCCCAGGGCCTGGGCTCGCCG
                ****************************** * *** * ******** ********* **

Ocedmrt1        GTGCCGCCGAACTTCAGCCTGGAAGACAATGACGGCACGGCGGCCTCGTTCTCCCCCGGC
OladmrtlbY      GTGCCGCCGTACTTCAGCCTGGAGGACAACGACGACGCAGCCGCCTCCTTCTTCCCCAGC
Oladmrt1a       GTGCCGCCGTACTTCAGCCTGGAGGACAACGACGGCGCCGCCGCCTCGTTCTCCCCCAGC
                ********* ************* ***** **** * * ** ***** **** **** **

Ocedmrt1        AGCATCGCCTCCACCCATGACTCCCCCCTGACCTGCCGCTCCATCAGCTCGCTGGTTAAC
OladmrtlbY      AGCCTCACCTCCACCCATGACTCCACCCTGACCTACCGCTCCATCAGCTCACTAGTGAAC
Oladmrt1a       AGCCTCACCTCCACCCATGACTCCACCCTGACCTGCCGCTCCATCAGCTCACTGGTGAAC
                *** ** ***************** ********* *************** ** ** ***
                          Ocedmrt1-R2
Ocedmrt1        G------TCAAGGCGGAGTTTGAGAGCGGCGGCCAAGTGTCCAGCTTCCCGGCCGACTCC
OladmrtlbY      GACGGCGTCAGGGCAGAGTTTGAGAGCGGCGGCCAGCCGCCCGTCTTCCCGGCCGACTCC
Oladmrt1a       GTCGGCGTCAAGGCGGAGTTTGAGAGCGGCGGCCAGCCGTCCGTCTTCCCGGCCGACTCC
                *      *** *** ********************   * **  ****************

Ocedmrt1        ATCAGCAGTGAAACTAAATGA
OladmrtlbY      ATGAGCAGTGAAACCAAATGA
Oladmrt1a       ATGAGCAGCGAGAGCAAATGA
                ** ***** ** *  ******
```

A.8.2. *Oce*Dmrt1, *Ola*Dmrt1a and *Ola*Dmrt1bY amino acid alignment

```
OlaDmrt1a       MSKEKQGRPVPEGPAPG--PQRSPRMPKCSRCRNHGFVSPLKGHKRFCRWKDCRCAKCKL
OlaDmrt1bY      MSKEKQCRP------------GPRVPKCSRCRNHGLKTPLKGHKRFCRWKDCQCFKCEQ
OceDmrt1        MSREGQCRPVPEGPGSGSDPQRSPRMPKCSRCRNHGFVSPLKGHKRFCRWKDCRCAKCKL
                **:* * **             .**:**********: :**************:* **:
```

```
OlaDmrt1a       IAEGQRVMAAQVALRRQQAQEEELGICSPEASSGPEVTVKNETGADCLFSMEGRSGTPGV
OlaDmrt1bY      IMVRQRVMAAQVADRRQQAQEEELGICSPEASSGPEVVVKNEAGADCLFSVEGRSGTLAI
OceDmrt1        IAERQRVMAAQVALRRQQAQEEELGICSPETSSGPEVMMKNEAGADCLFSMEGRSVTPAV
                *   ********* ****************:****** :***:*******:**** * .:
OlaDmrt1a       PP--NPLSAAGSCSASSSSPSAAARVYGEEASDLLLETSYYNFYQPSRYSSYYGNL--YQ
OlaDmrt1bY      PPSPNPLSAAGSYSASSSSPSAAARVYGEEASDCLLETSYYNFYQPSRYSSYYGNLHNYQ
OceDmrt1        TP--TPLSGSGSCSASSSSPLAAARVHAEEPSDLLLETSYYNFYQPSRYSSYYGNLYNYQ
                .*  .***.:** ******* *****:.**.** **********************  **

OlaDmrt1a       QY-QMPPSDGRLSGHSMPSQYRMHSFYPGTAYLPQGLGSPVPPYFSLEDNDGAAASFSPS
OlaDmrt1bY      QYQQMPPSDGRLSGHSMPSQYRMHSFYPGTTYLPQGLGSPVPPYFSLEDNDDAAASFFPS
OceDmrt1        QY-QMPHTDSRLSGHSMSSQYRMHSFYPGTPYFPQGLGSSVPPNFSLEDNDGTAASFSPG
                ** *** :*.*******.************.*:******.*** *******.:**** *.

OlaDmrt1a       SLTSTHDSTLTCRSISSLVNVGVKAEFESGGQPSVFPADSMSSESK
OlaDmrt1bY      SLTSTHDSTLTYRSISSLVNDGVRAEFESGGQPPVFPADSMSSETK
OceDmrt1        SIASTHDSPLTCRSISSLVN--VKAEFESGGQVSSFPADSISSETK
                *::*****.** ********  *:******** . *****:***:*
```

Appendix B

B.1. Original publications

Klüver, N., Kondo, M., Herpin, A., Mitani, H., Schartl, M. (2005): Divergent expression patterns of Sox9 duplicates in teleosts indicate a lineage specific subfunctionalization. ***Dev. Genes Evol.;****215(6):297-305.*

Klüver, N., Pfennig, F., Pala, I., Storch, K., Schlieder, M., Froschauer A., Gutzeit HO., Schartl M. (2007): Differential expression of anti-Müllerian hormone (amh) and anti-Müllerian hormone receptor type II (amhrII) in the teleost medaka. ***Dev Dyn.;*** *236(1):271-81*.

Klüver, N., Herpin, A., Braasch, I., Drießle, J., Schartl, M.: Regulatory Back-up Circuit of Medaka Wt1 Co-Orthologs Ensures PGC maintenance. (submitted).

Curriculum Vitae

Personal details

Name:	**Nils Klüver**
Date of birth:	January 10th, 1977
Place of birth:	Bremen, Germany
Nationality:	German
Marital status:	married, one child

Education

6/1996	Abitur (High School Exam), Gymnasium Horn, Schulzentrum am Vorkampsweg Bremen, Germany
10/1997 - 12/1999	Study of Biology at the Philipps-University of Marburg, Germany
10/1999 - 01/2003	Study of Biology at the Julius-Maximillians-University Würzburg. Diploma orals in Cell- and Developmental Biology, Genetics and Biochemistry. Diploma thesis "functional analysis of lamin-mutants *in vivo*" with Prof. Krohne, Department of Cell- and Developmental Biology, Division of Electron microscopy , University of Würzburg, Germany.
01/2003	Diploma degree, Dipl. Biol, at the University of Würzburg, Germany.
4/2003 – 8/2007	PhD thesis with Prof. Manfred Schartl, Institute of Physiological Chemistry I at the Julius-Maximilians-University of Würzburg, Germany. "Molecular analysis of gonad development in medaka (*Oryzias latipes*) and *Oryzias celebensis*"
4/2004 – 7/2007	PhD student of the Graduate college 1048 – Molecular Basis Of Organ Development – Graduate School of Life Science, University of Würzburg, Germany.

Scholarships

4/2004 – 7/2007 PhD-fellowship of the Graduate college 1048 – Molecular Basis Of Organ Development, Graduate School of Life Science, University of Würzburg, Germany.

Publications

Klüver, N., Kondo, M., Herpin, A., Mitani, H., Schartl, M. (2005): Divergent expression patterns of Sox9 duplicates in teleosts indicate a lineage specific subfunctionalization. ***Dev. Genes Evol.****;215(6):297-305.*

Veith, A.-M., Schäfer, M., Klüver, N., Schmidt, C., Schultheis, C., Schartl, M., Winkler, C., Volff J.-N. (2006): Tissue-Specific Expression of *dmrt* Genes in Embryos and Adults of the Platyfish *Xiphophorus maculates*. ***Zebrafish****, Vol. 3, No. 3: 325-337.*

Klüver, N., Pfennig, F., Pala, I., Storch, K., Schlieder, M., Froschauer, A., Gutzeit, HO., Schartl, M. (2007): Differential expression of anti-Mullerian hormone (amh) and anti-Mullerian hormone receptor type II (amhrII) in the teleost medaka. ***Dev Dyn.****; 236(1):271-81*.

Herpin, A., Rohr, S., Riedel, D., Klüver, N.**,** Raz, E., Schartl, M. (2007): Specification of primordial germ cells in medaka (*Oryzias latipes*). ***BMC Developmental Biology***, 7:3 **.**

Klüver, N., Herpin, A., Braasch, I., Drießle, J., Schartl, M. (2007): Regulatory Back-up Circuit of Medaka *Wt1* Co-Orthologs Ensures PGC maintenance. **submitted**

Poster

Nils Klüver and Manfred Schartl
Sex determining genes in medaka (*Oryzias latipes*). 1. Graduate day – Rudolf Virchow Centre – 20. July 2004 (Poster NK)

Nils Klüver, Mariko Kondo, Manfred Schartl
Expression pattern of *sox9a* and *sox9b* in Medaka (*Oryzias latipes*). GfE - School - Schloss Reisensburg - 23.-25. September 2004 (Poster NK)

N. Klüver, M. Kondo, A. Herpin, H. Mitani & M. Schartl

Sox9 duplicates in medaka (*Oryzias latipes*). Scientific meeting of the German society of developmental biology – 06.-09. April 2005, Münster (Poster NK)

Frank Pfennig, Nils Klüver, Manfred Schartl and Herwig O. Gutzeit

Anti-Müllerian-hormone (amh) and its receptor (amhrII) homologous genes of medaka (Oryzias latipes) are involved in the process of sex differentiation. 8th International Symposium on the reproductive physiology of fish Saint-Malo, June 3-8 2007 (Poster FP)

LEBENSLAUF

Persönliche Daten

Name:	**Nils Klüver**
Geburtsdatum:	10. Januar 1977
Geburtsort:	Bremen
Nationalität:	deutsch
Familienstand:	verheiratet, ein Kind

Schulausbildung

6/1996	Abitur, Gymnasium Horn, Schulzentrum am Vorkampsweg Bremen

Hochschulausbildung

10/1997 - 12/1999	Studium der Biologie (Grundstudium) an der Philipps-Universität of Marburg Vordiplom
10/1999 - 01/2003	Studium der Biologie (Hauptstudium) an der Julius-Maximillians-Universität Würzburg. Prüfungsfächer: Zell- und Entwicklungsbiologie, Genetik und Biochemie. Diplomarbeit "Funktionelle Analyse von Lamin-Mutanten *in vivo*" bei Prof. Krohne am Lehrstuhl für Zell- und Entwicklungsbiologie, Abteilung Elektronenmikroskopie, Julius-Maximillians-Universität Würzburg. Abschluss des Diploms.
4/2003 – 8/2007	Doktorarbeit am Lehrstuhl der Physiologischen Chemie I an der Julius-Maximillians-Universität Würzburg bei Prof. Manfred Schartl. Thema: “Molecular analysis of gonad development in medaka (*Oryzias latipes*) and *Oryzias celebensis*”
4/2004 – 7/2007	Promotions-Student des GKs 1048 – Molecular Basis Of Organ Development – Graduate School of Life Science, an der Julius-Maximillians-Universität Würzburg.

Stipendien

4/2004 – 7/2007 Promotions-Stipendium des GKs 1048 – Molecular Basis Of Organ Development, Graduate School of Life Science, an der Julius-Maximillians-Universität Würzburg.

Publikationen

Klüver, N., Kondo, M., Herpin, A., Mitani, H., Schartl, M. (2005): Divergent expression patterns of Sox9 duplicates in teleosts indicate a lineage specific subfunctionalization. ***Dev. Genes Evol.****;215(6):297-305.*

Veith, A.-M., Schäfer, M., Klüver, N., Schmidt, C., Schultheis, C., Schartl, M., Winkler, C., Volff J.-N. (2006): Tissue-Specific Expression of *dmrt* Genes in Embryos and Adults of the Platyfish *Xiphophorus maculates*. ***Zebrafish****, Vol. 3, No. 3: 325-337.*

Klüver, N., Pfennig, F., Pala, I., Storch, K., Schlieder, M., Froschauer, A., Gutzeit, HO., Schartl, M. (2007): Differential expression of anti-Mullerian hormone (amh) and anti-Mullerian hormone receptor type II (amhrII) in the teleost medaka. ***Dev Dyn.****; 236(1):271-81.*

Herpin, A., Rohr, S., Riedel, D., Klüver, N.**,** Raz, E., Schartl, M. (2007): Specification of primordial germ cells in medaka (*Oryzias latipes*). ***BMC Developmental Biology***, 7:3 .

Klüver, N., Herpin, A., Braasch, I., Drießle, J., Schartl, M. (2007): Regulatory Back-up Circuit of Medaka *Wt1* Co-Orthologs Ensures PGC maintenance. **submitted**

Poster

Nils Klüver and Manfred Schartl
Sex determining genes in medaka (*Oryzias latipes*). 1. Graduate day – Rudolf Virchow Centre – 20. July 2004 (Poster NK)

Nils Klüver, Mariko Kondo, Manfred Schartl
Expression pattern of *sox9a* and *sox9b* in Medaka (*Oryzias latipes*). GfE - School - Schloss Reisensburg - 23.-25. September 2004 (Poster NK)

N. Klüver, M. Kondo, A. Herpin, H. Mitani & M. Schartl
Sox9 duplicates in medaka (*Oryzias latipes*). Scientific meeting of the German society of developmental biology – 06.-09. April 2005, Münster (Poster NK)

Frank Pfennig, Nils Klüver, Manfred Schartl and Herwig O. Gutzeit
Anti-Müllerian-hormone (amh) and its receptor (amhrII) homologous genes of medaka (Oryzias latipes) are involved in the process of sex differentiation. 8th International Symposium on the reproductive physiology of fish Saint-Malo, June 3-8 2007 (Poster FP)

Acknowledgements

First of all, I would like to express all my gratitude to my mentor Prof. Dr. Manfred Schartl for providing me the opportunity to work in his Institute, and for his warm help and friendly concern. His many theories and researches are the basis and scientific guidance of my dissertation. I am greatly indebted to Prof. Dr. Ricardo Benavente for his acceptance to be an examiner of my PhD thesis. In addition, I am very grateful to Prof. Dr. Christoph Winkler for his support and kind help during my doctorate study.

I am deeply grateful to the GK 1048 “Molecular basis of organ development in vertebrates” for financial support. In addition Thanks a lot to the members for scientific events we have shared. Especially, I want to thank Gabriele Heilmann for her help during my time in the GK 1048.

I wish to express my appreciation to Dr. Amaury Herpin, Dr. Mariko Kondo, Ingo Braasch and all of the here unnamed colleagues in the department of Physiological Chemistry I for their kindly help and friendship.

I would like to give my personal thanks to all my friends for backing and cheering me up.

It is difficult to express in a few sentences the gratitude for my family, for their love and support. In addition, I am very grateful to my parents-in-law for their patience during the stay in their house.

The greatest acknowledgement I reserve for my wife Barbara and our daughter Amelie, supporting me every time either mentally or in reality, to whom I dedicate this dissertation.

Erklärung

Die vorliegende Arbeit wurde von mir selbständig und nur unter der Verwendung der angegebenen Quellen und Hilfsmittel angefertigt.

Diese Dissertation hat weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen.

Ich habe früher außer den mit dem Zulassungsgesuch urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Würzburg, den 22. August 2007

(Nils Klüver)