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Identification of sex markers by cDNA-AFLP in *Takifugu rubripes*

Jian-Zhou Cui [1], Xue-Yan Shen [1], Qing-Li Gong *, Guan-Pin Yang, Qian-Qun Gu

Division of Life Science and Technology, Ocean University of China, Qingdao 266003, PR China

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Abstract

We searched for sex markers in mature male and female *Takifugu rubripes* gonads and tailfins by cDNA-amplified fragment length polymorphism (AFLP) performed on pooled cDNA samples. Five sex markers (TDF1-5) were identified, cloned, characterized and confirmed on individual samples of cDNA and genomic DNA, showing good agreement with phenotypic sex. Among them, four markers (TDF1-4) derived from gonads were detected in immature *T. rubripes* (at months 6, 12, 18 and 24) testis or ovary cDNA, while the TDF5 from tailfins was detected in immature *T. rubripes* (at months 6, 12, 18 and 24) tailfin cDNA and *T. rubripes* tailfin genomic DNA. Therefore, the sex specific primer or probe of TDF5 could be used in quick detection of phenotypic sex in juvenile and even larval stages without the need to wait for the offspring to become sexually mature or dissect the fish. When tested on one closely related species from the *Takifugu* genus, TDF5 could amplify a specific fragment from *Takifugu pseudommus* tailfin genomic DNA, indicating that homologues of sex markers might exist in other species of the *Takifugu* genus.

Keywords: cDNA-AFLP; RT-PCR; *Takifugu rubripes*; *Takifugu pseudommus*; Testis; Ovary; Tailfin

1. Introduction

At present, several fish species including Chinook salmon (*Oncorhynchus tshawytscha*) (Devlin et al., 1991), medaka (*Oryzias latipes*) (Matsuda et al., 1997), Atlantic salmon (*Salmo salar*) (McGowan and Davidson, 1998), platyfish (*Xiphophorus maculatus*) (Cough-Coughlan et al., 1999), African catfish (*Clarias gariepinus*) (Kovacs et al., 2001), rainbow trout (*Oncorhynchus mykiss*) (Moran et al., 1996; Iturra et al., 1997), pufferfish (*Tetraodon nigroviridis*) (Li et al., 2002), Arctic charr (*Salvelinus alpinus*) and Brook Trout (*Salvelinus fontinalis*) (May et al., 1989; Woram et al., 2003), Nile tilapia (*Oreochromis niloticus*) (Lee et al., 2003), and threespine sticklebacks (*Gasterosteus aculeatus*) (Peichel et al., 2004) have been tested by PCR, RDA (Representational Difference Analysis), RAPD, AFLP, FISH and other molecular biology methods to search for sex markers. Several reports have demonstrated that many sex markers have essential roles in fish production (Price, 1984; Devlin and Nagahama, 2002). However, in an important fish model, *Takifugu rubripes*, no literature has been reported on searching for sex markers.

Puffer fishes of the genus *Takifugu* (Tetraodontiformes, Tetraodontidae) are mainly distributed in the Western Pacific Ocean. Two species of this genus, *T. rubripes* and *Takifugu pseudommus*, are distributed in

* Corresponding author. Tel.: +86 532 83898124; fax: +86 532 83886846.
E-mail addresses: jianzhou.cui@hotmail.com (J.-Z. Cui), qingli@vip.sina.com (Q.-L. Gong).
[1] The two authors contributed equally to this work.

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the coastal regions of China including the Bohai Sea, Yellow Sea and East China Sea. The muscle of *T. rubripes* is highly prized as food in China, Japan and Korea for its special flavor. Male fish are much sought after and are even more expensive than females, since there is a distinct difference between their growth rates and market values (Cheng et al., 1975). However, it is difficult to distinguish male fish from females by morphological characters at larval, juvenile and even adult stages. Therefore, efficient sex detection before maturity can be economically important for aquaculture of the species. On the other hand, *T. rubripes* with the most compact genome of all vertebrates is a key model organism for the identification and characterization of novel genes (Brenner et al., 1993). Polymorphic microsatellite DNA markers were isolated from the genome sequence of the species, which were used successfully to study the population genetic structure (Takagi et al., 2003; Cui et al., 2005) and construct a genetic linkage map (Kai et al., 2005). Despite all this information, until now, sex markers from *T. rubripes* have not been reported. Therefore, it is important to search for a technique for rapid detection of phenotypic sex without the need to wait for the offspring to become sexually mature.

We report here the results of a search for sex markers using the cDNA-amplified fragment length polymorphism (AFLP) (Bachem et al., 1996) to analyze differential mRNA expression between mature male and female *T. rubripes* gonads and tailfins. The objective of the present study was to search for cDNA or DNA sex markers, utilizing them for early confirmation of individual males or females and for the identification of genes associated with gonad development and differentiation.

2. Materials and methods

2.1. Pufferfish

T. rubripes (119 individuals) including immature (6, 12, 18 and 24 months old) and mature (30 months old) individuals, were randomly collected from Haiyang Sea Farming Center (Shangdong Province, P.R. China), and *T. pseudommus* (45 immature and mature individuals, a close relative of *T. rubripes* from the *Takifugu* genus) were collected from the wild in the coastal area of Qingdao, P.R. China. The total lengths of both species ranged from 17.5 to 38.1 cm. The individuals were temporarily cultured in the laboratory, and then dissected for identification of sex (52 males and 67 females for *T. rubripes*, 22 males and 23 females for *T. pseudommus*) (Nobuhiro et al., 1996; Nobuhiro, 1997). The tissues were frozen in liquid nitrogen and stored at −80 °C.

2.2. RNA extraction

Total RNA was extracted from *T. rubripes* tissues by using the Trizol reagent (Gibco BRL). The procedure was done according to instructions from the manufacturer with the following modifications: the RNA was precipitated in 0.2 volume of 1 M acetic acid and 0.7 volume of 100% ethanol, at −20 °C overnight. The pellet was washed twice in 3 M sodium acetate, pH 5.5, and once in 70% ethanol before being resuspended in diethyl pyrocarbonate treated water. Four pools (testis, ovary, male and female tailfins at month 30) were formed for each sex by mixing equal amount of RNA from 8 *T. rubripes* individuals and subjected to comparative cDNA-amplified fragment length polymorphism (AFLP) analysis.

2.3. cDNA-AFLP procedure

After DNase I treatment, reverse transcription was done with 2 μg total RNA using Superscript II reverse transcriptase (Gibco BRL). First-strand and second-strand cDNA syntheses were carried out according to standard protocol with 2-base anchored oligo(dT) primers. Double-stranded cDNA was used in the AFLP analysis, performed according to Bachem et al. (1998). Complementary DNA was digested with *Mse*I (Promega, USA) and *Eco*RI (Promega, USA). After ligation of adapters, preamplification of purified cDNA templates was performed with primers complementary to the adapter sequences without any additional selective nucleotide (Table 1). PCRs were performed in a 25 μl volume with 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 30 ng of each primer *Eco*RI1, *Mse*I1 (Table 1), 0.2 units of *Taq* DNA polymerase

Table 1
Sequences of primers and adaptors for cDNA-(AFLP) analysis

*Eco*RI adaptor	5′-CTCGTAGACTGCGTACC-3′ 3′-CTGACGCATGGTTAA-5′
*Mse*I adaptor	5′-GACGATGAGTCCTGAG-3′ 3′-TACTCAGGACTCAT-5′
*Eco*RI1 + 0 primer	5′-GACTGCGTACCAATTC-3′
*Mse*I1 + 0 primer	5′-GATGAGTCCTGAGTAA-3′
*Eco*RI2 + 2 primers	5′-GACTGCGTACCAATTCAorCorGorT/ CorGorTorA-3′
*Mse*I2 + 2 primers	5′-GATGAGTCCTGAGTAAAorCorGorT/ CorGorTorA-3′

(Promega, USA), and 5 μl diluted digested–ligated DNA fragments. Among the *Eco*RI2 and *Mse*I2 primers combinations, 64 different primer combinations were used for selective amplification (Table 1). We used 5 μl diluted preamplification template for each PCR reaction. Three replicates of the PCR reactions from 3 independent RNA extractions were performed to determine the accuracy of the analysis. Amplification products were separated by electrophoresis through a 6% denaturing polyacrylamide gel and visualized by silver-staining.

2.4. DNA extraction

DNA was extracted from pufferfish tailfin tissue following the method described by Sambrook et al. (1989) with some modifications. Approximately 100 mg of tissue was cut into small pieces. These pieces were then placed in 1 ml STE buffer (10 mM Tris/HCl, pH 8.0; 50 mM EDTA; 200 mM NaCl and 1.0% SDS) and digested with 1.0 mg/ml Proteinase K at 55 °C overnight. The resulting solution was centrifuged, and the DNA in supernatant was precipitated with ethanol and dissolved in TE buffer. DNA was purified using the phenol–chloroform extraction. The quality and concentration of DNA were assessed by agarose gel electrophoresis and stored at −20 °C until use.

2.5. Isolation and sequencing of fragments

The bands of interest were cut out from the gel with a razor blade. The gel slices were then hydrated in 50 μl water and incubated at 37 °C for 30 min. The eluted cDNA was reamplified with the same primers and under the same conditions as for the cDNA-AFLP selective amplifications, except that the PCR cycle consisted of 2 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C, and one final extension step of 3 min at 72 °C. The fragments were cloned into PMD-18 T vector (Promega, USA) and sequenced using Big Dye Terminator technology (Perkin–Elmer Applied Biosystems). The sequences were searched for homologous matches in NCBI National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) database using online BLAST tool.

2.6. Confirming the sex markers in cDNA and genomic DNA from individual T. rubripes

To confirm sequenced fragments, specific primers were designed using Primer Premier 5.0 (Primer Biosoft International, USA) software. Reverse Transcription (RT)-PCR analyses were performed in cDNA from individual *T. rubripes* (at 6, 12, 18, 24 and 30 months old; 7 females and 8 males at each stage) with primers of *MEST* for a control (Brunner et al., 2001). The RT-PCR primers, thermo-cycling conditions and reaction system were as follows (see also Table 2): an initial denaturation at 94 °C for 3 min was followed by 25 cycles of 1 min at 94 °C, 1 min at 56 °C, and 1 min at 72 °C with a final extension for 5 min at 72 °C. PCR was conducted in 25 μl volume containing 100 ng of template cDNA, 0.1 mM of primers (each), 0.2 mM dNTPs (each), 1× Taq buffer, 1.5–2.0 mM of $MgCl_2$. The products were analyzed by 1.5% agarose gel electrophoresis and molecular sizes were determined with 100 bp ladder (Promega, USA). Moreover, all the markers were tested in tailfin genomic DNA from individual mature *T. rubripes* (12 females and 9 males). The PCR primers and conditions were similar to those for the analyses in *T. rubripes* cDNA (Table 2).

Table 2
RT-PCR primers and conditions of five sex markers

Markers	Sequences (5′–3′)	$MgCl_2$ (mM)	T_m (°C)	Product size (bp)
TDF1	GCAGCAGCGGTAATGGCGGACT CGTTCCTGTCTCCGTCCCTC	1.5	56	202
TDF2	CAAAGATGGGAAGATGGA TGGCTACCCACAAACAAG	2.0	57	155
TDF3	CGACCAGTCTTGTCATAC CCAGAGGGTTACAAATAC	1.2	59	176
TDF4	GACGTAACGCTTGTTGATC TGGTTGGGCTGGCTTTCT	1.5	57	150
TDF5	CGCCACTTGAATCCCTA TAATGTGAGGAGCGACCA	1.5	58	87
MEST	AACCTGATTTCCCACGACTACGG TCGTCCAGGATGGTCAGTGTA	1.5	59	506

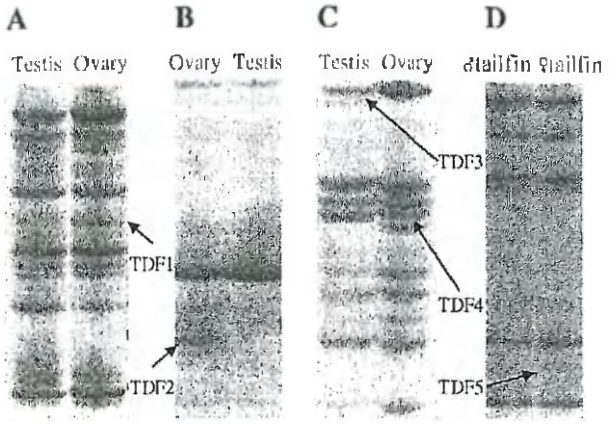

Fig. 1. Comparison of expression patterns of 5 TDFs by cDNA-AFLP. The arrow indicated the amplified fragments of TDF1, TDF2, TDF3 and TDF4 derived from testes or ovaries (A, B, and C), and TDF5 derived from female tailfins (D). The primers combinations were E7M1, E7M5, E5M8 and E5M9, respectively.

2.7. The homologues of the sex markers in adult *T. pseudommus*

The five sex markers from mature *T. rubripes* gonads and tailfins were tested in *T. pseudommus* individuals (23 females and 22 males) tailfin genomic DNA. The PCR primers and conditions were similar to that for the confirming analyses in *T. rubripes* (Table 2).

3. Results

3.1. Isolation and characterization of the five sex markers

Comparative cDNA-AFLP analysis was performed on testis versus ovary and male tailfin versus female tailfin cDNA pools generated from mature *T. rubripes* individuals. The cDNA expression profiles were determined by selective amplification using 64 different primer combinations, and 3000 cDNA fragments were screened. A total of 109 differentially accumulated TDFs (Transcript-Derived Fragments) from genes were identified. To further characterize the differentially expressed genes, 58 fragments were cloned, of which 45 clones were sequenced. Among them, five sex markers were confirmed by RT-PCR. The first marker (named TDF1) was amplified by primer E7M1 (Fig. 1A), and the other markers (TDF2-5) were amplified by primers E7M5, E5M8 and E5M9 (Fig. 1B, C, D). While TDF1-4 were isolated from testes or ovaries, TDF5 was derived from female *T. rubripes* tailfins. The nucleotide sequences of the five markers were determined and deposited in GenBank (Table 3).

BLAST searches in the NCBI database indicated that 26 sequences (57.8%) showed no similarity to known genes, while 19 sequences (42.2%) were highly homologous (>60%) with known genes. The homologies (>70%) of AFLP fragments to sequences in the databases are listed in Table 3. The AT/(AT + GC) ratio was ca. 60% for all the markers. At the DNA level, two markers (TDF1 and TDF5) showed significant homology (97%) with known sequences from *T. nigroviridis* egg RNAs in GenBank (CR718060, CR714542.2). TDF2, TDF3, and TDF4 showed high homology (80–94% identity) with an AFG3-like gene, calumenin gene, and Eu-HMTase gene, respectively. At the protein level, TDF1 was highly homologous (96% identity) with Wbscr1-prov protein from *Xenopus laevis* (AAH78074.1), and other markers showed high homology (95–98% identity) with unnamed proteins from *T. nigroviridis* (Table 3).

Table 3
Homologies (>70%) of AFLP fragments to sequences in the databases

cDNA fragment	Length (bp)	Accession numbers	Sequence similarity	% similarity (BLAST score)
TDF1	251	DR025656	Similar to Wbscr1-prov protein from *Xenopus laevis* (AAH78074.1)	(96%) 1e−09
TDF2	203	DR025657	Unnamed protein product from *Tetraodon nigroviridis* (CAF90270.1)	(95%) 5e−30
			Similar to AFG3-like protein 2 (paraplegin-like protein) from *Canis familiaris* (XP_547682.1)	(80%) 5e−22
TDF3	220	DR025658	Unnamed protein product from *Tetraodon nigroviridis* (CAG13044.1)	(98%) 4e−36
			Similar to calumenin from *Danio rerio* (NP_957376.1)	(94%) 7e−32
TDF4	192	DR025659	Unnamed protein product from *Tetraodon nigroviridis* (CAF93568.1)	(98%) 1e−32
			Eu-HMTase1 protein from *Homo sapiens* (AAH11608.2)	(88%) 8e−26
TDF5	126	DR025660	Unnamed protein product from *Tetraodon nigroviridis* (CAF90467.1)	(95%) 1e−14
			Similar to mitochondrial ribosomal protein from *Gallus gallus* (XP_415536.1)	(80%) 9e−06
TDF6	139	DR025661	Similar to hypothetical protein from *Gallus gallus* (XP_430089)	(88%) 5e−12
TDF7	100	DR025662	Unnamed protein product from *Tetraodon nigroviridis* (CAG08200.1)	(71%) 9e−1
TDF8	265	DR025663	Similar to KIAA0404 protein from *Homo sapiens* (AAH53596.1)	(75%) 7e−19
TDF9	99	DR025664	Unnamed protein product from *Tetraodon nigroviridis* (CAF94962.1)	(96%) 8e−12
			Similar to calpain-like protease from *Gallus gallus* (XP_417278.1)	(93%) 8e−10
TDF10	229	DR025665	Similar to ENSANGP00000026225 from *Anopheles gambiae* str. PEST (XP_555727.1)	(74%) 6e−7

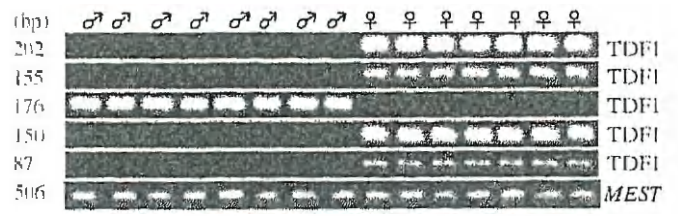

Fig. 2. The confirmed results of five sex markers in mature *T. rubripes* individuals by RT-PCR and *MEST* was used as a control. TDFs1-4 were confirmed in individual gonads and TDF5 was confirmed in female individual tailfins.

3.2. Confirming analysis of the five sex markers from T. rubripes

Testing on cDNA from individuals confirmed the results obtained with RT-PCR pools. We first tested the five markers on mature individuals. The results showed that TDF1, TDF2, TDF4 and TDF5 could be amplified in all seven female samples tested and did not show up in any of the eight male samples. TDF3 could be amplified from eight male samples tested, while cDNAs from seven females did not show the presence of the marker (Fig. 2). In immature individuals, the expression patterns of the five markers in gonad and tailfin cDNA were consistent with that in mature fish (partial results are shown in Fig. 3). However, at the DNA level, only TDF5 could be detected in tailfin genomic DNA from immature and mature *T. rubripes*, and there were no specific bands of TDFs1-4 (partial results are shown in Fig. 4A).

3.3. Search for homologues of the sex markers in T. pseudommus

Five primer pairs designed according to the sex markers of *T. rubripes* were used to search for homologous sequences in tailfin genomic DNA from 23 female and 22 male *T. pseudommus* individuals. The

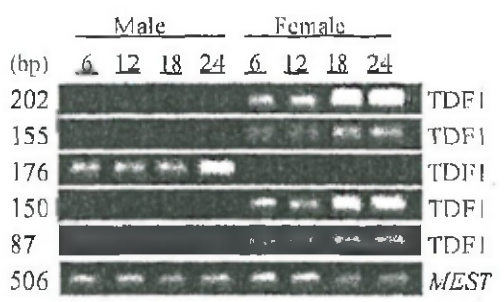

Fig. 3. The confirmed results of five sex markers in immature individuals of *T. rubripes* (6, 12, 18 and 24 months old) by RT-PCR and *MEST* was used as a control. TDFs1-4 were confirmed in individual gonads and TDF5 was confirmed in female individual tailfins.

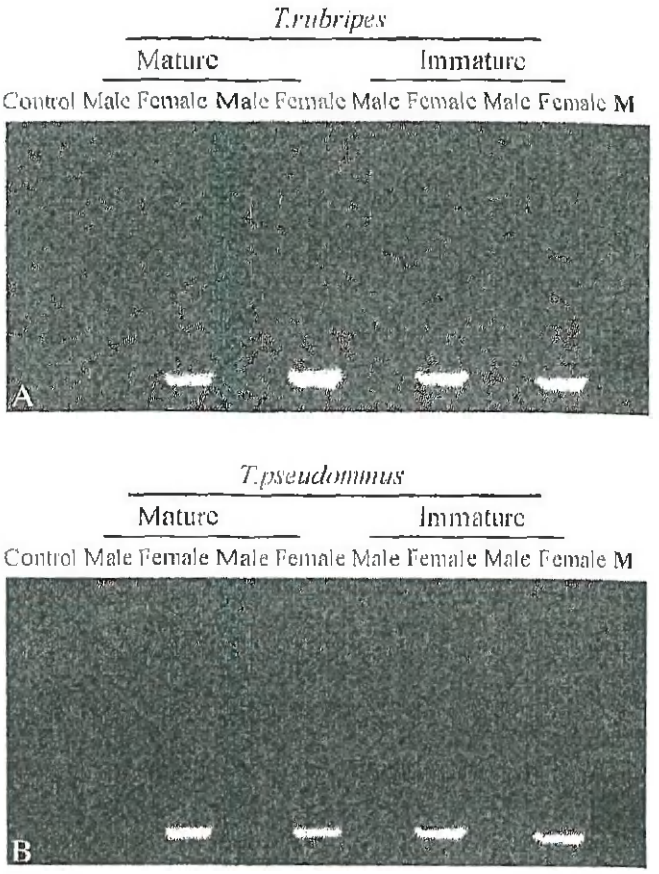

Fig. 4. The amplification results of TDF5 in *T. pseudommus* (A) and *T. rubripes* tailfin genomic DNA (B). Label M represents the molecular weight marker (100 bp DNA Ladder).

amplification of TDF5 was detected in females but not in males (partial results are shown in Fig. 4B).

4. Discussion

DNA markers provide useful tools for examining sex linkage in fish, since DNA structure is not anticipated to change with altered physiology or environments. Further, examination of DNA sequence organization on sex chromosomes can provide important insights into the evolutionary processes that are operating to influence sex-chromosome structure, and ultimately, can yield information on the conservation of sex-determination processes among species (Devlin and Nagahama, 2002). Moreover, isolation and characterization of sex markers could lead to improvements in fish production. At present, isolation of sex-linked genetic markers has been accomplished for several fish species by molecular marker technology such as RDA, RAPD and AFLP (see Introduction).

Such techniques, mentioned above, still suffer from problems of repeatability and uncertainty in the identification of specific fragments. However, the cDNA-AFLP method (Bachem et al., 1996) largely

overcomes these limitations and makes a simple and rapid verification of band identity possible. It is efficient, sensitive, reproducible, and can offer several advantages in gene expression analysis. In addition, the systematic screening of nearly all transcripts in a given biological system, using small quantities of starting material, is possible. In the present study, we used cDNA-AFLP technology to identify sex markers from mature *T. rubripes* gonads and tailfins. According to our knowledge these were the first sex markers isolated from *Takifugu* fish species.

Five sex markers (TDF1-5) were identified from mature *T. rubripes* gonads and tailfins by cDNA-AFLP analysis and confirmed in individuals at different developmental stages. Apart from TDF3, the other markers were all female-specific, and the expression patterns in male and female individuals were consistent with phenotypic sex. TDFs1-4 could be detected in gonad cDNA of immature *T. rubripes* (at 6, 12, 18 and 24 months old). More interestingly, TDF5 could not only be detected in tailfin cDNAs, but also in genomic DNA of *T. rubripes* tailfins. Therefore, the sex specific primer or probe of TDF5 could be used in quick detection of phenotypic sex at juvenile and even larval stages without the need to wait for the offspring to become sexually mature or dissect the fish.

Among five markers, BLAST search in NCBI database indicated that, at the protein level, TDF1 had high homology (96%) with Wbscr1-prov protein from *X. laevis*, indicating this marker may be related to gonad differentiation. Other markers (TDF2-5) showed high homology (95–98% identity) with unnamed proteins derived from *T. nigroviridis* egg ESTs, suggesting the function of these markers may be related to embryogenesis. Moreover, the amplification of TDF5 was detected in females only, suggesting that the *T. rubripes* may have a WZ/ZZ system. Kakimoto et al. (1994) concluded that *T. rubripes* has an XX-XY sex determination system on the basis of 93% of the meiotic gynogenetic progeny being female. However, if there is a WZ/ZZ sex determining gene located near to a telomere in this species, then a high level of recombination between this gene and the centromere would result in most of the gynogenetics being WZ (female) recombinants, with smaller proportions of WW (female) and ZZ (male) non-recombinants (the latter would be estimated at about 7% on the basis of this evidence, giving an overall estimated frequency of 14% non-recombinants and 84% recombinants, assuming complete interference). Similar situations have been found in tilapia (Lee et al., 2004), when analysing meiotic gynogenetics from WZ female *O. aureus* or XY neofemale *O. niloticus*, and very high rates of recombination have been observed for several other loci in meiotic gynogenetic fish of several species. Thus this earlier paper and the present data could both be interpreted to support a WZ/ZZ sex determination mechanism in this species. The data obtained here will provide the clues for guiding further studies on sex determination and/or differentiation in *T. rubripes*.

The number of sex-specific genes or markers found to date in fish is low in comparison to more recent vertebrate groups. Most markers do not seem to be applicable to more than one fish species or even strain (Iturra et al., 1997). The cross-species amplification results of the sex marker (TDF5) in *T. pseudommus* indicated that a homologue of this sex marker might exist in other closely related species in the *Takifugu* genus (Fig. 4).

There are a number of potential uses for sex markers, both in applied and basic research. In applied research for cultured *T. rubripes*, the identification of individual male or female requires two to four years during which time the fish reach sexual maturity. Molecular sexing allows for early confirmation of androgenesis or gynogenesis as well as hormonal sex reversal without the need to wait for the offspring to become sexually mature (Devlin and Nagahama, 2002). Moreover, since there is a distinct difference between the growth rates of the two sexes in *T. rubripes* (Cheng et al., 1975), improved genome manipulation by early molecular sexing could be economically important for the aquaculture of the species. In basic research, isolation and characterization of the putative sex markers from mature *T. rubripes* gonads and tailfins could provide valuable data for understanding the evolution of sex determination mechanisms and gonad differentiation in *T. rubripes* and the *Takifugu* genus.

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