Genomic Organization and Expression of the Doublesex-Related Gene Cluster in Vertebrates and Detection of Putative Regulatory Regions for DMRT1

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Genes related to the Drosophila melanogaster doublesex and Caenorhabditis elegans mab-3 genes are conserved in human. They are identified by a DNA-binding homology motif, the DM domain, and constitute a gene family (DMRTs). Unlike the invertebrate genes, whose role in the sex-determination process is essentially understood, the function of the different vertebrate DMRT genes is not as clear. Evidence has accumulated for the involvement of DMRT1 in male sex determination and differentiation. DMRT2 (known as terra in zebrafish) seems to be a critical factor for somitogenesis. To contribute to a better understanding of the function of this important gene family, we have analyzed DMRT1, DMRT2, and DMRT3 from the genome model organism Fugu rubripes and the medakafish, a complementary model organism for genetics and functional studies. We found conservation of synteny of human chromosome 9 in F. rubripes and an identical gene cluster organization of the DMRTs in both fish. Although expression analysis and gene linkage mapping in medaka exclude a function for any of the three genes in the primary step of male sex determination, comparison of F. rubripes and human sequences uncovered three putative regulatory regions that might have a role in more downstream events of sex determination and human XY sex reversal.

Key words: DMRT1, DMRT2, DMRT3, sex determination, medaka, Fugu rubripes, synteny, regulatory elements

INTRODUCTION

Studies on the evolution of the molecularly characterized genes involved in sex determination have revealed two categories of genes: those which are well conserved in divergent groups of organisms and those which are only found in a specific branch of the phylogenetic tree [1]. For the latter, a prototypic example is the primary sex determination gene Sry, which is only found on the Y chromosome of all mammals (with a few exceptions [2,3]), but not elsewhere. Other examples are the Drosophila melanogaster sex-determining genes Sxl and tra. From the other category, DMRT1, encoding a protein with a DNA-binding motif (called the DM domain) [4] involved in a certain type of XY sex reversal in humans, is conserved in a wide range of animals with diverse sex-determining mechanisms, including Caenorhabditis elegans, Drosophila, fish, reptiles, birds, and mammals [5–10]. Gene hierarchy studies in the worm and the fly revealed that the corresponding DMRT1 homologues, mab-3 and DSX, are furthest downstream in the sex-determination gene cascade. A hypothesis has been put forward proposing that the genes at the top of the hierarchy such as Sry or Sxl, which rule the mechanism of sex determination, have become involved in this process only relatively recently, whereas at least some of the genes downstream seem to have been present for a much longer time [1].

This has been challenged by findings that DMRT1 is Z-linked in chicken and it has been suggested that DMRT1 in birds has been recruited as an upstream regulatory sex-determining factor [11]. In chicken, DMRT1 expression precedes expression of all other potential sex-determination genes, is stronger in male than in female gonads, and is evident before the sex differentiation of the gonad anlage starts [7,8,12]. In
the turtle, DMRT1 expression is higher in the gonads of embryos incubated at the male determining temperature before sexual differentiation [13].

DMRT1 is a member of a family of genes that share the highly conserved DM domain but have no similarity outside this motif. Only for the fish homologue of DMRT1 expression is higher in the gonads of Fugu rubripes [14]. The function of the other genes and especially if and how the DMRTs are involved in sex determination and/or differentiation are unknown.

Fishes are an attractive group of organisms in which to study sex determination from the evolutionary point of view because members of this class exemplify a complete range of various types of sexuality, from hermaphroditism to gonochorism and from environmental to genetic sex determination [15]. This offers the possibility to compare the structure and expression of sex determination genes in such species and evaluate their upstream or downstream function in the sex-determination cascade and their conservation during evolution. Among fishes, the Japanese saltwater pufferfish Fugu rubripes is the model organism for many problems in biomedical research [28,29]. It has a stable genetic XX/XY sex-determination mechanism, but complete sex reversal can be experimentally achieved by sex hormone treatments, and even fully viable YY females and XX males have been produced [30].

We report here the cloning of genes from the DM gene family from F. rubripes and medaka, and their structural and functional characterization. Gene structure and gene order were found to be highly conserved within a larger region of synteny with human chromosome 9. While the chromosomal localization of the DMRT cluster argues against its role as a primary sex-determining locus, the comparative analysis uncovered putative regulatory elements that might be important during sex differentiation and sex reversal.

RESULTS

Conservation of DMRT Gene Cluster Organization and Synteny with Human Chromosome 9
A partial 365-bp chicken DMRT1 cDNA was used to screen two F. rubripes cosmid libraries, each covering four haploid genomes. F. rubripes cosmids ICRC65D0513Q7 and ICRC65H1799Q7 (Fig. 1) were subcloned and the contiguous sequence of 33 kb (GenBank acc. no. AJ295039) was determined. Additionally, the cosmid contig was extended by one walking step with the end clone of cosmid H1793. Analysis of the 33-kb fragment and the cosmid end sequences determined. Additionally, the cosmid contig was extended by one walking step with the end clone of cosmid H1793. Analysis of the 33-kb fragment and the cosmid end sequences revealed the presence of nine F. rubripes genes in the order DMRT2-KIAA0172 cluster from the FBP1–DAPK1 cluster in humans. However, available mapping data cannot exactly determine the orientation of genes in the human DAPK1–CTSL and FBP2–FBP1 clusters.

FIG. 1. Schematic map of the medaka and Fugu rubripes DMRT1 region and comparison with human chromosome 9. Selected F. rubripes cosmids ICRC65D0513Q7, ICRC66E1476Q1.3, ICRC66H1799Q7, and ICRC66J042Q1.3 comprise approximately 120 kb of genomic DNA and are shown by thin, vertical lines. From medaka the cosmids isolated from library 74 (strain Carbio) and 73 (strain Kaga) are shown (1, MPMGc74G13139Q2; 2, MPMGc74P03143Q2; 5, MPMGc74D1098Q2; 6, MPMGc74M0484Q2; 9, MPMGc75K2481Q2). They cover a region of approximately 100 kb. Cosmid sizes and distances between genes are not to scale. The bold arrow indicates the evolutionary breakpoint that separated the DMRT2–KIAA0172 cluster from the FBP1–DAPK1 cluster in humans. However, available mapping data cannot exactly determine the orientation of genes in the human DAPK1–CTSL and FBP2–FBP1 clusters.
[31]. Gene order and orientation in the DMRT1-DMRT2-DMRT3 cluster are conserved between human, *F. rubripes*, and medaka. No significant similarity to *MHCL* was identified in the available human genome databases. Human *FBP1*, *FBP2*, *CTSL*, and *DAPK1* map to the long arm of chromosome 9. *CTSL* and *DAPK1* sequences are present in the same PAC clone (GenBank acc. no. AL160279), and *FBP1* and *FBP2* are both contained in two overlapping PAC clones (GenBank acc. nos. AL358232 and AL161728). Human *CTSL* maps to 86.6–93.2 cM in 9q22.3 as specified in the human GeneMap '99 (http://www.ncbi.nlm.nih.gov/genemap/). Therefore, *CTSL* and *DAPK1* map proximal to the *FBP* genes. Based on available mapping data, however, the orientation of genes within the two groups cannot be precisely determined.

Cosmid libraries of medaka were screened with the human DMRT1 cDNA. Six cosmid were isolated, which could be arranged in a contig. Sequence analyses uncovered three genes from the DMRT gene family and the first exon of *MHCL* in the same order and orientation as in *F. rubripes*. Furthermore, one exon of the *KIAA0172* gene was identified in the end sequence of medaka cosmid 73K2481 (Fig. 1). Linkage analyses using the medaka reference DNA panel [32] revealed that these genes are located on one end of the autosomal linkage group 9. Hence, they are not on one of the sex chromosomes, which constitute linkage group 1 of medaka. Consistent with the mapping data, FISH on medaka metaphase chromosomes showed the DMRT1-DMRT2-DMRT3 cluster to be located close to the telomere of a medium-size acrocentric chromosome (Fig. 2).

We obtained multiple bands when genomic DNA from medaka was probed in a Southern blot experiment under conditions of low stringency with the human DMRT1 cDNA probe. Using the cloned fragments of medaka DMRT1, DMRT2, and DMRT3 containing the DM domain, several of these bands were assigned to the medaka genes (Fig. 3). Of the remaining bands, one corresponds to DMRT4 (M.K., *et al.*, manuscript in preparation) and one represents a so-far-unidentified DMRT5.

### Comparative Sequence Analysis

The *F. rubripes* DMRT1 protein sequence was deduced from a testis cDNA clone (DKFZp581G1133Q2) and aligned to the medaka (GenBank acc. no. AF319994) and other known DMRT1 proteins (Fig. 4). A longer stretch of very high similarity in all species is only found throughout the 57 amino acids spanning the amino-terminal DM domain, which represents the DNA-binding domain with the two intertwined zinc fingers. Shorter, conserved segments are found in the middle (50% identity/69% similarity between human amino acids 202–231 and *F. rubripes* amino acids 150–179) and the carboxy-terminal domain (59% identity/66% similarity between human amino acids 274–302 and medaka amino acids 177–208) of the protein. A P/S domain, rich in proline and serine residues, is found at a corresponding position in the C-terminal part of all DMRT1 proteins. The highest proline abundance is observed for human and *F. rubripes* DMRT1 with five residues. Serines are most frequent in the mouse, pig, and human orthologues with 14 and 13 residues, respectively. The rainbow trout DMRT1 protein contains a LGQ (leucine-glycine-glutamine)-like domain (amino acids 220–249), which is absent from all other species (Fig. 4).

Comparison of the *F. rubripes* and medaka genomic sequences with the corresponding DMRT1 cDNA sequences disclosed the existence of five exons. A sixth, 5' untranslated exon was identified in *F. rubripes* by RT-PCR. In contrast, human DMRT1 does not possess a 5' untranslated exon, thus it consists of five exons. The additional 5' exon is not observed in medaka. Splice sites 1 and 4 (Fig. 4) in the DM and P/S domains are conserved between human and *F. rubripes*, even though sequence similarity is rather low in the P/S stretch. DMRT1 is the only member of the DM domain gene family found so far in which the putative DNA-binding domain sequence is interrupted by an intron. The position of the second exon–intron junction in human is roughly conserved in *F. rubripes*, being just two triplet codons apart. Splice site 3 is different between humans and *F. rubripes*.
This is due to the addition of a 42-amino-acid stretch in human, pig, mouse, and chicken DMRT1 that is absent from fish. Analysis of the partial genomic medaka DMRT1 sequence (GenBank acc. no. AF319991) showed identical exon–intron boundaries 1–3 when compared with the F. rubripes sequence. Multiple comparisons of the F. rubripes, medaka, and human DMRT1 promoter regions did not reveal any significant sequence similarity.

In medaka (GenBank acc. no. AF319992) and F. rubripes DMRT2, sequence conservation with human DMRT2 is also present outside the DM domain, although to a lower degree. Sequence analysis predicted that DMRT2 consists of three exons with identical exon–intron boundaries. This was confirmed in both fish by sequencing of the corresponding RT-PCR fragment. The same splice sites are also present in human DMRT2. Contrary to DMRT1, the DM domain of DMRT2 ends exactly with the exon–intron 1 boundary.

F. rubripes DMRT3 has two exons and resides between DMRT1 and DMRT2 (Fig. 1). The same organization is found in medaka (GenBank acc. no. AF319993) and in humans, where the human DMRT3 open reading frame is deduced from partial, overlapping cDNA sequences AF193872 and AF193873 [33]. The DMRT3 DM domain is—like in DMRT2—present in exon 1. Sequence similarity is also found outside the DM domain. Exon 1 of the Tetraodon nigroviridis DMRT3 orthologue was found in genomic sequence AJ251456, which is 94% identical to the first exon of F. rubripes DMRT3. However, the annotation of AJ251456 defines this sequence as “partial T. nigroviridis DMRT4.” Here, the priority nomenclature from Ottolenghi, et al. [33], is used, hence T. nigroviridis DMRT4 corresponds to F. rubripes, medaka, and human DMRT3. Similarly, the annotated “partial T. nigroviridis DMRT3” (GenBank acc. no. AJ251455) sequence stands for DMRT4 (M.K., et al., manuscript in preparation). It was not possible to map a human DMRT4 by database searches because AJ251455 gave matches with two overlapping PAC clones (GenBank acc. nos. AC026928 and AL049637) that are described to originate from human chromosomes 9 and 1p32.3–p34.2, respectively. Possibly, one of the two PAC clones is chimeric or has a wrong chromosomal assignment.

Identification of Conserved, Noncoding Sequences in the DMRT1 Region
A percentage identity plot (PIP) analysis was carried out for a global search of sequence similarities between the 33-kb F. rubripes and the human sequence. The sequence of overlapping PAC clones AL136979, AL136365, and AC036116, which comprise the KIAA0172–DMRT1–DMRT3–DMRT2 region, was used in the comparison as the human counterpart. The PIP points out the presence of three noncoding regions with the nucleic acid level was obtained to a T. nigroviridis BAC end sequence from clone COAB012CB02C1 (http://www.genoscope.cns.fr/externe/English/Projets/Proj et_C/C.html). However, no human MHCL orthologue was identified in the region covering KIAA0172–DMRT1 or elsewhere in the human genome. A putative exon 1 of medaka MHCL was identified in sequence AF319991 with only 59% nucleotide sequence identity to F. rubripes MHCL. In medaka, the MHCL–DMRT1 intergenic segment is only ~500 bp, less than half of the distance that both genes have in F. rubripes.

| TABLE 1: PCR conditions for amplification of conserv ed, noncoding regions A–C |
|---------------------------------|-----------------|-----------------|
| Conserved region               | Primer no.      | Sequence (5’→3’) |
| A                               | 877             | GAGTTAAGGACGCGGAACAG^a |
| B                               | 878             | GTATTCAACACGAGCTGG^a |
| C                               | 835             | CCCTTAATTCGTACCGTGG |
|                                 | 836             | CGGCAATTTTAAACTTTCTTG |
|                                 | 879             | AGTCACTTGAATATCCCT |
|                                 | 880             | GTTATTCAAGCAMATAATGGG^a |

^aR = A/G, Y = C/T, M = A/C.
sequence conservation (Figs. 5A and 5B, regions A, B, and C). Region A maps to the last DMRT1 intron in F. rubripes and humans and is 82% conserved over 229 bp. This intron is very large in both species, with 7 kb in F. rubripes and 51 kb in humans. We found that 263 bp and 66 bp 3’ to the respective, putative poly(A) signals of F. rubripes and human DMRT1, both sequences are 89% similar over 385 bp (region B). Segment C (79%, 220 bp) also maps to the intergenic DMRT1–DMRT3 region, 985 bp and 2707 bp 5’ to the F. rubripes and human DMRT3 initiation codons, respectively. Regions A, B, and C are most likely not translated, because no significant matches to expressed sequences were found and no extended open reading frame was predicted by NIX for any of the three segments in both species. The DM domain is boxed and overlining marks the proline-serine-rich domain. F. rubripes and human splice sites are numbered from 1 to 4, whereas solid triangles describe the F. rubripes splice sites and open triangles the human exon-intron junctions.


rubripes and human (Table 1). Almost all tested jawed chordates were positive in the PCR analysis of the three segments (Fig. 6). Exceptions were zebrafish for region A and medaka and shark for region C. No PCR products were obtained with DNAs from lamprey, hagfish, sea urchin, Drosophila, C. elegans, and fission yeast. The failure to detect these regions in these organisms might be due to either their absence or more divergent sequences. Because segments A and B are present in chondrichthyes (that is, shark), but were not found in agnathans (that is, lamprey and hagfish), and the respective divergence time to humans is estimated to be 450 and 500 million years [34], the appearance of both elements dates back at least 450 million years. As segment C is not conserved in shark, but is found in many fish and all higher vertebrates, it might be therefore approximately 430–450 million years old.

Expression of DMRT1, DMRT2, and DMRT3
Because of the limited availability of F. rubripes tissues for expression analysis, we isolated a partial T. nigroviridis DMRT1 cDNA (GenBank acc. no. AJ295040). RT-PCR was carried out with RNAs from several adult female and male tissues.

FIG. 5. Sequence conservation of the DMRT1–DMRT2–DMRT3 cluster between F. rubripes and human. (A) Percentage identity plot (PIP) of the F. rubripes DMRT1 genomic region compared with the equivalent human sequence. Genes identified within the F. rubripes sequence (GenBank acc. no. AJ295039) are annotated and transcriptional orientations are shown by arrows. Exons of genes are numbered, whereas black exons are protein-coding and gray exons are noncoding. Simple repetitive elements present in F. rubripes are shown by white, vertical boxes. Exonic parts within the PIP are highlighted by gray shading. Horizontal lines and dots represent similarities between the F. rubripes and human (GenBank acc. nos. AL136979, AL136365, AC036116) sequences. Three blocks of similarity in noncoding regions were identified, which are boxed and lettered from A to C. (B) BESTFIT alignment of the three noncoding DMRT1 regions A–C that are highly similar between F. rubripes (top sequence) and human (bottom sequence).
DMRT1 transcripts were detected in testis and at a much lower level in ovary, consistent with earlier findings in the rainbow trout [9], but not in liver, eye, brain, skin, heart, gills, or head kidney (Fig. 7A).

In adult medaka (Fig. 7B), DMRT1 is expressed only in testes. No expression above background was seen in any other organ, including ovary. DMRT2 is expressed in testes as well, but at equal levels in ovary and gills of both sexes. DMRT3 is also expressed in testes. The level of the transcript, however, is lower than of the two other DMRT genes. During embryogenesis (Fig. 7C) expression of DMRT1 is barely detectable. Notably, there were no transcripts found around the hatching stage, which in medaka is the critical period of development and early larval stages.

**DISCUSSION**

_F. rubripes_ is a widely accepted model for a comparative approach to contribute to an understanding of human gene structure and function, and for evolution of the vertebrate genome. We have found that human chromosome 9 (HSA9), which is syntenic over two-thirds of its length to the chicken Z sex chromosome, has a syntenic counterpart in fish. So far eight orthologues to genes from HSA9 have been found. The consmid contig analyzed in _F. rubripes_ might contain even more genes, because the region between KIAA0172 and DAPK1 was only partially sequenced. For MHCL from _F. rubripes_ and medaka, the corresponding orthologue could not be identified in humans. Either it was lost during evolution from the tetrapod lineage or it has evolved only in fishes. The third possibility is that it has diverged to an extent that orthology is no longer evident from the DNA sequence alone. The latter possibility is supported by the fact that the sequence conservation of MHCL between both pufferfishes is only 80% and to the available sequence from medaka is 60%.

Conservation of synteny, the presence of orthologous genes on interspecific homologous chromosomes of evolution-ary distant groups of organisms like mammals and teleost fish, has been observed in several instances [36,37]. With a few exceptions [17,19,20], however, the position of genes within the syntenic groups is not conserved, and gene order seems often to be at random. The finding that the order and orientation of the three DMRT genes is conserved between human and fish is reminiscent of situations with the _Hox_ cluster, in which the gene order of a set of paralogous genes is conserved in all vertebrates and even further in the phylogenetic tree [reviewed in 38]. For the _Hox_ cluster, the genomic organization is most obviously a result of the need for a coordinated and tightly regulated expression of the gene family members. All three DMRT genes are expressed in testes of fish. For DMRT1, testis expression is known from all other vertebrates looked at so far. At least some isoforms of human DMRT2 transcripts are found in testes. Human DMRT3 is also expressed in the male gonad. Thus this concerted expression of the three paralogues may require their colocalization in one gene cluster. In the human genome several other testes-expressed genes have been found in the PACs spanning the DMRT cluster [33]. This suggests the presence of a common regulatory element. However, no sequences that could be the orthologues of the other testes genes from HSA9 could be identified in the vicinity of the DMRT cluster in fish.

Besides the conserved expression and, probably, a conserved function in testes, there are some differences in the gene expression pattern of human and fish DMRT2. The expression in gills is fish-specific and can hardly be interpreted as the equivalent to the weak expression in human lung. The expression found in ovary in medaka is not seen in humans [39]. Conversely, the expression in human kidney and skeletal muscle was not observed in fish.

Data propose that an extra round of genome duplication in the fish lineage had a major impact on the evolution of gene families and shaping the genome of fish. Unlike for the _Hox_ clusters in medaka [32] we found no evidence that the DMRT1–DMRT2–DMRT3 cluster is duplicated. Also, _T. nigroviridis_ DMRT4 has a clear orthologue in human and therefore does not seem to be the result of a fish-specific genome duplication. The status of the unidentified DMRT5 from medaka is unclear, but the pufferfish seems to contain only DMRT1–4.

A large part of the recent interest in the DMRT genes

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**FIG. 6.** PCR analysis of the conserved, noncoding segments A–C present in the DMRT1 region with DNA from various species. *Sizes of PCR products are given, whereas the upper value is for the human and the lower value for the _F. rubripes_ product. Conservation of segments A–C is observed in all jawed chordates tested with exceptions for region A in zebrafish (very faint band in clawed frog) and for region C in medaka and shark.
Article

**Materials and Methods**

*Fish.* All medaka fish used here were taken from closed colony breeding stocks. These are derived either from the highly inbred medaka lines HNI, HB32C [41], and Kaga (Asuko Shimada and A.S., unpublished data), or from the non-inbred Carbio strain (Carolina Biological Supplies). *T. nigroviridis* were purchased from a local dealer.

Isolation and sequencing of cosmids and cDNAs. Initially, two *F. rubripes* genomic cosmid libraries (nos. 65 and 66 from the German Resource Center of the Human Genome Project, RZPD) and a *F. rubripes* testis cDNA library (no. S81) were screened with a 32P-labeled, 365-bp chicken DMR1 probe containing the DM domain. The hybridization and washing of the filters was at low stringency according to standard protocols. Positive clones were isolated, DNA purified, and cDNAs inserts sequenced. *F. rubripes* cDNA clone DKFZp581G1133Q2 contains the entire DMR1 open reading frame and clone DKFZp581K2020Q2 is a truncated DMR1 cDNA. True positive cosmid clones were identified by screening with a probe from the 3’ UTR of the DMR1 cDNA. To extend the cosmid contig, libraries 65 and 66 were rescreened at high stringency with a genomic *F. rubripes* FBP1 (GenBank acc. no. AJ295036) probe, which originates from an end clone sequence of *F. rubripes* cosmid ICRF65H1793Q7 (Fig. 1). The additional *F. rubripes* cosmids were end sequenced, which identified parts of KIAA0172 (ICRFc651674Q4 cosmid end sequence, GenBank acc. no. AJ295036), CTSI (ICRFc65A041Q7 cosmid end sequence, GenBank acc. no. AJ295037), and DAPK1 (ICRFc66N212QI.3 cosmid end sequence, GenBank acc. no. AJ295038). Outside of the 33-kb contiguous *F. rubripes* sequences, gene order was determined by analysis of the cosmid end sequences compared with the hybridization patterns of DMR1, FBP1, KIAA0172, CTSI, and DAPK1 probes to all isolated cosmids D0513 and H1793, which overlap by 3 kb, to cosmid clones digested with EcoRI. A PstI digestion of the cosmid DNAs and hybridization with the FBP1 probe disclosed the existence and position of the *F. rubripes* gene FBP2. *F. rubripes* cosmids D0513 and H1793, which overlap by 3 kb, were digested with EcoRI, HindIII, and PstI, subcloned, and sequenced as described [42]. Cosmid end sequences were determined by use of primers Lawr16f, 5’-CGCCCTCGAGTTGCTTATC-3’, and Lawr16r, 5’-GCCAGATGCTACACATGTACG-3’. *F. rubripes* cDNA clones in pAMPl were sequenced with standard M13 primers.

Filters of arrayed medaka genomic cosmid libraries (RZPD no. 73 and 74) were screened with the human DMR1 cDNA under conditions of low stringency. Positive clones were arranged in groups by restriction fragment analysis and Southern blotting. End fragment probes were generated by a method derived from the ligation-mediated PCR. Restriction fragments cross-hybridizing with the human DMR1 probe were subcloned and sequenced.

For isolation of a medaka (strain Carbio) DMR1 cDNA, total RNA was isolated from testes. A combination of RT-PCR and 3’-RACE with medaka testis specific primers was used with primers based on the sequence of the genomic clone. For RT-PCR, first strand cDNA was primed with oligo(dT)12-18 from the SuperScript II system following the supplier’s protocol (Gibco BRL). We identified 383 bp of the 5’ end and the domain by RT-PCR with the primers mdMR1-5’ (5’-AGCATTTTTATACCTTGAC-3’) and Cos1pst-1r (5’-ACCTCGCGCTCACATGAC-3’). For 3’-RACE, two nested primers, Cos1pst-1f (5’-ACCGATTCC-
TABLE 2: RT-PCR primers and PCR conditions

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</tbody>
</table>


CAACACGGCGCATA-3') and Cos1pst-2f (5'-TGGGCTCTCCGCTGATGAGGAGC-3') were designed from the identified cDNA sequence. One primer (5'-CAAATAGCTCCAATAGGGGTTGCGAATCCC-3') was used for reverse transcription of medaka testis total RNA following the standard protocol (Gibco BRL). Two rounds of nested PCR were carried out. The first one was performed with primers Cos1pst-1f and AP1 (5'-CCATCTCATAAGCTCATATAGGGGTTGCGAATCCC-3'). The first PCR product (3 µl) was applied for the second one with primer Cos1pst-2f and AP2 (5'-ACTCAATAGGGGTTCGATTGGGTTGCGAATCCC-3'). The PCR cycling condition was the same for both reactions except that the annealing Ta was 62°C for the first PCR and 60°C for the second. The PCR cycles were 50 s at 94°C, 60 s at 62°C, and 90 s at 72°C. The 1-kb product was cloned into the pCIG-T-Easy vector for sequence analysis. Sequence assembly and comparisons were carried out using the HUSAR program at the German Cancer Research Center (DKFZ, Heidelberg). The sequence from the 3'-RACE experiments was assembled with the previously identified cDNA sequence to the full-length sequence file of the medaka DMRT1 cDNA (GenBank acc. no. AF519994). Primers from this sequence were used to amplify the medaka testis full-length cDNA clone.

**Sequence analysis.** Sequences were analyzed with the NIX software tool (http://www.hgmp.mrc.ac.uk/NIX/). Putative exons and genes were compared at the nucleotide and amino acid level with known genes by standard GCG programs and BLAST [43] and Fasta [44] database searches. The DMRT1 multiple alignment was carried out with PILEUP (GCC) with a reduced gap extension penalty value of 1. We used PipMaker (http://bioinfo.cse.psu.edu/pipmaker/) for pairwise comparison between the 33 kb F. rubripes sequence and corresponding human PAC sequences. For PIP analysis, the F. rubripes sequence was masked by RepeatMasker (http://repeatmasker. genome.washington.edu/cgi-bin/RM2_req.pl) and in the advanced PipMaker menu the options “single coverage” and “search both strands” were chosen.

**Southern blot analysis.** DNA from individual fish was obtained from pooled organs and AA was used for Southern blot analysis. Membranes were hybridized either under conditions of low stringency (hybridization in 35% formamide, 0.1% Na-pyrophosphate, 50 mM Tris-HCl pH 7.5, 5% SSC, 1% SDS, 5x Denhard's, 100 µg/ml call thymus DNA at 42°C, washing in 1x SSC/1% SDS at 60°C) or high stringency (hybridization at 42°C in the same buffer except the formamide concentration was 50% washing in 0.1x SSC/1% SDS at 68°C) with the following probes: OlaDMRT1, 4-kb EcoRI fragment from cosmid 73K2481; OlaDMRT2, 1.1-kb EcoRV/PstI fragment from cosmid 74ID0189; OlaDMRT3, 1.7-kb EcoRI fragment from cosmid 73K2481; HSADMRT1, 1.5-kb EcoRI fragment from the human DMRT1 cDNA.

**Expression analysis.** Total RNA was extracted from pooled organs of several adult medakafish or 30-40 pooled total embryos of defined stages [45] using the TRIZol reagent (Gibco BRL). After DNase treatment, reverse transcription was done with 2 µg total RNA using Superscript II reverse transcriptase (Gibco BRL) and random primers. DNase-treated total F. rubripes testis RNA was reverse transcribed as described [46]. For primers, annealing temperatures, MgCl₂ concentrations, and product sizes see Table 2. PCR products for DMRT1 and MHCL were sequenced to confirm all exon-intron boundaries.

For T. nigroviridis DMRT1, a multiple alignment of the available DMRT1 coding sequences was used to design a primer pair (714/808) conserved in all species. In a RT-PCR reaction with DNase-treated total adult T. nigroviridis samples were as described in Table 2, and 96°C for 1 min, annealing for 1 min, 72°C for 1 min. PCR products were separated on 2% agarose gels.

**PCRs of the conserved, noncoding regions.** We used 100-200 ng genomic DNA, 10 pmol of each primer (Table 1), 200 µM dNTPs, 2-2.5 mM MgCl₂, and 1 U of AmpliTaq Gold DNA polymerase (Perkin Elmer) for PCR amplification in a 25 µl volume. Initial denaturation was 9 min at 95°C, followed by 35 cycles each consisting of 94°C for 1 min, annealing for 1 min, and 72°C for 1 min. PCR products were separated on 2% agarose gels.

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was denatured, preannealed with excess sonicated genomic DNA, and hybridized overnight at 37°C to denatured medaka mitotic chromosomes. Following post-hybridization washing under moderate stringency conditions (1 × SSC/60°C), hybridization sites were detected with fluorescein-labeled avidin (Oncor) followed by signal enhancement using biotinylated anti-avidin and FITC-conjugated avidin. Slides were examined on a Zeiss fluorescence microscope equipped with a CCD camera. Applied spectral imaging software was used to obtain digitized image presenting hybridization signal on DAPI-stained chromosomes.

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