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Differential expression of a retrotransposable element, *Rex6*, in *Colossoma macropomum* fish from different Amazonian environments

Cassiane Martins Barbosa[1], Edson Assunção Mareco[1], Maeli Dal Pai Silva[1], Cesar Martins[1], and Fernanda Antunes Alves-Costa[2,*]

[1]UNESP—São Paulo State University (Universidade Estadual Paulista); Instituto de Biociências de Botucatu; Departamento de Morfologia; Botucatu, SP, Brazil;
[2]UNIP—University of São Paulo (Universidade Paulista); Instituto de Ciências da Saúde; Bauru, SP, Brazil

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Transposable elements (TEs) are DNA sequences that have the ability to move and replicate within the genomes. TEs can be classified according to their intermediates of transposition, RNA (retrotransposons) or DNA. In some aquatic organisms, it has been observed that environmental factors such as pH, temperature and pollution may stimulate differential transcription and mobilization of retrotransposons. In light of this information, the present study sought to evaluate the expression of *Rex6* TE transcripts in *Colossoma macropomum*, which is a very commercially exploited fish in Brazil. In order to establish a comparative analysis using real-time PCR, the samples were collected from Amazonian rivers with different physical and chemical characteristics (distinguished by clear water and black water). Quantitative RT-PCR analyses revealed a differential pattern of expression between tissues collected from different types of water (clear and black waters). When it came to the hepatic and muscle tissues sampled, the levels of *Rex6* transcripts were significantly different between the two Amazonian water types. These results suggest that environmental conditions operate differently in the regulation of *Rex6* transcription in *C. macropomum,* results which have implications in the reshaping of the genome against environmental variations.

Introduction

The Amazon basin is considered the largest drainage system in the world at approximately 700,000 km^2, and it is composed of approximately 7,000 tributaries.[1] Throughout this large area, very different environments exist, including different water types.[2]

Based on physical and chemical factors, a classification system of the Amazonian waters has been suggested. This system distinguishes between white, clear and black waters.[3] White water presents a high concentration of minerals, a neutral pH (6.5 to 7.0) and high conductivity. Clear water presents variable pH (4.5 to 7.0) and relatively low conductivity. Finally, black water presents a high concentration of humic acids, which result in dark hues and acidic pH levels. Black water also has a low concentration of minerals and an absence of calcium and magnesium ions.[4]

In spite of the environmental variations found among the types of Amazonian waters, some fish species, such as *Colossoma macropomum*, may be found in all of these environments.[5-7] This species occupies a prominent position in Brazilian fishery and aquaculture, and it is known for its features such as the flavor of the meat, their large body size, and their easy adaptation to captivity.[5,8,9]

In general, some factors such as disease, pollution, temperature change and other environmental adversities that expose an organism to stress lead to the mobilization of transposable elements (TEs).[10-14] Transposable elements (TEs) are DNA sequences that have the intrinsic ability to move and replicate within genomes. They share characteristics such as promotion of duplication, polymorphism of insertion sites, and copy number variability within and between species.[15] TEs can be classified according to their transition intermediates: RNA (Class I or retrotransposons) or DNA (Class II or DNA transposons). TE mobilization is subject to a complex set of regulatory mechanisms that involve protein encoded not only by the transposon, but also by the transposon hosts.[16]

In recent decades, several studies have revealed the extent of the participation of the environment in the expression and/or activation of retrotransposons in studies on various organisms such as plants,[11,17] insects of the genus *Drosophila*,[18] shrimp,[14] fish[19] and mice.[20,21] Fish have a particularly large number of active retrotransposons, and many are unique to this group.[22,23] Thus, fish are an important model for studies on the evolution, dynamics, and especially the influences of the environment on retrotransposon expression. Even so, studies involving the expression of retrotransposons in fish are scarce.

*Correspondence to: Fernanda Antunes Alves-Costa; Email: fa_alves2003@yahoo.com.br
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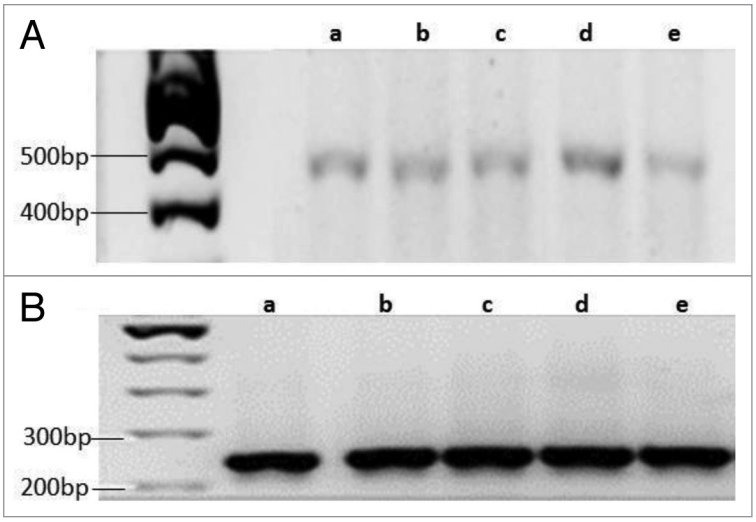

Figure 1. cDNA amplification patterns of *Colossoma macropomum*, visualized in 1.5% agarose gel, obtained through the use of the primer sets *Rex6* F1, *Rex6* R1 (**A**) and 18S1, 18S2 (**B**), and different tissues samples from the gills (**a**), liver (**b**), skeletal muscle (**c**), kidney (**d**), and brain (**e**). Left: 1 Kb DNA ladder.

Some retrotransposable elements such as *Rex1, Rex3* and *Rex6* seem to be abundant among teleosts,[24,25] and they are being widely investigated in fish in order to better understand the evolution, structural characterization, and location in the chromosomes, but there is still no data available regarding the transcription and the mechanisms regulating the expression of these elements in these organisms.[26-31] The *Rex* family corresponds to non-LTRs repetitions that have remained active during the evolution of different fish species. *Rex1* and *Rex3* encode reverse transcriptases and apurinic/apyrimidinic endonucleases, which are required for cleavage of the target sequence. *Rex6* also encodes a reverse transcriptase, as well as an endonuclease, which is similar to a restriction enzyme (REL).[27]

Considering the ecological and commercial importance of *C. macropomum,* as well as the fact this species evidence physiological traits in the different clear and black water environments,[32] the study about differential *Rex6* expression patterns may reveal important data on the potential influence of different natural conditions on expression of this retrotransposon in fishes. Thus, the present study sought to evaluate the expression of *Rex6* transcripts in different tissues of *C. macropomum*, and then to develop a comparative analysis using samples collected from different Amazonian waters (clear and black waters). Based on the idea that different environments could generate differential patterns of expression of *Rex6* transcripts, the analysis of expression levels between samples from clear and black waters may represent the first step to further investigation using controlled experiments related to *Rex6* expression.

Results

Identification of *Colossoma macropomum Rex6* transcripts and sequence analysis

The primer sets, which were used in RT-PCR reactions for *Rex6* and *18S rRNA* amplifications, yielded fragments in all tissues sampled that were approximately 450 base pairs (bp) and 230 bp in size, respectively (**Fig. 1A and B**).

Nucleotide sequencing of selected fragments from *C. macropomum* tissues, which had been obtained with rRNA 18S1 and 18S2 primers, allowed for the characterization of a distinct fragment with 197 bp. BLAST searches of GenBank at the National Center for Biological Information (NCBI) indicated that the isolated fragment presented a high identity (95%) to the 18S rRNA sequences of other fish species, including *Leiopotherapon unicolor* (HQ615553.1), *Philypnodon macrostomus* (HQ615580.1), *Porochilus rendahli* (FJ710880.1), *Tandanus tandanus* (FJ710890.1), and *Ictalurus punctatus* (GQ465834.1).

RT-PCR products were obtained with the primers *Rex6* F1 and *Rex6* R1 from different tissues of *C. macropomum*. The nucleotide sequencing revealed a 348 bp fragment. Database searches for nucleotide similarity revealed that a 31 bp fragment of this the nucleotide sequence was highly conserved relative to another retrotransposon *Rex6* fragment that has been described in the fish species, *Oryzias latipes* (AB465503.1). The qRT-PCR primers were designed for the amplification of this specific region.

Quantification of *Rex6* transcript levels

Gills, liver, skeletal muscle, kidney and brain tissues collected from *C. macropomum* in black water were processed for qRT-PCR analysis in order to analyze differences in *Rex6* transcript levels among these tissues. Gills, liver and skeletal muscle tissues collected from *C. macropomum* in clear water were processed for qRT-PCR analysis in order to compare the differences in *Rex6* transcript levels of these tissues from the two water types. Real Time PCR data was recorded as an expression of the different tissues and water types relative to the calibrator sample, normalized to 18S rRNA expression values.

The presence of *Rex6* transcripts in all *C. macropomum* tissues sampled indicated that this transcript is widely distributed among the tissues of this species. However, the quantification of the relative expression of *Rex6* transcripts revealed a differential pattern of expression among the tissues sampled from *C. macropomum* (**Fig. 2**). Significantly higher levels of *Rex6* transcripts were found in gill and kidney tissues, and lower levels of these transcripts were found in liver and brain tissues. In skeletal muscle tissues, the levels of *Rex6* transcripts did not differ from the gill and kidney *Rex6* transcripts levels, but the variation in muscle tissue was considerably lower than in the other sampled tissues (**Fig. 2**).

When the levels of *Rex6* transcripts in gill, liver and skeletal muscle tissues of *C. macropomum* from different Amazon environments (clear and black waters) were compared, a differential pattern of relative expression was found. The gill tissue was found to have high levels of *Rex6* transcripts in both environments, and no statistical difference was found between these levels when the specimens from the two water types were compared (**Fig. 3**). *Rex6* transcripts isolated from skeletal muscle and liver tissue of

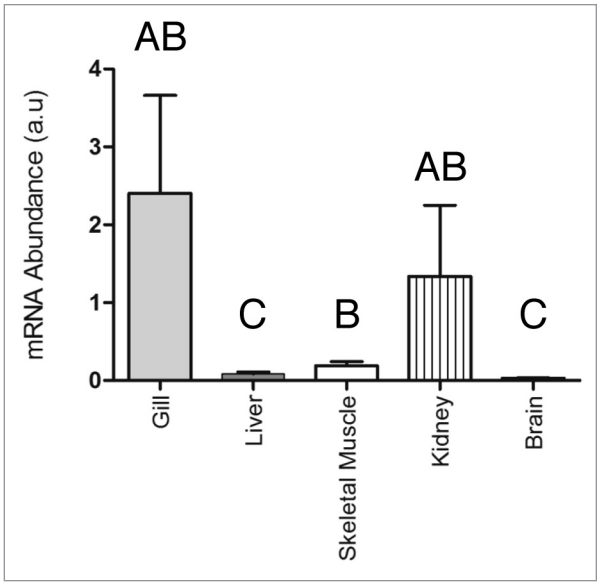

Figure 2. Relative *Rex6* retrotransposon expression, in arbitrary units, of diferent tissues of *C. macropomum*. Data are expressed as Mean ± SDM. (**A–C**): *same letters*, no statistical difference; *differente letters*, statistical difference ($P < 0.05$).

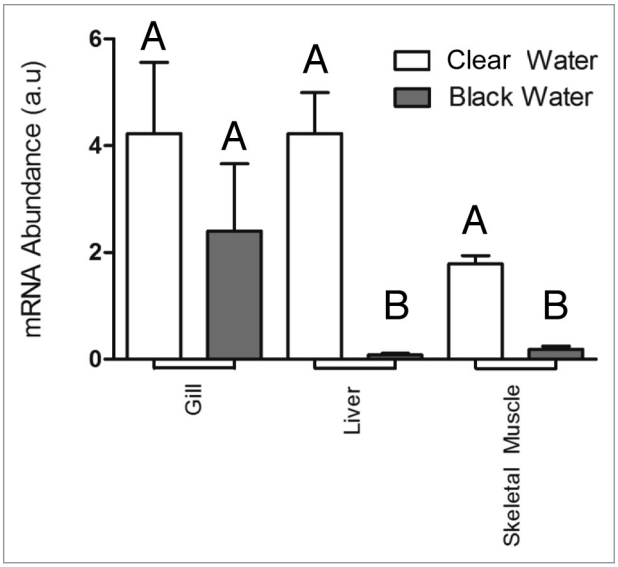

Figure 3. Comparative data on relative *Rex6* retrotransposon expression of diferent tissues of *C. macropomum,* collected from different water types. The data is expressed as as Mean ± SDM, in arbitrary units. (**A and B**): *same letters*, no statistical difference; *difference letters*, statistical difference ($P < 0.05$).

C. macropomum were found to have statistically significant differences between the relative expression levels of this retrotransposon ($P = 0.0118$) when the two Amazon environments (clear and black waters) were compared (**Fig. 3**).

Despite the increased *Rex6* expression in gill tissue in fish from both water types (clear and black waters), the variations in the values obtained from this tissue were also higher compared with those of the liver and muscle tissues (**Fig. 3**).

Discussion

This study is the first report on analyses of retrotransposon expression in *C. macropomum* (a fish species of great economic and ecological importance), and it has addressed the differential expression of the *Rex6* between different Amazonian environments. The results support hypotheses about the possible influence of the environment on the regulation of expression of this retrotransposon.

Among the *Rex* retrotransposon family, *Rex6* is the most rarely studied of the elements, and the GenBank at NCBI shows a limited amount of data on the transcript sequence of this transposable element, a fact that is reflected by the low number of similar sequences available. Additionally, the low similarities found among *Rex6* sequences may be due to major changes in its nucleotide sequence. A previous study suggests that this retrotransposon has undergone a significant divergence, which has led to several processes of segment loss or horizontal transfer mechanisms, and is argued these changes may be causing the low levels of similarity among the nucleotide sequences of *Rex6* and may also explain the absence of these sequences in some vertebrate groups, such as in the human genome.[28]

The relative expression of *Rex6* in brain tissue, sampled from black water, showed the lowest levels of transcripts, compared with the levels of *Rex6* transcripts in gill, liver, skeletal muscle and kidney, sampled in the same type of water. The work of differential expression *Rex6*, particularly in brain tissues of fishes, are scarce, but the data suggest that the factors influencing the expression of this retrotransposon in gill, liver, muscle and kidney tissues, acted differently in the brain, which may be related to the influences of several sets of metabolites and hormones that act in this tissue. The results of relative expression of *Rex6* in different tissues of *C. macropomum* from different Amazonian environments suggest that the environment is an important factor in the regulation of the expression of *Rex6*. This result implies that different environmental factors between clear and black waters may be promoting the unequal transcription of *Rex6* in the skeletal muscles and livers of *C. macropomum*.

Environmental changes such as contamination, temperature alterations, hypoxia and other changes that represent an environmental stress condition may stimulate the transcription of retrotransposons. In aquatic organisms such as shrimp and fish, water conditions seem to be determinant in the differential expression of retrotransposons.[11] As an example, retrotransposons were being differentially expressed in the shrimp *Penaeus monodon* when specimens were exposed to different temperature and osmolality conditions and different oxygen concentrations in the water, all of which simulated situations of environmental stress.[14]

Black water in Amazonian rivers is characterized by a lack of nutrients, little sunlight penetration, a lack of aquatic plants, a low concentration of mineral salts, and a high incidence of small-sized fish species.[4,33-35] These features may represent stress conditions for the fish species that are commonly found in this environment, but expression data was inconclusive after this attempt to detect a

decrease in the levels of *Rex6* transcripts in hepatic and muscular tissues of *C. macropomum* from black water.

Studies on the population structures of several fish species found in black water environments show that there are approximately 40 species of miniature fish that reach sexual maturity at greatly reduced sizes, and this phenomenon has been associated with a low concentration of nutrients in these waters.[34,36] The lower expression of *Rex6* in skeletal muscle of *C. macropomum* in this type of water may be associated with mechanisms of skeletal muscle growth; thus, it may be inferred that this retrotransposon exerts some kind of regulatory effect during the processes of fish muscle growth. The results of the present study show that the differential expression of *Rex6* in skeletal muscle may be related to environmental factors, a finding which provides important data for studies on fish muscle growth and the participation of transposable elements in the regulation of muscle development. Interestingly, *C. macropomum* from black water showed a decrease in relative expression of factors that regulated muscle growth, including *myoD, myogenin, myf5* and *mrf4*.[32] So far, however, there are no reports that address this issue in the literature, and further studies need to be developed to validate and characterize the association between *Rex6* and molecular skeletal muscle growth control.

The *Rex6* expression in gills resulted in high levels of this transcript in both aquatic environments. These results may represent the greater susceptibility of this tissue to environmental changes and, consequently, this tissue may receive more stimuli for the transcription of *Rex6*.

The increased expression and subsequent mobilization of retrotransposons may result in changes to the expression of flanking genes. Thus, retrotransposons may play a role in the evolution of gene functions.[37] From this perspective, *Rex6* may be related to processes that have allowed this species to better adapt to different Amazonian environments.

In addition to expanding the knowledge on both the genetics of this fish species and transposable elements, the data obtained may contribute to future studies that can lead to a better understanding the mechanisms of interference involved in the environmental regulation of the expression of these elements. A series of controlled experiments to tease out pH, minerals and oxygen concentrations, will be an interesting and useful issue in strengthening the present results, and represent the next step of this investigation approach.

A better understanding of the dynamics, control and action of transposable elements may aid in the insertion and selection of sequences in the genome of fishes, the attainment of transgenic species and the identification of transposable elements and their application to fish farming.[38]

Materials and Methods

Animal samples

A total of 20 adult specimens of *Colossoma macropomum* (Characiformes, Characidae) were obtained from private fishery stations in the Brazilian States of Pará and Amazonas (Costa do Tapará Fish Farm—city of Santarém, Pará, Brazil; and Fazenda Santo Antônio Fish Farm, city of Rio Preto da Eva, Amazonas, Brazil), where they had been kept under the same temperature and feeding conditions. The fishery stations, Costa do Tapará and Fazenda Santo Antônio, use water from two different rivers: the Tapajós River (clear water) and the Urubu River (black water), respectively. The photoperiod and temperature were the same in both conditions because the fish were collected during the same time of the year, and thus experienced similar natural conditions. The pH of the water systems were 6.0 to 7.0 for clear water and 4.0 to 5.0 for black water.

Fish specimens were euthanized using benzocaine (0.1 gL^{-1}), and the gills, liver, skeletal muscle, kidney and brain tissues of the animals from the black water, and the gills, liver and skeletal muscle of the animals from the clear water were collected and immediately stored at –80 °C until RNA extraction.

All experiments were approved by Ethics Committee of the Bioscience Institute, São Paulo State University (UNESP) (Protocol nº 178-CEEA).

RNA isolation and Reverse Transcription

Approximately 100 mg of the tissue samples were mechanically homogenized with 1 mL of TRizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and total RNA extraction was performed following the manufacturer's protocol. Total RNA samples were incubated with DNase I—Amplification Grade (Invitrogen Life Technologies, Carlsbad, CA, USA) to remove DNA contamination. RNA samples were eluted in RNase-free water, small aliquots of each sample were loaded into agarose gel to check for RNA integrity, and the samples were then quantified (Thermo Scientific NanoDrop 1000 Spectrophotometer) by measuring the optical density (OD) at 260 nm. RNA purity was ensured by obtaining a 260/280 nm OD ratio ≥ 1.80.

Two micrograms of total RNA were reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), and the reaction was characterized as 10 μL of reverse transcriptase buffer (10× RT Buffer), 4 μL of dNTP (25×), 10 μL of Randon Primers (10×), 2.5 μL of MultiScribe Reverse Transcriptase (50 U/μL), and 2.5 μL of Recombinant Ribonuclease Inhibitor RNaseOUT (40 U/μL).

RT-PCR, Sequencing and Sequence analysis

The cDNA samples were amplified using primer pairs specific to the amplification of 18S rRNA genes (18S1: 5′-TACCACATCC AAAGAAGGCA G-3′; 18S2: 5′-TCGATCCCGA GATCCAACTA C-3′)[39] and the retrotransposon *Rex6* (*Rex6* F1: 5′-AGGAACATGT GTGCAGAATA TG-3′; *Rex6* R1: GGTGGTTTCT CCTCCAAGCT CG).[31] The constitutively expressed gene for 18S rRNA was used as a positive control to assay the integrity of RNA extracted from each tissue. Each cDNA amplification reaction consisted of 0.2 μg of cDNA (10%), 0.2 mM of each primer, 25 mM of a $MgCl_2$ PCR buffer, 0.2 mM of dNTPs, and 0.2 unit of Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), with a final volume of 25 μL. The RT-PCR conditions were performed accordingly to the protocol previously described.[40] Amplification products (10 μL) were fractionated on 1.5% agarose gel, stained with Gel Red (Life Technologies, Carlsbad,

CA, USA),[41] and visualized under UV light (Hoefer UV-25). The molecular weight of the amplified fragments was assigned through a comparison with a 1 Kb DNA ladder (Invitrogen Life Technologies, Carlsbad, CA, USA).

RT-PCR products were purified and used in automated sequencing in an ABI 377 Automated DNA Sequencer (Applied Biosystems, Foster City, CA, USA) with a DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare Life Sciences, Piscataway, NJ, USA), following the manufacturer's instructions and using the same primers described previously. Nucleic acid sequence database searches were performed using BLAST/N on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast). Sequence alignments were obtained using Clustal-W function, and the consensus sequences were determined manually.[42,43]

Real-time PCR and statistical analysis

Quantitative RT-PCR (qRT-PCR) was performed using an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and a Power SYBER Green PCR Master Mix Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Standard reactions (25 μL) were assembled using 12.5 μL of Power SYBER Green PCR Master Mix 2× , 2 μL of each primer (5 μM), 2 μL of template cDNA treated with DNase I (Invitrogen Life Technologies, Carlsbad CA, USA) (20 ng/μL), and 6.5 μL of ultrapure water. Primers were designed using the Primer Express v.2.0 software (Applied Biosystems, Foster City, CA, USA) based on the previously obtained DNA sequences (*Rex6* F2: 5′-TGAGAGGGCA AAGATCCTGT-3′; *Rex6* R2: 5′-CTGGTTGGTT TGCCATTACC-3′). cDNA templates were 1:10 diluted, and cDNA samples were replaced by DEPC water in the negative controls. Real-time assays were performed in duplicate. Forty amplification cycles were performed, and each cycle was performed at 94 °C for 15 s, followed by 60 °C for 1 min. Amplification and dissociation curves generated by the 7300 System/Sequence Detection Software version 4.0 (Applied Biosystems, Foster City, CA, USA) were used for gene expression data analysis. The qRT-PCR signals were normalized to a segment of the 18S rRNA housekeeping gene using the primers 18S3 (5′-CGGAATGAGC GTATCCTAAA CC-3′) and 18S4 (5′-GCTGCTGGCA CCAGACTTG-3′), which had been designed based on consensus sequences of this gene described for several fish species.

Standard curves for target and reference genes, which had been created on the basis of a linear relationship between the Ct value and the log of the starting cDNA quantity, had acceptable slope values: between –3.8 and –3.3.[44] Standard curves were obtained using serial dilutions of cDNA samples.

The Ct values were used to calculate a relative gene expression value for each transcript according to the $2^{-\Delta\Delta Ct}$ method. Because this method was used, data was recorded as the fold-change transcript levels normalized with the reference gene and relative to the calibrator sample.[45]

The relative gene expression values were used in statistical analysis with the Kruskal-Wallis Test, which was supplemented by Student Newmwn-Keuls Test with pairs of multiple comparisons. In addition, Student's *t* test for normal distribution was applied to the data.[46] Differences were considered significant when $P < 0.05$, and a 95% confidence level of the difference was used.

The qRT-PCR products were cloned into a pGEM-T vector (Promega, Madison, WI, USA) and used to transform competent cells of the *E. coli* strain DH5α (Invitrogen Life Technologies, Carlsbad, CA, USA). Clones were purified and used in automated sequencing in an ABI 377 Automated DNA Sequencer (Applied Biosystems) with a DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare Life Sciences, Piscataway, NJ, USA) following the manufacturer's instructions and using primers complementary to vector arms. Nucleic acid sequence database searches were performed using BLAST/N on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast), which confirmed the retrotransposon *Rex6* fragment's identity.[42]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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