Tc1-like transposase Thm3 of silver carp (Hypophthalmichthys molitrix) can mediate gene transposition in the genome of blunt snout bream (Megalobrama amblycephala).

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Abstract

Tc1-like transposons consist of an inverted repeat sequence flanking a transposase gene that exhibits similarity to the mobile DNA element, Tc1, of the nematode, Caenorhabditis elegans. They are widely distributed within vertebrate genomes including teleost fish; however, few active Tc1-like transposases have been discovered. In this study, 17 Tc1-like transposon sequences were isolated from 10 freshwater fish species belongs to the families, Cyprinidae, Adrianichthyidae, Cichlidae and Salmonidae. We conducted phylogenetic analyses of these sequences using previously isolated Tc1-like transposases and report that 16 of these elements comprise a new subfamily of Tc1-like transposons. In particular, we show that one transposon, Thm3 from silver carp (Hypophthalmichthys molitrix; Cyprinidae), can encode a 335 a.a. transposase with apparently intact domains, containing 3-5 copies in its genome. We then co-injected donor plasmids harboring 367 bp of the left end and 230 bp of the right end of the non-autonomous silver carp Thm1 cis-element along with capped Thm3 transposase RNA into the embryos of blunt snout bream (Megalobrama amblycephala; 1-2 cell embryos). This experiment revealed that the average integration rate could reach 50.6% in adult fish. Within the blunt snout bream genome, the TA dinucleotide direct repeat, which is the signature of Tc1-like family of transposons, was created adjacent to both ends of Thm1 at the integration sites. Our results indicate that the silver carp Thm3 transposase can mediate gene insertion by transposition within the genome of blunt snout bream genome, and that this occurs with a TA position preference.

Key words: Tc1-like transposase; transposition; Hypophthalmichthys molitrix; Megalobrama amblycephala
Transposable elements (TEs) can move within their host genomes by changing their positions of insertion in a process called transposition (McClintock, 1953; Rubin and Spradling, 1982; Craig et al., 2002). Their wide distribution among all major branches of life, their diversity, and their intrinsic biological features have made TEs a considerable source of many genetic innovations during species evolution (Radice et al., 1994; Kazazian, 2004; Feschotte and Pritham, 2007; Janicki et al., 2011). DNA transposons constitute one of the two sub-classes of Class II TEs (Craig, 1995). In eukaryotic genomes, DNA transposons have been divided into at least 17 super-families on the basis of sequence similarities between their element-encoded transposases (Yuan and Wessler, 2011). The transposition of DNA transposons follows the ‘cut-and-paste’ mechanism and is catalyzed by functional transposase and that allows the transposon to excise itself from a donor chromosomal site and then to re-insert at a different chromosomal locus (McClintock, 1953; Kidwell and Lisch, 2000).

*Tc1* transposon was first discovered in the nematode genome (*Caenorhabditis elegans*) and was shown to encode an active transposase (Emmons and Yesner, 1984; Eide and Anderson, 1985). *Tc1*-like transposons are approximately 1.3 kb long and contain a single gene without an intron encoding an ~350-amino acid transposase. The transposase gene is flanked by inverted terminal repeats (ITRs) and is characterized by a DNA binding domain and a catalytic domain which can catalyze the DNA cleavage and TA dinucleotide target-joining steps of transposition (Plasterk et al., 1999). To date, six autonomously active *Tc1*-like elements have been isolated from eukaryotes, i.e. *Minos* from *Drosophila hydei* (Franz and Savakis, 1991), *Mos1* from *Drosophila mauritiana* (Medhora et al., 1991), *Famar1* from *Forficula auricularia* (Barry et al., 2004), *Mboumar-9* from *Messor bouvieri* (Munoz-Lopez et al., 2008), and *Passport* from seawater flatfish (*Pleuronectes platessa*) (Clark et al., 2009). Moreover, four active *Tc1*-like elements have been reconstructed according to the bioinformatic consensus sequences, i.e. *Sleeping Beauty (SB)* from salmonid
(Ivics et al., 1997), *Frog Prince* (FP) from *Rana pipiens* (Miskey et al., 2003), Himar1 from *Haematibia irritans* (Lampe et al., 1996), and Hsmar1 from humans (Robertson and Zumpano, 1997). These works have shown the ability of Tc1-like transposases to catalyze transposition in a variety of cells and species.

DNA transposons can be developed as valuable tools for genetic analyses (Izsvak et al., 2000; Kawakami et al., 2004; Kotani and Kawakami, 2008). Identification fish-derived Tc1-like transposase will provide techniques for transgenesis and insertional mutagenesis and might provide some clues to explain the fish genome evolution (Hickman et al., 2005). In teleost fish, Tc1-like transposons are found to distribute widely within the genomes. For instance, between 6% and 10% of the genome of Atlantic salmon (*Salmo salar*) is occupied by Tc1-like and PiggyBac subfamily transposons (Davidson et al., 2010), and approximately 4.2% of the genome of channel catfish (*Ictalurus punctatus*) is composed of Tc1-like transposons (Nandi et al., 2007). Similarly, the Tc1-like transposon accounts for nearly 1.7% of the genome of common carp (*Cyprinus carpio*), which is second only to that of the hAT transposon superfamily (approximately 2.3%) (Wang et al, 2011). However, the vast majority of Tc1-like transposons that have been identified in teleost fish are defective as they contain frame-shifts, insertions/deletions, and premature termination codons within the coding regions of their transposase genes during their evolution (Lohe et al., 1995).

Since multiple copies of Tc1-like transposons have accumulated in the genomes of freshwater fish during evolution (Davidson et al., 2010; Nandi et al., 2007; Wang et al., 2011), the goal of this study is to screen a few fish Tc1-like elements to see whether any copy may still retain transposition function. On the basis of 17 novel Tc1-like transposon sequences isolated from 10 freshwater fish species, we show that one of these can encode a transposase with apparently intact domains, which can mediate DNA transposition of cis-element of silver carp Thm1 in another fish species.
MATERIALS AND METHODS

Experimental animals

A total of 18 freshwater fish species belonging to seven families were sampled for PCR analysis at different years from 2011-2014 (see Table 1 for details). The common carp, crucian carp, spotted steed, topmouth gudgeon, silver carp, grass carp, blunt snout bream, Fugu, yellowhead catfish and Chinese longsnout catfish were collected at Qingpu Quanjie Fish Farm, Shanghai, China. The medaka, Nile Tilapia, Russian sturgeon, goldfish, common angelfish and Denison barb were obtained from the Aquaculture Center of Shanghai Ocean University, Shanghai, China. The brown trout and naked carp were obtained at the Yadong Fish Farms, Tibet, China. The tail fin of each fish was cut and stored in 95% ethanol at -25°C. All experiments were conducted following the guidelines approved by the Shanghai Ocean University Committee on the Use and Care of Animals.

Analysis of genomic DNA

Total genomic DNA was isolated from the tail fin samples (0.1 to 0.2 g) using a standard phenol-chloroform procedure as detailed by Sambrook et al. (1989). Two microliters of genomic DNA was used as the template in PCR amplification with a single primer Tc1-like A: 5'-TACAGTTGAAGTCGGAAGTTTACATAC-3', which was designed according to the inverted repeats of Tc1-like transposon (Izsvak et al., 1995; Liu et al., 2009; Nandi et al., 2007). PCR reaction conditions were as follows: pre-denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, and elongation at 72°C for 2 min; and a final elongation step at 72°C for 10 min. PCR products were gel-purified, ligated into the T/A cloning vector pMD-19T (Takara, Dalian, China) and transformed into Escherichia coli DH5α competent cells. Positive clones were examined by PCR and direct sequencing.
**Sequence analysis**

The nucleotide sequences of Tc1-like transposons were analyzed using BioEdit 7.0.0.1 (Hall, 1999). The complete inverted terminal repeat (ITR) sequence and the transposase open reading frame were filtered by comparing the left and right end ITR sequence and the transposase open reading frame region. The sequences of Tc1-like transposon from different fish species were obtained using the BLASTN NCBI (http://ncbi.nlm.nih.gov) search program. Alignment of the putative amino acid sequences of the Tc1-like transposases was performed with the Clustal X 1.81 program (Thompson et al., 1997). After eliminating positions with gaps the gene genealogies were assessed by maximum likelihood using the software package PAUP*4.0b10 (Swofford, 2003). For maximum likelihood, the best model and parameters were estimated by MrModeltest v2.2 (Nylander et al., 2004), based on the Akaike Information Criterion (AIC), and the following parameters: 500 bootstrap replications; ConLevel = 50; search = heuristic; brlens = yes.

**Southern blot analysis**

The 625bp silver carp Thm3 probe was designed with primer pairs Thm3-f: 5’-AGATGCTGGCTGAAACTGGTAA-3’ and Thm3-r: 5’-ATCAGGTTTGTAAGCCCTTCTC G-3’, which cover the major open reading frame (ORF) frame from 425 bp to 1049 bp of Thm3. The probes were digoxin-labeled in vitro using the DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche, Basel, Switzerland), according to the manufacturer’s instructions. Southern blot analysis was performed as described previously by Koga et al (2000), with the following modifications. High molecular weight DNAs (20-30 µg for each gel slot) were digested with BglII restriction enzyme, fractionated on 0.8% agarose gels and transferred to nylon membranes (Hybond-N; Amersham), followed by fixation via baking at 80°C. The membranes were then hybridized with a digoxin-labeled silver carp Thm3 probe. Finally, stripping and re-probing DNA blots were performed after immunological detection.
**Plasmid constructs**

To generate the pCS2-CMV-Thm3TP construct, the ORF of the silver carp Thm3 transposase cDNA, which encodes 335 aa residues, was amplified by PCR using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) and the primers 5’-CCGCTCGAGATGGGAATTCAAGAGAA-3’ and 5’-GCTCTAGATTAGTATTTGGTAGAATT-3’. This was then digested with *Xho*I and *Xba*I and sub-cloned into the *Xho*I-*Xba*I sites of the pCS2+ vector. The sequences of all plasmids were confirmed by DNA sequencing. The left (367 bp) and right (230 bp) Thm1 inverted terminal repeats (ITRs) were amplified from the silver carp using PCR primers for the left ITRs (sense, 5’-GGACTAGTTACAGTTGAAGTCGGAAG-3’; antisense, 5’-CCGCTCGAGCTCTGTGTTTGAGGTGTG-3’) and right ITRs (sense, 5’-GAAGATCTGAAGTGTATGTATACTTCTGACTT-3’; antisense, 5’-GGGGTACCTACAGTTGAAGTCGGAAGTTTACA-3’). To generate the pThm1-Mlyz2-RFP (red fluorescent protein) construct, the left ITR PCR product was digested with *Spe*I and *Xho*I, and the right ITR PCR product was digested with *Bgl*II and *Kpn*I. These two fragments were then sub-cloned into the *Spe*I-*Xho*I and *Bgl*II-*Kpn*I sites, respectively, of pTgf2-Mlyz2-RFP that contained the zebrafish myosin light chain 2 (Mlyz2) gene promoter (Guo et al., 2013).

**In vitro transcription and microinjection**

Capped silver carp Thm3 transposase mRNAs were synthesized in vitro using the mMESSAGE mMACHINE kit (Ambion, Austin, TX, USA) using *Not*I linearized pCS2-CMV-Thm3TP plasmid DNA as a template. The microinjection volume was estimated at 1 nL/embryo, and contained 50 pg of circular donor plasmid pThm1-Mlyz2-RFP co-injected with 50 pg of Thm3 transposase capped mRNA. This was injected into blunt snout bream embryos at the 1-2 cell stage. The linearized donor plasmid pThm1-Mlyz2-RFP was
injected without *Thm3* transposase capped mRNA as a control. After injection, the embryos were placed in an embryo rearing medium and maintained at room temperature. Fluorescent expression in the embryos was analyzed using a Nikon SMZ1500 fluorescence microscope.

**Transgene efficiency and insertion site analysis**

The primers used for RFP PCR were: RFP-f, 5′-GCATGGAGGGCTCCGTGAACG-3′; RFP-r, 5′-GGTGTAGTCCTCGTTGTGGG-3′. PCR amplification, cloning and sequencing were conducted as previously described (Jiang et al., 2012). The flanking sequences of the transposon insertion sites were analyzed using the GenomeWalker™ Universal Kit (Clontech, California, USA). For splinkerette PCR, 25 µg genomic DNA was digested for 12-16 h at 37°C with 80 U of *Stu* I and *EcoR* V in a 100 µL reaction volume, purified by ethanol precipitation, and 4 µL of the digestion mix was ligated with the splinkerette adaptor overnight at 16°C. The linker ligation was used as a template for two rounds of PCR to amplify the transposongenome junction. The nested primers for the 5′ flanking sequences were 5′-AGGTGTGCCTTTATATTCATCCACAGGCGT-3′ and 5′-ATCAATTAACCTATCAGAAGCTTCTT-3′. The nested primers for the 3′ flanking sequences were 5′-TAAGGAAGTGTATGTATACTTCTGACTTTGA-3′ and 5′-TCCCTCGCTCGATTCTGACATTTAACA-3′. The amplified fragments were cloned into the pMD19-T vector (TaKaRa, Dalian, China), transformed into *DH5α E. coli* cells and the positive clones examined by PCR and direct sequencing.

**RESULTS**

**PCR screening and sequence analysis of Tc1-like elements from different fish species**

A total of 34 specific nucleotide sequences (ranging from 907 bp to 2036 bp) were amplified from the 18 fish species using the single *Tc1*-like A primer complementary to ITRs (Table 1). Seventeen of these sequences exhibited structural features of the *Tc1*-like
transposon comprised of the ITRs and the transposase coding region. The remaining 17 sequences coded for other genes or were unknown sequences. The 17 Tc1-like transposons were distributed in ten species belonging to four families (Table 1). Like most members of the Tc1-like family, the 17 cloned Tc1-like transposons were always flanked by a TA target site (data not shown). All Tc1-like transposon sequences we obtained are available in GenBank under Accession nos. KJ742716 – KJ742730. Since brown trout Tbt1 and Tbt2 cloned from the samples collected at 2014 were >99% similarities to the sequences identified at 2011 (Guo et al., 2014), previous GenBank accession numbers (JQ782178- JQ782179) were used for both sequences in this study (Table 1).

We used the 17 cloned Tc1-like transposons DNA sequence as queries for BLAST n searches of the GenBank databases, all sequences matched similarity above 50% were used for phylogenetic analysis. Our results indicate the C. elegans Tc1 does not appear as a monophyletic group but clusters together with TC-TSS3 Salmo salar as outgroup with a high value of bootstrap (Fig. 1A). The isolated Tc1-like sequences seem to cluster as a distinct subfamily of the Tc1-like transposon with 98% of bootstrap, except for brown trout Tbt1. As shown in Fig.1A, the Tpd1 and Tpd2 from Denison barb do not cluster to the Tc1-like sequences from other Cyprinidae fish species, but cluster well with the medaka Tmf1 from Adrianichthyidae and the common angelfish Tpc1 from Cichlidae. Even for the isolated 13 Tc1-like sequences from 7 species of Cyprinidae family, their phylogenetic relationship is patchy clustering (Fig. 1A). This means that these Cyprinidae Tc1-like sequences are not in line with the vertical evolutionary relationships (Fig. 1B). By pair-wise comparisons, Tc1-like transposons of same size (1473bp) isolated from grass carp (Tci2), common carp (Tcc2), crucian carp (Tca2) and brown trout (Tbt2) shared >99% identity to quite distantly related groups (Fig. 1A; Table 1). The closely related Tc1-like transposons described here are distributed among divergent lineages of Cyprinidae and Salmonidae (diverged for >250 million years). The patchy distribution Tc1-like elements, coupled with the extreme level of
similarity between Tc1-like elements in the distantly divergent host species are incompatible with vertical inheritance, but strongly indicative of multiple horizontal introductions.

The 17 Tc1-like elements screened from different fish species could be divided into three structural patterns based on their ITRs and their transposase ORF. One pattern involved silver carp Thm1, naked carp Tgpp1 and brown trout Tbt1, and preserved complete left and right ITRs, while the transposase regions were interrupted by frameshifts or stop codons (Fig. 2A).

A second pattern involved silver carp Thm2, grass carp Tci1, Tci2, Denison barb Tpd1, Tpd2, crucian carp Tca2, common carp Tcc2, Tcc4, medaka Tmf1, common angelfish Tpc1 and brown trout Tbt2, which had incomplete ITRs as well as interrupted transposases (Fig. 2B).

The third pattern involved silver carp Thm3, common carp Tcc3 and blunt snout bream Tma2, which have lost some nucleotides in the ITRs, but have preserved uninterrupted transposases encoding 335 aa, 280 aa and 238 aa Tc1-like transposases, respectively (Fig. 2C).

Uninterrupted Tc1-like transposase Thm3 screening from silver carp

The ORF of silver carp Thm3 encodes 335 amino acids of the transposase, but preserves incomplete ITRs (Fig. 3). Meanwhile, the silver carp Thm1 has long ITRs of ~210 bp with internal repeats/directed repeats (IRs/DRs), an internal structure described by Izsvak et al. (1995), but preserves interrupted transposases. The silver carp Thm3 and Thm1 elements share a high similarity (91%), except for some insertion/deletion nucleotides (Fig. 3). Our further Southern blot analyses reveal that there are only 3-5 copies of the Thm3 transposon in the silver carp genome (Fig. 4). However, the positions of these Thm3 copies are relatively constant in the silver carp genome, which indicates the silver carp Thm3 transposase may lose its autonomous transposition activity. Although this may also be attributed to the endogenous inhibition of host cell (Castaneda et al., 2011), our efforts to detect mRNAs encoded by the Thm3 element indeed failed to demonstrate the presence of the putative transposase in multiple tissues of silver carp (data not shown).
The 335 aa Thm3 transposase from silver carp exhibits greater than 80% sequence identity with reconstructed transposase SB from salmonids, but only 46% and 44% sequence identity with reconstructed transposase FP from *Rana pipiens* and natively active flatfish *PPTN*, respectively (Fig. 5). As seen in salmonid SB transposase, the silver carp Thm3 transposase contains the main functional domains of *Tc1*-like transposases, i.e. a paired-like domain with leucine-zipper for DNA binding, a bipartite nuclear localization signal (NLS), a glycine-rich box and a complete DD(34)E domains in the C-terminal (Fig. 5). The DD (34) E domain is the essential site for catalytic transposition of *Tc1*-like transposase. There are two Aspartates (D, D) separated by 90 amino acids, the second of which is separated from Glutamate (E) by 34 amino acid residues (Fig. 5). The silver carp Thm3 transposase retains its intact functional domains, which suggests that it could be used to construct gene transfer systems in other teleost fish.

**Silver carp Thm3 can mediate gene transposition in blunt snout bream genome**

To explore whether the silver carp Thm3 transposase has transposition activity, a binary transposon vector system was developed based on Thm3 transposase and ITRs from the silver carp non-autonomous element Thm1. We constructed the donor plasmid pThm1-Mlyz2-RFP with a 367 bp left-end and 230 bp right-end of the Thm1 transposon (Fig. 6A). In addition, the pCS2-CMV-Thm3TP plasmid was constructed and used to *in vitro* synthesize capped RNA encoding 335 a.a. residues Thm3 transposase (Fig. 6B). We then co-injected 50 pg of donor plasmid DNA with 50 pg of capped RNA into the embryos (1-2 cell stage) of blunt snout bream. We found that the red fluorescence expression was mainly focused in the myofibrils in blunt snout bream embryos at 72 hour post-fertilization (hpf), indicative of tissue-specific expression by the zebrafish myosin light chain 2 (Mlyz2) promoter (Fig. 6C – D). In 200-day-old adult blunt snout bream, the red color could be observed in the dorsal skeletal muscles of transgenic individuals (Fig. 6E – F). PCR analysis of the transposition rate of RFP
at adult blunt snout bream was done as previously described (Jiang et al., 2012). The integration rate of RFP gene into the blunt snout bream genome when fish were 200 days old ranged from 47.0% to 59.5% (average = 50.6%, 519/1015; Table 2). In control embryos by injected linearized donor plasmid alone, only 20.2% embryos showed weak and high rate of the mosaic expression of RFP and few fish expressed RFP at 200-day-old stage (data not shown). Moreover, PCR analysis showed the average RFP-transgenic rate in control adult fish injected with the linearized donor plasmid pThm1-Mlyz2-RFP alone was 5.0% (8/184; Table 2).

To precisely characterize insertions at a sequence level, we cloned the junctions of the integrated Thm1 cis-element and the surrounding genomic DNA using inverse PCR. Since the endogenous Tc1-like element Tma2 was also present in the genome of blunt snout bream (Fig.1, Table 1), its similarity with sequences of the left- (367 bp) and right-end (230 bp) of silver carp Thm1 were 54% and 65%, respectively (Supplemental Fig.1). To avoid false positive, we chose low similarity (<50%) regions to design the nested primers to clone the 5’ or 3’ flanking sequences (Supplemental Fig.1). Among five RFP-transgenic positive individuals, there were 1 – 4 genomic integration sites in the genome of the blunt snout bream, including the specific end sequences of Thm1 DNA (Table 3; Supplemental Fig.1), while no PCR products during splinkerette PCR were amplified in genome of the negative control (data not shown). Moreover, a TA dinucleotide direct repeat of the target DNA at the integration site (which is the signature of Tc1-like family of transposons) was created adjacent to both ends of Thm1 at the integration sites in all individuals (Table 3). These results indicate that the silver carp Thm1 transposon insertions occurred by transposition mediated by the Thm3 transposase. It also suggests that during DNA transposition the silver carp Thm transposon system exhibits a TA insertion position preference. Furthermore, the RFP-transgenic fish were raised to maturity and crossed with the opposite sex blunt snout bream of wild type, and F1 embryos were analyzed for RFP expression under a fluorescent microscope at 48 hpf.
Among 11 RFP-transgenic positive fish we randomly selected, 10 of 11 founders were able to express RFP in their F1 embryos from 13% to 64% (Table 4), indicating that the silver carp transposon vector inserted into the blunt snout bream genome could be transmitted to the F1 progeny.

DISCUSSION

In this study, we isolated 17 Tc1-like transposons from 10 freshwater fish species and most (16) of which clustered as a new subfamily of Tc1-like transposons. Like most members of the Tc1-like family (Eide and Anderson, 1985; Emmons and Yesner, 1984; Izsvak et al., 1995), the 17 cloned Tc1-like transposons were always flanked by a TA target site. Of these transposons, we have shown that the Thm3 transposon of silver carp (Hypophthalmichthys molitrix) contains the main functional domains of Tc1-like transposases, and is present at relatively low copy numbers. A binary transgene vector system was developed based on the 335 amino acids Thm3 transposase and terminal inverted repeats (ITRs) from silver carp non-autonomous element Thml. Although Thm3 is not an autonomous transposon in the silver carp, our results show that the Thm3 transposase can efficiently mediate gene insertion in the genome of one of its Cyprinidae relatives, the blunt snout bream (Megalobrama amblycephala) and can mediate transposition in the blunt snout bream germ lineage. We have shown that the silver carp Thm transposon system has a TA insertion position preference during DNA transposition in blunt snout bream.

DNA transposons transferred by the horizontal approach have been found in multiple prokaryotic and eukaryotic species (Leaver, 2001; de Boer et al., 2007; Pocwierz-Kotus et al., 2007; Pace et al., 2008; Kuraku et al., 2012; Jiang et al., 2012). In the present study, the isolated Tc1-like sequences from different fish species do not conform the vertical evolutionary relationships, and their phylogenetic relationship is patchy clustering. This means that the isolated Tc1-like transposons might be horizontally transmitted among species,
because of inconsistency with the accepted phylogenetic relationships of their host organisms. Also, the idea that horizontal transmission has played a role in the dissemination of cloned Tc1-like transposons in this study is supported by the presence of a complete copy in the fish genome. An almost identical 1473bp Tc1-like transposon is found to distribute in the diverged Cyprinidae and Salmonidae, and their nucleotide sequence similarity is markedly greater than some of the most conserved protein-coding genes in vertebrates (e.g., RAG-1) (Venkatesh et al., 2001). Another convincing example is seen in the transposons, Tc1-1Neo from round goby and Tc1-2Per from European perch, which have virtually identical nucleotide sequences and have been found in fish belonging to quite distantly related groups (Fig. 1A and see Pocwierz-Kotus et al., 2007). Despite the lack of direct evidence, our results suggest that horizontal transmission of Tc1-like transposons seems to occur in some divergent fish lineages. Interestingly, evidence has been found that horizontal transfers of Tc1-like elements can occur between teleost fishes and lampreys, their vertebrate parasites (Gilbert et al., 2010; Kuraku et al., 2012).

Transposons are believed to have evolved via three processes: horizontal transmission, vertical inactivation, and stochastic loss (Lohe et al., 1995). An autonomous DNA transposon usually encodes an endogenous functional transposase in the host cell, which is expected to have originated relatively recently via horizontal transfer (Eide and Anderson, 1985; Koga and Hori, 2000; Clark et al., 2009; Jiang et al., 2012). However, most transposons, especially those in teleost fish, undergo vertical inactivation or stochastic loss to produce a large number of different copies of un-autonomous transposons that have mutations in their transposase regions (Leaver, 2001; Ahn et al., 2008; Wang et al., 2011; Pujolar et al., 2013). For example, in the channel catfish genome the Tip1 transposon exists at approximately 150 copies per haploid genome, while the Tip2 transposon exists at approximately 4,000 copies per haploid genome (Nandi et al., 2007), with 32,000 copies of the non-autonomous element Tipnon present (Liu et al., 1999). However in the silver carp genome, our results showed that low
numbers of 3-5 copies Thm3 transposon were existed. This suggests that horizontal transmission of Thm3 transposon into the silver carp genome might not be too long, and yet vertical inactivation may not occur in the host cell. In fact, the silver carp Thm3 transposase retains its intact functional domains that it may be used to construct gene transfer systems.

The Tc1-like transposons are powerful molecular tools for transgenesis in vertebrate cells (Gallardo-Galvez et al., 2011; Garrels et al., 2014). Most Tc1-like transposons identified in fish are considered defective as they contain insertions/deletions that result in either sequence frame-shifts or point mutations, which lead to a premature stop codon or a nonsense codon in the transcribed mRNA (Radice et al., 1994). Recently, several novel Tc1-like transposable elements have been found to include all the functional domains of Tc1-like transposons, e.g. CCTN in the genome of the common carp, which contains an intact 331 a.a. Tc1-like transposase (Wang et al., 2011), e.g. the MMTS transposon that contains motifs including DDE from mud loach (Ahn et al., 2008), and e.g. the Tanal transposase composed of 341 amino acids in the genome of sturgeons (Pujolar et al., 2013). However, further assays should be conducted to test if the catalytic activity of the DDE motif remains active. In this study, a binary silver carp Thm vector system was developed to enhance production of transgenic blunt snout bream as an alternative to methods of transgenesis involving the injection of donor plasmid DNA. We have shown that the silver carp Thm3 transposase can efficiently catalyze the integration of donor DNA into a TA dinucleotide site of a recipient genome. In future, it would be good to compare the efficiency of transposition of the new element with sleeping beauty or some previously isolated Tc1-like element, although these Tc1-like elements such as SB and FP were seldom used for fish transgenesis.

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REFERENCES


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<td>Fugu, <em>Takifugu obscurus</em> (2012)</td>
<td>1044bp, 907bp, 1089bp</td>
<td>---</td>
</tr>
</tbody>
</table>
Table 2: The transposition efficiencies of silver carp *Thm* transposon systems in 200-day-old blunt snout bream by PCR analysis

<table>
<thead>
<tr>
<th>Batches</th>
<th>Numbers of survival injection individuals</th>
<th>No. of individuals integrated RFP</th>
<th>Integration rates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>66</td>
<td>31</td>
<td>47.0</td>
</tr>
<tr>
<td>2</td>
<td>147</td>
<td>64</td>
<td>43.5</td>
</tr>
<tr>
<td>3</td>
<td>121</td>
<td>72</td>
<td>59.5</td>
</tr>
<tr>
<td>4</td>
<td>87</td>
<td>41</td>
<td>47.1</td>
</tr>
<tr>
<td>5</td>
<td>212</td>
<td>107</td>
<td>50.5</td>
</tr>
<tr>
<td>6</td>
<td>187</td>
<td>96</td>
<td>51.3</td>
</tr>
<tr>
<td>7</td>
<td>195</td>
<td>108</td>
<td>55.4</td>
</tr>
<tr>
<td>Average</td>
<td>145</td>
<td>74</td>
<td>50.6 **</td>
</tr>
</tbody>
</table>

Control1 115 5 4.3
Control2 69 3 5.7
Average 92 4 5.0

Control is injected pThm1-Mlyz2-RFP plasmid only. ** *p*<0.01.
Table 3: Exogenous *Thm1* end regions and the surrounding transposon insertion site sequences in the genome of 200-day-old blunt snout bream

<table>
<thead>
<tr>
<th>Sample numbers</th>
<th>Copies</th>
<th>Sequence of target integration sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>i</td>
<td>(143nt)GTACTTTTATAACAGTT----AACTGTATACACGAT(11nt)</td>
</tr>
<tr>
<td></td>
<td>ii</td>
<td>(180nt)CAGACTTCTACAGTT----AACTGTAACCTGTCAC(92nt)</td>
</tr>
<tr>
<td></td>
<td>iii</td>
<td>(75nt)ACATATATTACAGTT----AACTGTAATGTGTC(138nt)</td>
</tr>
<tr>
<td></td>
<td>iv</td>
<td>(85nt)CCTTTGTATAACAGTT----AACTGTATAAATGG(175nt)</td>
</tr>
<tr>
<td>2</td>
<td>i</td>
<td>(92nt)TTATGACCTTACAGTT----AACTGTAATTCATATA(127nt)</td>
</tr>
<tr>
<td></td>
<td>ii</td>
<td>(38nt)TAAGGTTGTACAGTT----AACTGTAAGGTTGTAC(95nt)</td>
</tr>
<tr>
<td>3</td>
<td>i</td>
<td>(28nt)AACTGCTCTTACAGTT----AACTGTAACTGAGTA(134nt)</td>
</tr>
<tr>
<td></td>
<td>ii</td>
<td>(73nt)GTTGTAAGTTACAGTT----AACTGTACAGTACTA(117nt)</td>
</tr>
<tr>
<td></td>
<td>iii</td>
<td>(92nt)GCGTAAGTTACAGTT----AACTGTATAGTGACG(83nt)</td>
</tr>
<tr>
<td>4</td>
<td>i</td>
<td>(177nt)GGATAGTTTACAGTT----AACTGTAATGAGTGAA(76nt)</td>
</tr>
<tr>
<td>5</td>
<td>i</td>
<td>(58nt)AGACTTGGTACAGTT----AACTGTAAGCGATTT (154nt)</td>
</tr>
<tr>
<td></td>
<td>ii</td>
<td>(14nt)GTAGGGTTTACAGTT----AACTGTAACGGTAAAT(217nt)</td>
</tr>
<tr>
<td></td>
<td>iii</td>
<td>(73nt)GGTATAGGGTACAGTT----AACTGTAAGGTTATACG(312nt)</td>
</tr>
</tbody>
</table>

The TA dinucleotide direct repeats of the target DNA are marked in gray, and the end sequences of *Thm1* DNA are underlined.
### Table 4: RFP expression in F1 embryos of eleven RFP-transgenic positive blunt snout bream crossed with the opposite sex of wild-type

<table>
<thead>
<tr>
<th>ID of RFP-transgenic positive fish&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gender</th>
<th>No. of F1 embryos examined</th>
<th>No. of RFP positive embryos</th>
<th>RFP positive/total F1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>690000116601632</td>
<td>female</td>
<td>89</td>
<td>57</td>
<td>64</td>
</tr>
<tr>
<td>690020042302853</td>
<td>female</td>
<td>254</td>
<td>123</td>
<td>48</td>
</tr>
<tr>
<td>690000116602112</td>
<td>male</td>
<td>275</td>
<td>40</td>
<td>15</td>
</tr>
<tr>
<td>690020042302431</td>
<td>male</td>
<td>212</td>
<td>27</td>
<td>13</td>
</tr>
<tr>
<td>690020042302497</td>
<td>female</td>
<td>238</td>
<td>38</td>
<td>16</td>
</tr>
<tr>
<td>690020042303065</td>
<td>male</td>
<td>152</td>
<td>78</td>
<td>51</td>
</tr>
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<td>690020042303056</td>
<td>male</td>
<td>96</td>
<td>55</td>
<td>57</td>
</tr>
<tr>
<td>690020042302805</td>
<td>female</td>
<td>321</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>690020042303004</td>
<td>female</td>
<td>186</td>
<td>27</td>
<td>15</td>
</tr>
<tr>
<td>690000116601753</td>
<td>female</td>
<td>141</td>
<td>51</td>
<td>36</td>
</tr>
<tr>
<td>690000116602026</td>
<td>male</td>
<td>311</td>
<td>50</td>
<td>16</td>
</tr>
<tr>
<td>Control&lt;sup&gt;b&lt;/sup&gt;</td>
<td>—</td>
<td>215</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>The ID of RFP-transgenic positive fish were labeled with passive integrated transponder (PIT) tags (Hongteng Barcode Corporation, Guangzhou).  
<sup>b</sup>The wild-type female mating with male was used as control.
Figure 1: (A) Maximum likelihood (ML) consensus tree for Tc1-like transposases from a variety of teleost fish species. Bootstrap percentages are shown by numbers at the interior nodes. The C. elegans Tc1 sequence was used as the outgroup. The isolated 17 Tc1-like transposon sequences of this study are shown in gray in the background. (B) Phylogenetic hypotheses proposed by Nelson (2006) and Wang et al. (2007) for fish species in this study.
Figure 2: Analysis of inverted terminal repeat sequences (ITRs) and coding regions of *Tc1*-like transposon from different fish species. Patterns contain (A) a complete left and right ITRs and an interrupted transposase, (B) an incomplete left and right ITRs and an interrupted transposase, and (C) an incomplete left and right ITRs and an intact transposase.
**Figure 3:** Sequence alignment of *Thm3* with *Thm1* from silver carp. The ITRs are shown in open boxes. The gray background sequences represent internal repeats/directed repeats (IRs/DRs). Dashed lines represent missing nucleotides.
**Figure 4**: Southern blot hybridization analysis of the Thm3 transposon in the silver carp genome. Genomic DNA of 9 individuals was sampled from the tail fins of silver carp. Genomic DNA was digested with BglII and hybridized with a digoxin-labeled Thm3 probe. The marker sizes are indicated along the right margin.
Figure 5: Alignment of the amino acid sequences of Tc1-like transposases from the silver carp Thm3, reconstructed transposase SB from salmonid, reconstructed transposase FP from *Rana pipiens*, and natively active PPTN transposase from flatfish. The major functional domains are highlighted according to the structural domains of the SB transposase (Ivics et al., 1997).
Figure 6: Results of transposition efficiency analysis of silver carp Thm3 transposase in blunt snout bream. (A) The donor plasmid construct pThm1-Mlyz2-RFP harbors the left (367 bp) and right (230 bp) silver carp Thm1 inverted terminal repeats (ITRs). This also contains the zebrafish myosin light chain 2 (Mlyz2) promoter, the red fluorescent protein (RFP) and the SV40 poly(A) signal. (B) The transposase plasmid construct pCS2-CMV-Thm3TP. Capped silver carp Thm3 transposase mRNAs were synthesized in vitro, using linearized pCS2-CMV-Thm3TP plasmid DNA as a template. Light (C) and fluorescence (D) microscopy images of RFP expression in blunt snout bream embryos at 72 hpf after coinjection of the donor plasmid pThm1-Mlyz2-RFP and capped silver carp Thm3 transposase mRNAs in embryos at the 1-2-cell stage. Optical images of RFP expression in the negative control (E) and in transgenic positive individuals (F) in 200-day-old blunt snout bream. Arrows indicate RFP expression signals. White scale bars = 600 µm. Dark scale bars = 2 cm.
Supplemental Figure 1: Sequence alignment of the 367 bp-left-end (A) and 230 bp-right-end (B) silver carp Thm1 with blunt snout bream Tma2. The nested primers for the 5’ or 3’ flanking sequences are marked in gray. Dashed lines represent missing nucleotides and dots mean similar nucleotides. The double underlined sequences are the end sequences of Thm1 DNA at target integration sites in the genome of the blunt snout bream as shown in Table 3.