**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 belonging 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-aa transposase with apparently intact domains, containing three to five copies in its genome. We then coinjected donor plasmids harboring 367 bp of the left end and 230 bp of the right end of the nonautonomous silver carp Thm1 cis-element along with capped Thm3 transposase RNA into the embryos of blunt snout bream (Megalobrama amblycephala; one- to two-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.

**KEYWORDS** 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 subclasses of Class II TEs (Craig 1995). In eukaryotic genomes, DNA transposons have been divided into at least 17 superfamilies on the basis of sequence similarities between their element-encoded transposases (Yuan and Wessler 2011). DNA transposons follow the “cut-and-paste” mechanism and is catalyzed by functional transposase, and allows the transposon to excise itself from a donor chromosomal site and then to reinsert 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 that can catalyze the DNA cleavage and TA dinucleotide target-joining steps of transposition (Plasterk et al. 1999). To date, six autonomously active Tc1-like transposons have been isolated from eukaryotes, i.e., *Mnos* from *Drosophila hydei* (Franz and Savakis 1991), *Mos1* from *Drosophila mauritiana* (Medhora et al. 1991), *Famar1* from *Forficula auricularia* (Barry et al. 2004), *Mboumar9* from *Messor bouvieri* (Monoz-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), *Hsmar1* from *Haematobia 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 of 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 majority of Tc1-like transposons that have been identified in teleost fish are defective because they contain frameshifts, insertions/deletions, and premature termination codons within the coding regions of their transposase genes during their evolution (Lohe et al. 1995).

Because 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 during different years from 2011 to 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’-TACAGTTGAATGCGAGATTTACATAC-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 sec, annealing at 51°C for 30 sec, 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 625-bp silver carp *Thm3* probe was designed with primer pairs *Thm3*-f: 5’- AGATGCTGGCTGAAACTGGTAA-3’ and *Thm3*-r: 5’-ATACAGTTGTGAAGCCTATTCTC-3’, which cover the major open reading frame (ORF) 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

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**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.
modifications. High-molecular-weight DNA (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 reprobing 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'-CCGCTCGAGATGGAGAATCTCAAGAAGA-3' and 5'-GGTACCTAGATCTCAAGAAGA-3'. The linearized donor Thm3 of NotI linearized pCS2-CMV-Thm3TP plasmid DNA as a template. The PCR product was digested with XhoI and XbaI and subcloned into the XhoI-XbaI 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'-GGACTAGTTAGTATTTGGTAGAATT-3' and antisense, 5'-CGGCTCGAGATGGAGAATCTCAAGAAGA-3') and right ITRs (sense, 5'-GAAGATCTGAAGTGTATGTATACTTCTGACTT-3' and antisense, 5'-GGGTTACCTACAGTITAGCTCAGGAAGA-3'). To generate the pThm1-Mylz2-RFP (red fluorescent protein) construct, the left ITR PCR product was digested with SpeI and XhoI, and the right ITR PCR product was digested with BglII and KpnI. These two fragments were then subcloned into the SpeI-XhoI and BglII-KpnI sites, respectively, of pTgf2-Mlyz2-RFP that contained the zebrafish myosin light chain 2 (Mlyz2) gene promoter (Guo et al. 2013).

In vitro transfection and microinjection

Capped silver carp Thm3 transposase mRNA were synthesized in vitro using the mMESSAGE mMACHINE kit (Ambion, Austin, TX) using NotI linearized pc2-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 one- to two-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'-GATGGAGGGCTCCGTGAAG-3' and RFP-r, 5'-GTACCTAGATCTCAAGAAGA-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). For splinkerette PCR, 25 μg genomic DNA was digested for 12–16 hr at 37°C with 80 units 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 transposon-genome junction. The nested primers for the 5' flanking sequences were 5'-AGGTGTCGCGCCTCATATGACACAGGCGT-3' and 5'-ATCAATTAACCTATCGAGCTTCTT-3'. The nested primers for the 3' flanking sequences were 5'-TAAGAGATTTGATGATCTAATCTCTGACTTTTG-3' and 5'-TACCTCGCTGATGATCTGAGCATTTAACA-3'. The amplified fragments were cloned into the pMD19-T vector (TaKaRa, Dalian, China) and transformed into DH5α E. coli cells, and the positive clones were examined by PCR and direct sequencing.

Data availability

Figure S1 contains supporting figures. Further information on all plasmids and more detailed data are available upon request.

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 comprising 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 10 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 numbers (JQ782178–JQ782179). Previous GenBank accession numbers (KJ742716–KJ742730). Because brown trout Tbt1 and Tbt2 cloned from the samples collected in 2014 had >99% similarities to the sequences identified in 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 bootstrap value (Figure 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 Figure 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 Adrianichthys and the common angelfish Tpc1 from Cichlidae. Even for the isolated 13 Tc1-like sequences from seven species of the Cyprinidae family, their phylogenetic relationship is patchy clustering (Figure 1A). This means that these Cyprinidae
Tc1-like sequences are not in line with the vertical evolutionary relationships (Figure 1B). By pairwise comparisons, Tc1-like transposons of the same size (1473 bp) isolated from grass carp (Tci2), common carp (Tcc2), crucian carp (Tca2), and brown trout (Tbt2) shared ~99% identity to quite distantly related groups (Figure 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 are 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, which 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 (Figure 3). Our further Southern blot analyses reveal that there are only three to five copies of the Thm3 transposon in the silver carp genome (Figure 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).

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 (Figure 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 (Figure 3). Our further Southern blot analyses reveal that there are only three to five copies of the Thm3 transposon in the silver carp genome (Figure 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 fish PPTN, respectively (Figure 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 complete DD(34)E domains in the C-terminal (Figure 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 (Figure 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 nonautonomous 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 (Figure 6A). In addition, the pCS2-CMV-Thm3TP plasmid was constructed and used to *in vitro* synthesize capped RNA encoding 335 aa residues Thm3 transposase (Figure 6B). We then coinjected 50 pg of donor plasmid DNA with 50 pg of capped RNA into the embryos (one-to-two-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 hr postfertilization (hpf), indicative of tissue-specific expression by the zebrafish myosin light chain 2 (Mlyz2) promoter (Figure 6, C and D). In 200-d-old adult blunt snout bream, the red color could be observed in the dorsal skeletal muscles of transgenic individuals (Figure 6, E and F). PCR analysis of the transposition rate of RFP at adult blunt snout bream was performed as previously described (Jiang et al. 2012). The integration rate of RFP gene into the blunt snout bream genome when fish were 200 d 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% of embryos showed weak and high rates of the mosaic expression of RFP and few fish expressed RFP at the 200-d-old stage (data not shown). Moreover, PCR analysis showed that 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. Because the endogenous Tc1-like element *Tma2* was also present in the genome of blunt snout bream (Figure 1, Table 1), its similarity with sequences of the left-end (367 bp) and right-end (230 bp) of silver carp *Thm1* were 54% and 65%, respectively (supporting information, Figure S1). To avoid false positives, we chose low-similarity (<50%) regions to design the nested primers to clone the 5' or 3' flanking sequences (Figure S1). Among five RFP-transgenic positive individuals, there were one to five RFP-transgenic positive individuals, there were one to...
four genomic integration sites in the genome of the blunt snout bream, including the specific end sequences of Thm1 DNA (Table 3; Figure S1), whereas no PCR products during splinkerette PCR were amplified in the 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 that 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 Thm 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; Izsák 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 acid Thm3 transposase and terminal inverted repeats (ITRs) from silver carp nonautonomous element Thm1. 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.

#### Table 2 The transposition efficiencies of silver carp Thm transposon systems in 200-d-old blunt snout bream by PCR analysis

| Batches | No. of Survival Injection Individuals | No. of Individuals Integrated RFP | Integration Rates (%) |
|---------|--------------------------------------|----------------------------------|-----------------------|
| 1       | 66                                   | 31                               | 47.0                  |
| 2       | 147                                  | 64                               | 43.5                  |
| 3       | 121                                  | 72                               | 59.5                  |
| 4       | 87                                   | 41                               | 47.1                  |
| 5       | 212                                  | 107                              | 50.5                  |
| 6       | 187                                  | 96                               | 51.3                  |
| 7       | 195                                  | 108                              | 55.4                  |
| Average |                                      | 92                               | 50.6*                 |
| Control 1 |                                    | 115                              | 4.3                   |
| Control 2 |                                    | 69                               | 3                    |
| Average  |                                      | 92                               | 5.0                   |

Control is injected pThm1-Mlyz2-RFP plasmid only.

*P < 0.01.
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 to 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 1473-bp 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 (Figure 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 and 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 unautonomous 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, whereas the Tip2 transposon exists at approximately 4000 copies per haploid genome (Nandi et al. 2007), with 32,000 copies of the nonautonomous element Tipnon present (Liu et al. 1999). However, in the silver carp genome, our results showed that low numbers of three to five copies of Thm3 transposon 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

### Table 3 Exogenous Thm1 end regions and the surrounding transposon insertion site sequences in the genome of 200-d-old blunt snout bream

| Sample No. | Copies | Sequence of Target Integration Sites |
|------------|--------|-------------------------------------|
| 1          | i      | (143nt)GTACCTTTGATACGTT—AACTTGGTA TACAGCATT(11nt) |
|            | ii     | (180nt)CAGACTCTCTCACTGTT—AACTGTA TACGTCATT(92nt) |
|            | iii    | (75nt)ACATATATGTACGTT—AACTGTA TAAATGCTC(138nt) |
|            | iv     | (85nt)CCTTGGTATACGTT—AACTGTA TTTAAATGG(175nt) |
| 2          | i      | (92nt)TTATGACCTGATACGTT—AACTGTA TTATTACATATA(127nt) |
|            | ii     | (38nt)TAAGCTGTTACGTT—AACTGTA CGCCAAAC(95nt) |
| 3          | i      | (28nt)AACTGCTCTACGTT—AACTGTA ACTGACATT(134nt) |
|            | ii     | (73nt)GGTTGTCGTACGTT—AACTGTA CCTGACTA(117nt) |
|            | iii    | (92nt)CGCTAAGGTACGTT—AACTGTA ATGTACGCG(83nt) |
| 4          | i      | (177nt)GGATAGGTGTACGTT—AACTGTA TGAGTGAA(76nt) |
| 5          | i      | (58nt)AGACTGTGTAACGTT—AACTGTA GACGATTT(154nt) |
|            | ii     | (14nt)GTAGGGTTGTAACGTT—AACTGTA CGGTAAAT(217nt) |
|            | iii    | (73nt)GGTATAGGTACGTT—AACTGTA GTGTACGCG(121nt) |

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 11 RFP-transgenic positive blunt snout bream crossed with the opposite sex of wild-type

| ID of RFP-Transgenic Positive Fisha | Gender | No. of F1 Embryos Examined | No. of RFP Positive Embryos | RFP Positive/Total F1 (%) |
|-----------------------------------|--------|-----------------------------|-----------------------------|--------------------------|
| 690000116601632                   | Female | 89                          | 57                          | 64                       |
| 690020042302853                   | Female | 254                         | 123                         | 48                       |
| 690000116602112                   | Male   | 275                         | 40                          | 15                       |
| 690020042302431                   | Male   | 212                         | 27                          | 13                       |
| 690002042302497                   | Female | 238                         | 38                          | 16                       |
| 690020042303065                   | Male   | 152                         | 78                          | 51                       |
| 690020042303056                   | Male   | 96                          | 55                          | 57                       |
| 690020042302805                   | Female | 321                         | 0                           | 0                        |
| 690020042303004                   | Female | 186                         | 27                          | 15                       |
| 69000116601753                   | Female | 141                         | 51                          | 36                       |
| 690000116602026                   | Male   | 311                         | 50                          | 16                       |
| Controlb                         | —      | 215                         | 0                           | 0                        |

* The IDs of RFP-transgenic positive fish were labeled with passive integrated transponder (PIT) tags (Hongteng Barcode Corporation, Guangzhou).

* The wild-type female mating with male was used as control.
intact functional domains and 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 because 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 aa 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 Tana1 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 injection of donor plasmid DNA. We have shown that the silver carp bream as an alternative to methods of transgenesis involving the in

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LITERATURE CITED

Ahn, S. J., M. S. Kim, J. H. Jang, S. U. Lim, and H. H. Lee, 2008 MMTS, a new subfamily of Tc1-like transposons. Mol. Cells 26: 387–395.

Barry, E. G., D. J. Witherspoon, and D. J. Lampe, 2004 A bacterial genetic screen identifies functional coding sequences of the insect mariner transposable element Fumari1 amplified from the genome of the earwig, Forficula auricularia. Genetics 166: 823–833.

Castaneda, J., P. Genez, and A. Bortvin, 2011 piRNAs, transposon silencing, and germline genome integrity. Mutat. Res. 714: 95–104.

Clark, K. J., D. F. Carlson, M. J. Leaver, L. K. Foster, and S. C. Fahrenkrug, 2009 Passport, a native Tc1 transposase from flatfish, is functionally active in vertebrate cells. Nucleic Acids Res. 37: 1239–1247.

Craig, N. L., 1995 Unity in transposition reactions. Science 270: 253–254.

Craig, N. L., R. Craige, M. Gellert, and A. M. Lambowitz, 2002, pp. 12–23 in Mobile DNA II, ASM Press, Washington, DC.

Davidson, W. S., B. F. Koop, S. J. Jones, P. Utrera, R. Vidal et al., 2010 Sequencing the genome of the Atlantic salmon (Salmo salar). Genome Biol. 11: 403.

de Boer, J. G., R. Yazawa, W. S. Davidson, and B. F. Koop, 2007 Bursts and horizontal evolution of DNA transposons in the speciation of pseudotetraploid salmonids. BMC Genomics 8: 422.

Eide, D., and P. Anderson, 1985 Transposition of Tc1 in the nematode Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA 82: 1756–1760.

Emmons, S. W., and L. Yesner, 1984 High-frequency excision of transposable element Tc1 in the nematode Caenorhabditis elegans is limited to somatic cells. Cell 36: 599–605.

Feschotte, C., and E. J. Pritham, 2007 DNA transposons and the evolution of eukaryotic genomes. Annu. Rev. Genet. 41: 331–368.

Franz, G., and C. Savakis, 1991 Minos, a new transposable element from Drosophila hydei, is a member of the Tc1-like family of transposons. Nucleic Acids Res. 19: 6646.

Gallardo-Galvez, J. B., T. Mendez, J. Bejar, and M. C. Alvarez, 2011 Endogenous transposases affect differently Sleeping Beauty and Frog Prince transposons in fish cells. Mar. Biotechnol. (NY) 13: 695–705.

Garrels, W., S. Holler, U. Taylor, D. Herrmann, H. Niemann et al., 2014 Assessment of fetal cell chimerism in transgenic pig lines generated by sleeping beauty transposition. PLoS One 9: e96673.

Gilbert, C., S. Schack, J. K. Pace, P. J. Brindley, and C. Feschtte, 2010 A role for host-parasite interactions in the horizontal transfer of transposons across phyla. Nature 464: 1347–1350.

Guo, X., C. Huang, R. Shen, X. Jiang, J. Chen et al., 2013 Insertion efficiency of Tgfl2 transposon in the genome of Megaleobrama amblycephala. Hereditas 35: 999–1006.

Guo, X., F. Li, X. Jiang, and S. Zou, 2014 Evolution analysis of Tc1-like transposon in Salmo trutta fario genome. J. Shanghai Ocean Univ. 23: 15–21.

Hall, T.A., 1999 BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41: 95–98.

Hickman, A. B., Z. N. Perez, L. Zhou, P. Musingarimi, R. Ghirlanda et al., 2005 Molecular architecture of a eukaryotic DNA transposase. Nat. Struct. Mol. Biol. 12: 715–721.

Ivics, Z., P. B. Hackett, R. H. Plasterk, and Z. Iszvav, 1997 Molecular re-construction of Sleeping Beauty, a Tc1-like transposase from fish, and its transposition in human cells. Cell 91: 501–510.

Izsvak, Z., Z. Ivics, and P. B. Hackett, 1995 Characterization of a Tc1-like transposable element in zebrafish (Danio rerio). Mol. Gen. Genet. 247: 312–322.

Izsvak, Z., Z. Ivics, and R. H. Plasterk, 2000 Sleeping Beauty, a wide host-range transposon vector for genetic transformation in vertebrates. J. Mol. Biol. 302: 93–102.

Janicki, M., R. Rooke, and G. Yang, 2011 Bioinformatics and genomic analysis of transposable elements in eukaryotic genomes. Chromosome Res. 19: 787–808.

Jiang, X., X. Du, Y. Tian, R. Shen, C. Sun et al., 2012 Goldfish transposase Tgfl2 presumably from recent horizontal transfer is active. FASEB J. 26: 2743–2752.

Kawakami, K., H. Takeda, N. Kawakami, M. Kobayashi, N. Matsuda et al., 2004 A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. Dev. Cell 7: 133–144.

Kazazian, H. H., 2004 Mobile elements: drivers of genome evolution. Science 303: 1626–1632.

Kidwell, M. G., and D. R. Lisch, 2000 Transposable elements and host genome evolution. Trends Ecol. Evol. 15: 95–99.

Koga, A., and H. Hori, 2000 Detection of de novo insertion of the medaka fish transposable element Tol2. Genetics 156: 1243–1247.

Koga, A., A. Shimada, A. Shima, M. Sakaiizumi, H. Tachida et al., 2000 Evidence for recent invasion of the medaka fish genome by the Tol2 transposable element. Genetics 155: 273–281.

Kotani, T., and K. Kawakami, 2008 Misty somites, a maternal effect gene identified by transposon-mediated insertional mutagenesis in zebrafish that is essential for the somite boundary maintenance. Dev. Biol. 316: 383–396.

Kuraku, S., H. Qiu, and A. Meyer, 2012 Horizontal transfers of Tc1 elements between teleost fishes and their vertebrate parasites, lampreys. Genome Biol. Evol. 4: 929–936.

Lampe, D. J., M. E. Churchill, and H. M. Robertson, 1996 A purified mariner transposase is sufficient to mediate transposition in vitro. EMBO J. 15: 5470–5479.

Leaver, M. J., 2001 A family of Tc1-like transposons from the genomes of fishes and frogs: evidence for horizontal transmission. Gene 271: 203–214.

Liu, D., C. You, S. Liu, L. Liu, W. Duan et al., 2009 Characterization of a novel Tc1-like transposon from bream (Cyprinidae, Megalobrama) and
its genetic variation in the polyploidy progeny of bream-red crucian carp crosses. J. Mol. Evol. 69: 395–403.

Liu, Z. J., P. Li, H. Kucuktas, and R. A. Dunham, 1999 Characterization of nonautonomous Tc1-like transposable elements of channel catfish (Ictalurus punctatus). Fish Physiol. Biochem. 21: 65–72.

Lohe, A. R., E. N. Moriyama, D. A. Lidholm, and D. L. Hartl, 1995 Horizontal transmission, vertical inactivation, and stochastic loss of mariner-like transposable elements. Mol. Biol. Evol. 12: 62–72.

McCIntock, B., 1953 Induction of instability at selected loci in Maize. Genetics 38: 579–599.

Medhora, M., K. Maruyama, and D. L. Hartl, 1991 Molecular and functional analysis of the mariner mutator element Mos1 in Drosophila. Genetics 128: 621–632.

Miskey, C., Z. Izsvak, R. H. Plasterk, and Z. Ivics, 2003 The Frog Prince: a reconstructed transposon from Rana pipiens with high transpositional activity in vertebrate cells. Nucleic Acids Res. 31: 6873–6881.

Nandi, S., E. Peatman, P. Xu, S. Wang, P. Li et al., 2007 Repeat structure of the catfish genome: a genomic and transcriptomic assessment of Tc1-like transposon elements in channel catfish (Ictalurus punctatus). Genetica 131: 81–90.

Nelson, J. S., 2006 Fishes of the World, Ed. 4th. Wiley, New York.

Nylander, J. A., F. Ronquist, J. P. Huelsenbeck, and J. L. Nieves-Aldrey, 2004 Bayesian phylogenetic analysis of combined data. Syst. Biol. 53: 47–67.

Pace, J. K., C. Gilbert, M. S. Clark, and C. Feschotte, 2008 Repeated horizontal transfer of a DNA transposon in mammals and other tetrapods. Proc. Natl. Acad. Sci. USA 105: 17023–17028.

Plasterk, R. H., Z. Izsvak, and Z. Ivics, 1999 Resident aliens: the Tc1/mariner superfamily of transposable elements. Trends Genet. 15: 326–332.

Pocierz-Kotus, A., A. Burzynski, and R. Wenne, 2007 Family of Tc1-like elements from fish genomes and horizontal transfer. Gene 390: 243–251.

Pujolar, J. M., L. Astolfi, E. Boscari, M. Vidotto, F. Barbisan et al., 2013 Tana1, a new putatively active Tc1-like transposable element in the genome of sturgeons. Mol. Phylogenet. Evol. 66: 223–232.

Radice, A. D., B. Bugaj, D. H. Fitch, and S. W. Emmons, 1994 Widespread occurrence of the Tc1 transposon family: Tc1-like transposons from teleost fish. Mol. Gen. Genet. 244: 606–612.

Robertson, H. M., and K. L. Zumpano, 1997 Molecular evolution of an ancient mariner transposon, Hsmar1, in the human genome. Gene 205: 203–217.

Rubin, G. M., and A. C. Spradling, 1982 Genetic transformation of Drosophila with transposable element vectors. Science 218: 348–353.

Sambrook, J., E. F. Fritsch, and T. Maniatis, 1989 Molecular Cloning: A Laboratory Manual, Ed. 2. New York, Cold Spring Harbor, USA.

Swofford, D., 2003 PAUP: Phylogenetic analysis using parsimony and other methods, Version 4, Handbook and Software. Sunderland, M. A., Sinauer Associates, USA.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins, 1997 The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25: 4876–4882.

Venkatesh, B., M. V. Erdmann, and S. Brenner, 2001 Molecular synapomorphies resolve evolutionary relationships of extant jawed vertebrates. Proc. Natl. Acad. Sci. USA 98: 11382–11387.

Wang, Q., P. Ji, P. Xu, and X. Sun, 2011 Identification and characterization of a novel Tc1-like transposon in the Cyprinus carpio genome. J. Fish. Sci. China 18: 1392–1398.

Wang, X., J. Li, and S. He, 2007 Molecular evidence for the monophyly of East Asian groups of Cyprinidae (Teleostei: Cypriniformes) derived from the nuclear recombination activating gene 2 sequences. Mol. Phylogenet. Evol. 42: 157–170.

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