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Inventing a Sex-Specific Gene: A Conserved Role of DMRT1 in Teleost Fishes Plus a Recent Duplication in the Medaka *Oryzias latipes* Resulted in DMY

Georges Lutfalla,[1] Hugues Roest Crollius,[2] Frédéric G. Brunet,[2,3] Vincent Laudet,[3] Marc Robinson-Rechavi[3]

[1] Défenses Antivirales et Antitumorales, UMR CNRS 5124, 1919, Route de Mende, 34293 Montpellier Cedex 5, France
[2] Genoscope, UMR CNRS 8030, 2, rue Gaston Crémieux, CP 5706, 91057 Evry Cedex, France
[3] Laboratoire de Biologie Moléculaire et Cellulaire, Ecole Normale Supérieure de Lyon, UMR CNRS 5665, 46 allée d'Italie, 69364 Lyon Cedex 07, France

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Abstract. DMY, the first sex-determining gene to be described in a nonmammal vertebrate was recently characterized in the medaka fish (*Oryzias latipes*). It is homologous to DMRT1, a conserved gene of the sex determination cascade in vertebrates. We have checked the near complete genomes of two other percomorph fishes, *Tetraodon nigroviridis* and *Takifugu rubripes*, for supplementary homologs of DMRT1 and DMY. We also compared the new gene, DMY, to its homolog DMRT1 from all available vertebrates. Finally, we found evidence for sex-specific expression and alternative splicing of the homolog from *T. nigroviridis*. Our results show that DMY is a recent duplicate of DMRT1 in the medaka. Its role in sex determination was not acquired through an acceleration of evolutionary rates, but by translocation to the Y chromosome and possibly changes at key positions.

Key words: Teleost fish — *Oryzias latipes* — DMY gene — DMRT1 gene

Introduction

The first sex-determining gene to be described in a non-mammal vertebrate was recently characterized in the medaka fish (*Oryzias latipes*) (Matsuda et al. 2002). This gene, called DMY, is homologous to DMRT1, a conserved gene of the sex determination cascade in vertebrates. Although sex determination in vertebrates follows different rules including male heterogamety, female heterogamety, and environmental sex determining systems, the DMRT1 gene appears to play a central role (Marshall Graves and Shetty 2001). In birds, its localization on the heterogametic Z chromosome (Nanda et al. 1999) makes it a good candidate as a sex determining gene. In mammals it is downstream of the SRY gene in the sex determining/differentiation cascade; DMRT1 is on human chromosome 9. The situation in human where haploinsufficiency could lead to sex reversal is clearly different from that in mice where DMRT1 +/− males are normal but DMRT1−/− have severely hypoplastic testes (Moniot et al. 2000; Raymond et al. 2000; Ottolenghi and McElreavey 2000). DMRT1 has already been cloned from different fish species (Brunner et al. 2001; Guan et al. 2000; Marchand et al. 2000), leading to either complete or partial protein sequences and qualitative results showing preferential expression in male gonads.

This poses several very interesting evolutionary questions: how did DMY arise? How general is this discovery to other fishes? Is a sex-specific function present in DMRT1? To answer these questions, we have compared by phylogenetic analysis the new

Correspondence to: M. Robinson-Rechavi; *email:* marc@sdsc.edu

gene, DMY, to its homolog DMRT1 from all available vertebrates. We also took advantage of the near complete genomes of two other percomorph fishes, *Tetraodon nigroviridis* (Roest Crollius et al. 2000) and *Takifugu rubripes* (Aparicio et al. 2002), to check for supplementary homologs of DMRT1 and DMY, by similarity searches in the respective assemblies of these genomes. In addition, we have experimentally characterized the homolog predicted in *T. nigroviridis*, showing sex-specific expression.

Materials and Methods

Sequence Dataset

The sequence of the medaka gene for DMY was obtained from GenBank (AB071534) and translated into amino-acids. This was used for a similarity search against all vertebrate genes in release 42 (26 April 2002) of Hovergen (Duret et al. 1994), by BLASTP (Altschul et al. 1990). All the most significant hits were from the same homology family (HBG005035), which contains proteins defined as "DMRT1." Medaka DMY was added to the alignment of all 15 proteins from this family using Seaview (Galtier et al. 1996).

To identify the *T. nigroviridis* DMRT1 gene, the medaka DMRT1 and DMY sequences were used to search the assembled *T. nigroviridis* genome sequence available at http://genoscope.cns.fr/tetraodon/. This assembly represents approximately 83% of the genome at 5.6× coverage. Using Genewise (Birney et al. 1996), a gene model with four exons was constructed by aligning the medaka DMRT1 and DMY sequences on the Tetraodon genomic contig that showed the highest sequence similarity to DMY (FS_CONTIG_1918_2). The translated peptide (TnDMRT1 hereafter) was then compared with BLASTP to the human International Protein Index (IPI) and identified DMRT1 with the highest E-value (E = 1.2e−50), while in return human DMRT1 identifies TnDMRT1 with the highest E-value (E = 1.2e−31) among all Tetraodon predicted proteins (manuscript in preparation).

Tetraodon nigroviridis *Samples*

Wild *T. nigroviridis* imported from Thailand were purchased at a local dealer in France. They included mature and juvenile animals from both sexes. Abdominal dissections allowed sex typing. The male gonads from juveniles to mature individuals (gonad size 1 mm to almost 1 cm) are white. The female gonads are from very pale orange in juveniles to bright orange in adults and oocytes can easily be distinguished either by eye or under the binocular (juveniles). Tissue samples were used to prepare RNA using the High Pure RNA Tissue Kit from Roche.

Amplification and Cloning of TnDMRT1

Spl2XhoT18 primed reverse transcriptions were performed on total RNAs using the M-MuLV RT from Invitrogen. PCR primers are the following: RNP31, GTGTCCTCCTTAATTCCACCA; RNP51, TTCATCGGAGGTCTGAGCTT; DME2, GTTTCAC GGTGGAAGGACGA; DME3, CAGTCAGACCTGCTGCT GGA; DME3R, GTACAGGTTGCTGTAGTAGGA; DME4R, GACACAAGCAGTGGAGCTCA. Spl2XhoT3, CAAGGATG GATGCGTGGTGCTCGAGTTT; Spl2XhoT18, same as Spl2XhoT3 but with a 3′ extension of 18 Ts instead of 3.

Conventional PCR was performed using Platinium Taq DNA polymerase in a Biometra Tgradient thermocycler. Real time Quantitative PCR (Q-PCR) were performed using SYBR GREEN technology in a LightCycler Instrument from Roche. For the measure of TnDMRT1 expression primers DME2 and DME3R present on exons 2 and 3 respectively were used. For the splice regulator hnRMPA2 primers RNP31 and RNP51 were used.

3′RACE, Spl2XhoT18 primed first strand cDNA were PCR amplified using DME3 (from the third coding exon of TnDMRT1) and Spl2XhoT3. Amplification products were separated on an agarose gel and blotted on a nylon membrane. Hybridization using labeled oligonucleotide DME3R revealed the presence of amplified fragments of 1.1 and 1.4 kb. PCR fragments were cloned in pCR2.1-TOPO vector and transformed in Top10 bacteria. Clones were screened using probe DME3R and positive clones were sequenced.

Evolutionary Analyses

All analyses were done using only complete sites (no gap, no X in any sequence).

Phylogenetic reconstruction was done with neighbor-joining (Saitou and Nei 1987) as implemented in Phylo_win (Galtier et al. 1996), modified by M.R.R. to correct for rate heterogeneity between sites with a gamma law (Yang 1996). The alpha parameter of the gamma law was estimated in Tree-Puzzle (Schmidt et al. 2002), with eight categories. Robustness of nodes was assessed with 2000 bootstrap replicates (Felsenstein 1985). Phylogenetic reconstruction was also done using maximum likelihood as implemented in ProtML (Adachi and Hasegawa 1992), without bootstrap. *A priori* defined alternative positions of medaka DMY in the tree were also compared using a KH likelihood test (Kishino and Hasegawa 1989), as implemented in Tree-puzzle (Schmidt et al. 2002) with a JTT evolution model (Jones et al. 1992) plus rate heterogeneity (gamma law).

Evolutionary rates were compared using the relative-rate test (Wu and Li 1985) as implemented in RRTree (Robinson-Rechavi and Huchon 2000), correcting for multiple substitutions with the LPB model of synonymous (Ks) and nonsynonymous (Ka) substitutions (Li 1993; Pamilo and Bianchi 1993), on coding sequence alignments deduced from the alignment of amino-acid sequences. Positive selection was also tested for by branch-specific and site-specific Ka/Ks maximum likelihood models in PAML (Yang 1997).

Results

Sequence of the TnDMRT1 Protein

Clearly the genome of *T. nigroviridis* harbors a single homolog for the two medaka genes, TnDMRT1. A unique contig spanning this gene from its 5′ end to 3 kb downstream of the fourth coding exon was found in the current genome assembly (Fig. 1). The available data from the fugu genome project (http://www.jgi.doe.gov/fugu) allows the detection of the previously reported fugu DMRT1 gene (Brunner et al. 2001), but no supplementary DMY ortholog could be detected. We can thus state that in both genomes the only close homolog to the medaka DMY is the DMRT1 gene, although other members of the DM family are found.

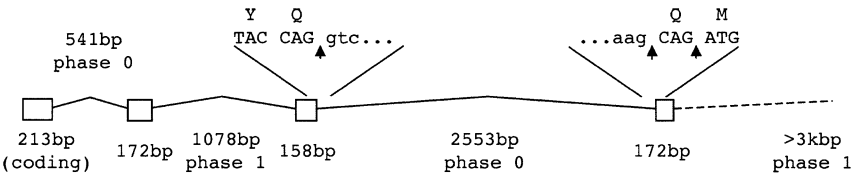

Fig. 1. Structure of the *Tetraodon nigroviridis* DMRT1 gene: The first four exons (open boxes) of the gene are represented in contig FS_CONTIG_1918_2. The sequence terminates before the fifth and last exon of the gene. Size of exons and introns, as well as phase of introns, are indicated. The sequence at the exon-intron junctions around intron 3 is shown; intron sequence is in small caps and exon sequence in capitals with the amino-acid translation. Arrows indicate splice junctions, with two alternative acceptor sites for exon 4.

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                                                13        2325
DMY Oryzias          MSKEKQCR----------P-G----PRVPKCSRCRNHSLKTPLKGHKRFCRWKDCHCLKC
DMRT1 Oryzias        ......G.PVPE--GP-A.-.PQRS..M.......N.GFVS.............R.A..
Tetraodon            .....KSKALS--T.TVT.SKGLKP..M.........GFVST.......S.R..Q.P..
Takifugu             .T....SKAS---A.T.T..KGPKP..M.........GFVS.........N.R..Q....
Tilapia              ..QD..SKQ..DCS..MS.TKA.K...M.........GYVS.........N.R..Q.P..
Oncorhynchus         ..DDE.TKLL-..A..PSA.PGKKP..M.........GYVS.........N.R..Q.Q..

                          50
DMY Oryzias          KLIVDRQRVMAAQVALRRQQAQEEELGICSPEASSGPEVVVKNEAGADCLFSVE-GRSGT
DMRT1 Oryzias        ...AEG.................................T..N.T.......M.-.....
Tetraodon            ....E.....................W.LGPLP.AG.M....P..E.C.T..-...Q.
Takifugu             ....E.....................VPL..AGMM.......E.F..A.-..NQA
Tilapia              ...AE.....................VSL..S.MM....V.........-...P.
Oncorhynchus         ...AE.....................M.L...ATL.SQ.......PTG...S..SG...P.

                                                        161-2
DMY Oryzias          PA--PPPNPNPLSVAGSYSASSSSPSAAAR-VYSEEASDQPLETSYYNFYQPSRYS-SYY
DMRT1 Oryzias        .G--V..N.--..A...C.............-..G.....LL.....N.........-...
Tetraodon            ST--SG.SS--AVATA.RTVP..Q...G..-AHH.GQ..LL..S.F.H.....C.P-...
Takifugu             AT--STSSS--SV.PA.R.VT..R...G..-AANDGQ..LL..S.F..L....C.P-A..
Tilapia              .---TSHAT--SA.T.TR....P.......-AHT.GP..LL...P..........P-T..
Oncorhynchus         CGNTSAGTS--P.N...R.GLA...T.FS.GQSTDGTA.LLVD............PTA..

                     alternative splicing
DMY Oryzias          GNLYNYQQYQQMPPSDGRLSGHSMPSQYRMHSFYPGTAYLPQGLGSP-------------
DMRT1 Oryzias        .N..-----.-------------------------------------------------
Tetraodon            S...N.....-..HG....QN.NVS--.-..TYC..AP..SP..S.TA------------
Takifugu             .............HG....QN.NVSP..CV..YCS.GP..S...S.ST------------
Tilapia              ......S...-..HG....PS..VS.......Y..AAT..T.....TS------------
Oncorhynchus         S...K.....-..NGES...S.NVSP......Y.SSAS..S....QGLGQGLGQVLGQGL

                                                  225
DMY Oryzias          ------------------VPPYFSLEDNDG--------AAASFFPSSLTSTHDSTLTYRS
DMRT1 Oryzias        ------------------.........N..----------....S.............C..
Tetraodon            -----------------C...I..M...NNGS--CAQTLGTT.S....STGP..SM.C--
Takifugu             -----------------....I..V...NSNNNS.P..L.TA.S...PSTGP..SM.C.P
Tilapia              -----------------....F...D..NNS----.SE.M....S.G.ISAG....MVC..
Oncorhynchus         GQGLGHGLGQGLGTTAA....M......TCH-----D.KQT..S.V.GGANGHDG.SCL.

DMY Oryzias          ISSLVN-DGVKAEFESGGQP---PSSRPTP-----
DMRT1 Oryzias        .....N-V.............SVF.ADSMSS-ESK-
Tetraodon            --------------.ATSEMAN.TI.STVDGDATN
Takifugu             ..TM.T-SDLHP.C.AT.ETAN.TI.SIMD.DAG.
Tilapia              .......-GDA...C.ASS.AAG.TVDAIEG.AT.-
Oncorhynchus         ......SSEG.T.CDGQD.GQG.TVDSIIE.NH.-
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Fig. 2. Alignment of DMRT1 and DMY protein sequences: Amino acids identical to medaka (*Oryzias*) DMY (or to the first sequence if DMY has a gap) are represented by a dot; gaps are represented by hyphens. Sites unique to medaka DMY are boxed, with the position in the DMY sequence (without gaps) indicated; bold boxes indicate radical amino-acid changes. The DM domain is indicated by a grey background. The alternative splicing discovered in *Tetraodon nigroviridis* is indicated by two arrows.

Due to the weak similarity of the DMRT1 proteins at their COOH extremity and to the large size of the last intron of the gene, the identification of the last coding exon of the TnDMRT1 gene was not obvious *in silico*. 3′RACE was therefore used to clone the 3′ end of the TnDMRT1 cDNA. cDNAs spanning from the third coding exon to their 3′ ends were cloned and sequenced, allowing the identification of the fifth coding exon which is also the last exon of the gene, that harbors two alternative polyadenylation sites.

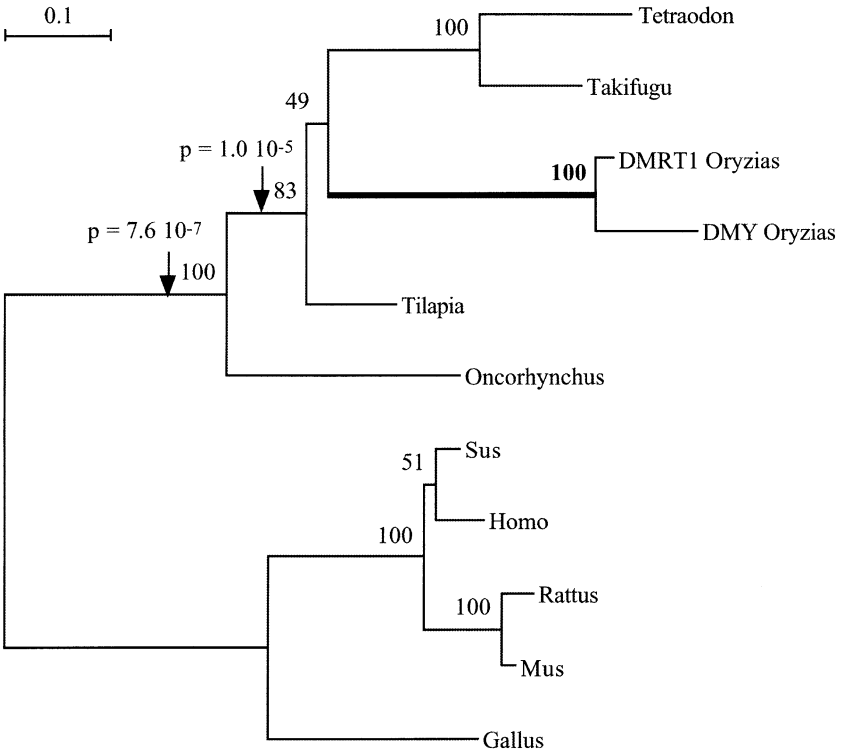

Fig. 3. Phylogenetic position of the medaka DMY relative to vertebrate DMRT1: Tree estimated by neighbor-joining with rate heterogeneity between sites corrected by a gamma law ($\alpha = 0.79$) on the amino-acid alignment; only complete sites were used; figures at nodes are bootstrap proportions from 2000 repetitions; branch lengths are proportional to evolutionary distance, the measure bar representing 0.1 substitutions per site. All sequences other than *Orizias* (medaka) are DMRT1 of the specified genus. The branch in bold supports a recent duplication leading to DMY. Arrows design alternative positions of *Orizias* DMY which were compared by a likelihood KH test, with the associated *p*-value of the test: the alternative positions are significantly less likely.

Thus we aligned the complete sequence of the TnDMRT1 protein with available DMRT1 and DMY protein sequences (Fig. 2). The sequence of the gene was deposited in GenBank, under the accession number AY135175. Of note, TnDMRT1 is flanked by the same genes in 5′ (MHCL and KIAA0172) and in 3′ (DMRT3 and DMRT2) as in medaka and fugu (Brunner et al. 2001).

Phylogeny and Evolutionary Rates

We present in Fig. 3 the tree obtained by Neighbor-Joining (Saitou and Nei 1987), but all methods (likelihood, parsimony) give the same results. This tree was obtained with 12 sequences and 149 complete sites, but results are unchanged by using more sequences (14 sequences, 82 complete sites) or more sites (nine sequences, 250 complete sites). The main result is that DMY groups with medaka DMRT1 with extremely strong support (100% bootstrap proportion in all cases). We also conducted a likelihood test (Kishino and Hasegawa 1989) between three hypotheses: DMY duplicated at the base of fishes, DMY duplicated at the base of percomorphs, or DMY grouped with medaka DMRT1; the latter hypothesis was the best with very significant differences ($p \leq 5 \times 10^{-5}$; Fig. 3).

Although the branch leading to DMY seems longer than its medaka paralog DMRT1 in the tree, the difference is not significant in a relative-rate test ($p > 0.05$ for Ka and Ks). There is also no significant Ka/Ks ratio difference (0.32 versus 0.40), and in all cases the Ka/Ks is under 0.40, indicating purifying selection on all genes in the analysis.

Structure and Splice Variants of the TnDMRT1 Gene

The position of the splice junction is indicated in the structure of the TnDNRT1 protein (Fig. 1). The junction between exons 3 and 4 is interesting in that it falls at a position where the medaka DMY has two glutamines whereas the medaka DMRT1 has only one. Interestingly *T. nigroviridis* coding exon 4 has two consensus splice acceptors, one leading to two glutamines (last of exon 3 + first of exon 4), the other leading to only one (from exon 3). We have therefore measured the use of both of these splice acceptor sites in different gonads. RT-PCR was performed using RNA from different male and female gonads (juveniles and adults from both sexes) with primers DME3 and DME4R, fragments were cloned and 15 independent clones from each RT-PCR were sequenced. The frequency of each splice variant was not statistically different from 50/50 despite the huge difference in expression levels between males and females (see next section). This is certainly indicative that this alternative splicing is not instrumental in sexual differentiation. Interestingly, according to the article describing the medaka DMY gene (Matsuda et al. 2002), only one cDNA has been sequenced, and the codon for the second glutamine (beginning of the fourth coding exon) is also a CAG that creates a potential alternative

splice acceptor. We can postulate that the DMY gene is also subject to alternative splicing at this position. The sequence of exon 4 from the zebrafish DMRT1 gene (partial gene from the NCBI Trace Repository: Z35724-a2940d09.p1c, zfish44906-2679e03.p1k, Zfish44625-1385c02.p1c) reveals that it can only code for the form with a single glutamine. This indicates that this alternative splicing is not a general situation in fishes, but may be specific to percomorphs.

Expression of TnDMRT1

The quantification of the expression of the TnDMRT1 using Q-PCR was performed in the following tissues: brain, liver, kidney, spleen, intestine, and gonads. All expressions were normalized to the expression of the splicing regulator hnRNPA2. The expression of DMRT1 is reproducibly detectable only in gonads, in all male gonads analyzed the ratio TnDMRT1/hnRNPA2 is 1.5–3, in female gonads, the ratio is $1–5 \times 10^{-4}$.

Discussion

The answer to the question of the evolutionary origin of DMY is extremely clear: DMY arose from a recent duplication of DMRT1, specifically in the medaka lineage (Beloniformes), after even the divergence from other percomorphs. While this paper was being edited, similar results were found by Kondo et al. (2003), who did not find DMY even in closely related *Oryzias* species. The consistency between phylogenetic conclusions and genome analysis is particularly striking, since only one ortholog of medaka DMRT1 and DMY is found in two almost complete percomorph fish genomes; of note, the sex of *Tetraodon* individuals used for sequencing is not known, but the individuals used for *Takifugu* sequencing were certainly males, since DNA was extracted from testis (Aparicio et al. 2002).

The novel evolutionary role of DMY does not appear to have been obtained by an increase in evolutionary rates, which would have implied positive selection or a relaxation of negative selection. This supports the notion that functional divergence of fish duplicate genes may have more to do with regulation or key amino acid positions than divergence of the sequence (Force et al. 1999; Robinson-Rechavi and Laudet 2001). This may be related to the observation that, whereas there is only one position in the alignment of fish proteins with an amino-acid specific to medaka DMRT1, there are seven positions specific to medaka DMY, of which four represent radical changes (change in chemical class of amino-acids); of special interest are positions 161 and 162 of DMY, with two radical changes clustered, unique to DMY (Fig. 2). These positions certainly represent prime targets for further characterization.

It is most probable that a duplicate of DMRT1 could become a sex-determining gene so rapidly due to not only the conserved role of DMRT1 in the sex determination cascade (Marchand et al. 2000; Moniot et al. 2000; Raymond et al. 2000), but also to expression in gonads of fishes. This expression has been observed in an eel (Huang et al. 2002), and has been shown to be testis-specific in medaka (Brunner et al. 2001). We show specific expression in gonads in another percomorph fish, the genomic model *T. nigroviridis*, with much higher expression in testis. Our results are consistent with a conserved role of DMRT1 in testis-formation in fishes. Thus, after duplication, the new gene is all set to become a sex-determining gene, and translocation to the Y chromosome was all that was needed, without a significant increase in evolutionary rates. Moreover, the existence of splice variants may have helped the establishment of this specific function, although further characterization of these in the medaka is needed before definitive conclusions can be drawn.

One point which emphasizes the importance of gene duplication in this scenario is the conserved linkage between DMRT1, DMRT2, and DMRT3: if there is a functional constraint to the order of these genes, as suggested by Brunner et al. (2001), it may well be because of the gene duplication that one copy of DMRT1 was able to translocate and become DMY. It is possible that in other cases of duplication of this gene, similar sex-determining genes have evolved independently in other fishes.

These results emphasize the high plasticity of fish genomes (Robinson-Rechavi et al. 2001; Venkatesh et al. 1999), but also of sex determination in animals, notably vertebrates (Baumstark et al. 2001; Soullier et al. 1998; Western and Sinclair 2001; Koopman and Loffler 2003). Evolution has selected a very original solution in medaka, where a recent duplication of the DMRT1 gene has lead to the insertion of a new gene, DMY, in the sex Y chromosome. Its early expression (compared to a late DMRT1 expression) is necessary for the establishment of a male phenotype (Matsuda et al. 2002).

Fishes in general, and medaka more specifically thus appear to be very interesting models for the establishment of sex determination in vertebrates. We would also like to point out the interest of obtaining the genome sequence of a fish species which is amenable to experiments in the laboratory, such as *Tetraodon nigroviridis*, whose genome is being sequenced. Finally, this result illustrates the caution which should be taken before generalizing results from a model species to a vast group such as euteleost fish.

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