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Full Length Research Paper

Identification of retrotransposon-like sequences in Iranian river buffalo

Borhan Shokrollahi[1], Cyrus Amirinia[2]* and Navid Dinparast Djadid[3]**

[1]Department of Animal Science, Islamic Azad University, Sanandaj, Iran.
[2]Department of Biotechnology, Animal Science Research Institute of Iran, Karaj, Iran.
[3]Malaria and Vectors Research Group, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran.

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Retrotransposon elements are peculiar genetic elements raised through copy and paste mechanism by retrotransposition. Their ability to move and/or replicate inside the genome is an important evolutionary force responsible for the increase of genome size and the regulation of gene expression. In this paper, molecular identification of retrotransposon-like elements including seven LTR and non-LTR (LINE and SINE) like sequences, which were characterised by cloning RAPD fragments in Iranian river buffalo, is reported. The analysis demonstrated the presence of partial sequences of SINEs (MIRb, Bov-A2, Bov-tA2, CHR-2_BT and CHR-2B), LINE (L1_Carn7) and LTR (ERVL-B4) in the target genome. The sequences of Bov-tA2 and CHR-2 like elements contain the whole promoter boxes of RNA polymerase III and tRNA-related region with few differences in their nucleotides. This may occur by mutations and extinction of elements during evolution. The identification of these retrotransposable elements for the first time in Iranian river buffalo represents an important step towards the understanding of mechanisms of genome evolution within the species and perhaps will be useful in other related studies on population genetics, speciation and genome manipulation of this species.

Key words: Retrotransposon-like elements, SINE, LINE, Iranian river buffalo, ***Bubalus bubalis.***

INTRODUCTION

Mobile elements are interspersed repetitive DNA sequences with the unique ability to move and make copies within the genome they occupy. They often comprise between 40 to 90% of a genome (Waterston et al., 2002). Mobile elements can be divided into two classes: Class I includes retrotransposons and class II includes DNA transposons (Ray, 2007). Class I elements utilizes the copy and paste method termed retrotransposition. With these elements, original copy of DNA in the genome is first transcripted to mRNA. This transcript is then reverse transcripted and the resulting DNA inserted to a new location in a process termed target primed reverse transcription (TPRT) (Luan et al., 1993).

Retrotransposons can be divided into two main classes (Feschotte et al., 2002): LTR (long terminal repeat) and non-LTR elements. LTR elements, when still active, increase their copy numbers via copy and paste mechanism. Non-LTR retrotransposons can be divided into two subclasses including LINE (long interspersed element) and SINE (short interspersed elements). Both SINE and LINE, in spite of their differences in structure and transposition mechanism, are ubiquitous components in eukaryotic genomes. SINEs are defined by the presence of a region homologous to 7SL RNA and tRNA, together with the promoter sequences designated the A and B boxes (Piskurek et al., 2006). They represent non-autonomous transposable elements and exploit the enzymatic retrotransposition machinery of LINEs (Kajikawa and Okada, 2002). SINEs are present in more than 10^4

*Corresponding authors. *E-mail: amirinya@gmail.com. Tel: +98 2614439214. Fax: +98 261 4413258. **E-mail: navid@pasteur.ac.ir or ndinparastdjadid@yahoo.com. Tel: +98 21 66480780. Fax: +98 21 66465132.

Abbreviations: RAPD, Random amplified polymorphic DNA; **LINE,** long interspersed element; **SINE,** short interspersed elements; **TPRT,** target primed reverse transcription; **LTR,** long terminal repeat; **PCR,** polymerase chain reaction; **IPTG,** Isopropyl β-D-1-thiogalactopyranoside; **X-Gal,** 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; **LB,** lysogeny broth; **bp,** base pair; **BMF,** bovine monomer family; **BDF,** bovine dimer family; **tRNA,** transfer ribonucleic acid; **MIRs,** mammalian-wide interspersed repeats.

copies per genome in multicellular animals from invertebrates to mammals and are present in plants as well (Shedlock and Okada, 2000).

Since retrotransposable elements are abundant, ubiquitous and highly conserved, they have drawn much attention for the development of genetic diversity and mapping markers. Ray (2007) suggested that SINEs have applications in population structure, conservation genetics, the genetics of speciation, phylogeny reconstruction, inbreeding, estimates of ancestral population size, heterozygosity and agreement with the expectation of Hardy-Weinberg equilibrium. Applications of retrotransposons in the mentioned fields have been restricted to species that their genomes have been sequenced such as human. As additional genome sequences become available, the identification of polymorphic retrotransposons in related species in the frame of comparative studies becomes more applicable.

In ruminants, for example in cattle, several SINEs have been characterized including the Bsu family of SINEs (Philippsen et al., 1975), Bovine ***Alu***-like sequences; BMF (Bovine Monomer Family) and BDF (Bovine Dimer Family) (Watanabe et al., 1982), PstI sequences (Majewska et al., 1988), Bov-A and Bov-B SINE elements (Lenstra et al., 1993). The cattle and goat C family of SINE elements are derived from tRNA and contain complete RNA polymerase III promoter (A and B) boxes (Rogers, 1985). A group of artiodactyl SINEs that are structurally related to the ***Alu*** and B1 and B2 families has been found in the intervening sequences of the beta globin gene in goat, sheep, and cow (Schimenti and Duncan, 1984; Spence et al., 1985; Kramerov et al., 1979). Mammalian-wide interspersed repeats (MIRs) are abundant in almost all mammalian species studied including dog, cat, horse, cattle, donkey, kangaroo, etc. (Jurka et al., 1995). RTE-1 is a non-long terminal repeat (non-LTR) retrotransposon first identified in ***Caenorhabditis elegans*** and subsequently found in bovine and ovine genomes (Malik and Eickbush, 1998). SINE species from hippopotamus are also found in small amounts in most of the animal species in Artiodactyla (Nomura et al., 1998). Shimamura et al. (1999) reported the CHR (Cetacea, Hippopotamidae and Ruminantia) family of SINEs from the genome of whale and described their genealogical relationships among almost all the families of SINEs present in the genome of cetaceans and artiodactyls.

Although several families of SINEs have been characterized in some members of the Bovidae, namely cattle and goat, few had been identified in buffalo (Mayfield et al., 1980; Philippsen et al., 1975; Watanabe et al., 1982; Jobse et al., 1995; Rogers, 1985). Sheikh et al. (2002) has described PstI (I and II) in cattle, goat and buffalo. Nijman et al. (2002) found Bov-tA, Bov-A2 and Bov-B in river buffalo and some of other Pecoran ruminants.

There are few studies on identification of retrotransposon elements in ***bubalus bubalis***, with no published data on identification of these elements in populations of Iranian river buffalo. Therefore, the current study was designed in order for the identification of retrotransposon elements by cloning of RAPD fragments in Iranian river buffalo. This, in turn will provide a baseline data for further applied studies on population genetics, molecular systematic and genetic manipulation in this species.

MATERIALS AND METHODS

Retrotransposon-like elements in Iranian river buffalo were identified by cloning and sequencing of RAPD (random amplified polymorphic DNA) amplified fragments, followed by bioinformatic analysis and detection of microsatellite loci.

Sampling

Blood samples (n = 80) were collected from jugular vein of four populations of Iranian river buffalo in Guilan, Mazandaran, Azarbaijan and Khuzestan provinces.

DNA extraction

DNA was extracted via the salting out procedure reported by Miller et al. (1988). Briefly, nuclei were isolated from 1 - 2 tubes of blood, collected in EDTA tubes. After the addition of 9 volumes of buffer A (containing 0.32 M sucrose [109.5 g sucrose], 10 mM Tris HCl [10 ml of 1 M Tris-HCl, pH 7.6], 5 mM $MgCl_2$ [5 ml of 1M $MgCl_2$] and 1% Triton-100), they were properly mixed and kept on ice for 2 min. The solution was centrifuged at 1500 rpm at 4°C for 15 min. The nuclei pellet was re-suspended in 5 ml buffer B (containing 25 mM EDTA [50 ml EDTA, pH 8.0] and 75 mM NaCl [40 ml of 5 M NaCl]) and transferred to a 15 ml polypropylene centrifuge tube. Following the addition of 500 µl of 10% sodium dodecyl sulfate (SDS) and 55µl proteinase K (10mg/ml stock), it was incubated on a low-speed orbital shaker at 37°C overnight. Then, 1.4 ml saturated NaCl solution (approximately 6 M) was added to each tube and it was shaken vigorously for 15 s, followed by centrifugation at 2500 rpm in the low-speed centrifuge for 15 min. The supernatant was transferred into another 15 ml polypropylene tube, leaving behind the precipitated protein pellet and then exactly two volumes of room temperature 100% ethanol was added and the tube inverted several times until the DNA precipitate was visible. The DNA strands were removed with a pipette tip and transferred to an eppendorf tube containing 200 µl TE. DNA was dissolved at 37°C for 2 h.

RAPD -polymerase chain reaction (PCR)

RAPD-PCR amplifications were performed by screening 10 RAPD primers in a 25 µl reaction volume containing 1X PCR buffer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 20 -100 ng of the template DNA, 10 pM of each primer, and 1 unit Taq DNA polymerase (Metabion Co., Germany). Amplifications were performed in a thermal cycler (Biorad Co., USA) under the following conditions: 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 50 s at 40°C, 1 min at 72°C; and a final extension of 10 min at 72°C.

Cloning RAPD fragments

The 10 RAPD primers generated different patterns in examined

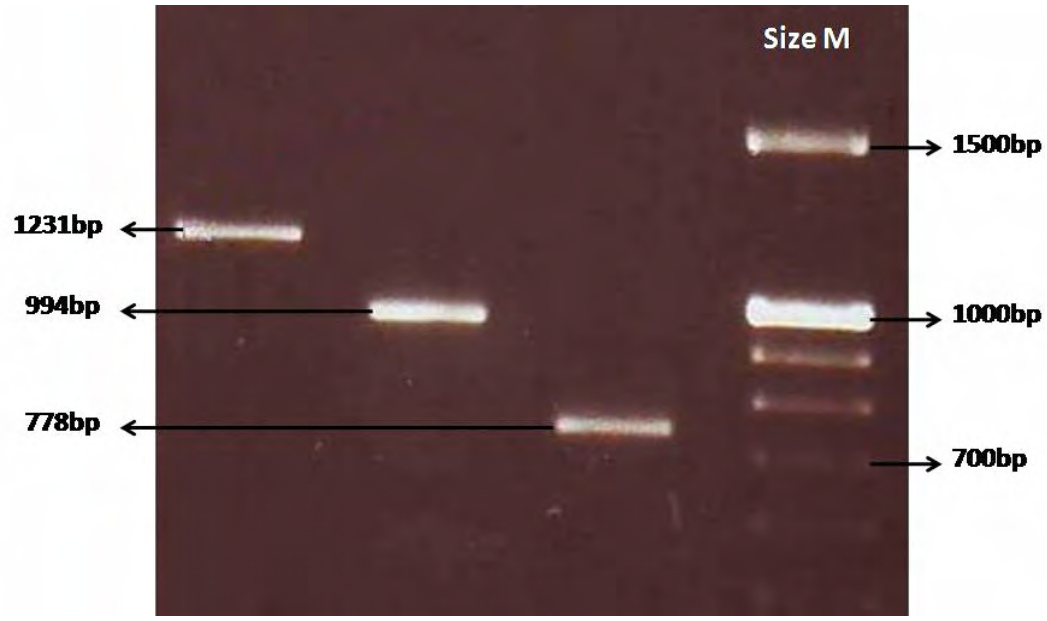

Figure 1. Three major sharp bands of 1231, 994 and 778 bp, which contain retrotransposon-like elements amplified with RAPD primer RP4. PCR products were excised from the gel and purified by gel extraction kit (Fermentas), followed by running on agarose gel stained with ethidium bromide. The 1231 bp fragment revealed to contain MbLS, BtA2LS_1, BtA2LS_2 and CHRbtLS, while CHR2Bls and ERVLB4LS were detected in the band of 994 bp. The 778 bp fragment shows BA2LS and L1C7LS.

specimens. Among these, three interested sharp bands amplified by a 10-mer primer, RP4 (5'-AAAGCTGCGG-3') were excised from agarose gel and purified with Qiagen gel extraction kit, following the manufacturer's specification. DNA fragments were ligated into pDrive TA cloning vector (Qiagen) with T4 DNA ligase at 4°C overnight, and transformed into ***Escherichia coli*** DH5α competant cells (heat shock at 42°C for 90 s). The cells were plated on LB-agar plates containing IPTG, X-Gal, and ampicillin to allow blue and white colony selection. Positive colonies were identified by colony PCR with M13 forward and reverse primers that may technically show greater than 262 bp bands on agarose gel electrophoresis if the inserted fragments exist in the colony. Plasmid from positively transformed clones was extracted using plasmid extraction kit (Fermentas) according to manufacturer procedure. Inserted fragments were sequenced in both directions using ABI Prism genetic analyzer.

Sequence analysis

After sequencing the plasmids, sequences were verified by alignment using the BioEdit software (version 7.0.9.0) to remove any redundant plasmids. High quality sequences were analyzed by repeat masker program for searching repetitive elements then, they were used as queries for database searching using BLAST tool. Sequence analysis and alignment were carried out using NCBI-BLASTN 2.2.14 version (Altschul et al., 1997) and CLUSTAL W 1.83 version for multiple sequence analysis (Gasteiger et al., 2003).

RESULTS

PCR amplification of RAPD primer (5'-AAAGCTGCGG-3') produced three cloned and sequenced major sharp bands of 1231, 994 and 778 bp (Figure 1). All fragments were amplified in eighty DNA samples of four buffalo populations. These segments were used as queries for repeat masker program to determine whether they contain any retrotransposon elements. Results showed that these sequences have a high similarity with 7 retrotransposons (mainly SINEs) reported previously. The list of retro-transposon-like elements, their length and GenBank accession numbers are shown in Table 1. Brief descriptions with the related sequences of RAPD derived retro-transposon-like element are as follows:

MIRb-like sequence (MbLS)

The first analyzed sequence is related to a 1231 bp RAPD fragment, within the positions 372-508. This 137 bp sequence contains the second promoter of RNA polymerase III and was revealed to be 57% similar to the MIRb, which is a tRNA-derived SINE (Figure 2).

Bov-tA2-like sequences (BtA2LS)

RAPD fragment at positions 528-712 and 713-834 (Figure 3). These sequences were consecutive and partial sequences of Bov-tA2. In both Bov-tA2-like sequences, we can see two promoter regions related to RNA polymerase III; BtA2LS_2 length was smaller than BtA2LS_1. BtA2LS_1 and BtA2LS_2 had 74 and 84%

Table 1. Retrotransposon-like-elements derived from the current study.

Retrotransposon-like element	Length	Type	GenBank accession number
MbLS	137 bp	SINE (MIRb)	GQ463458
BtA2LS	307 bp	SINE (Bov-tA2)	GQ463459
BA2LS	200 bp	SINE (Bov-A2)	GQ463463
CHRbtLS	172 bp	SINE (CHR-2_BT)	GQ463460
CHR2BLS	217 bp	SINE (CHR-2B)	GQ463462
ERVLB2LS	416 bp	LTR (ERVL-B4)	GQ463461
L1C7LS	565 bp	LINE (L1_Carn7)	GQ463464

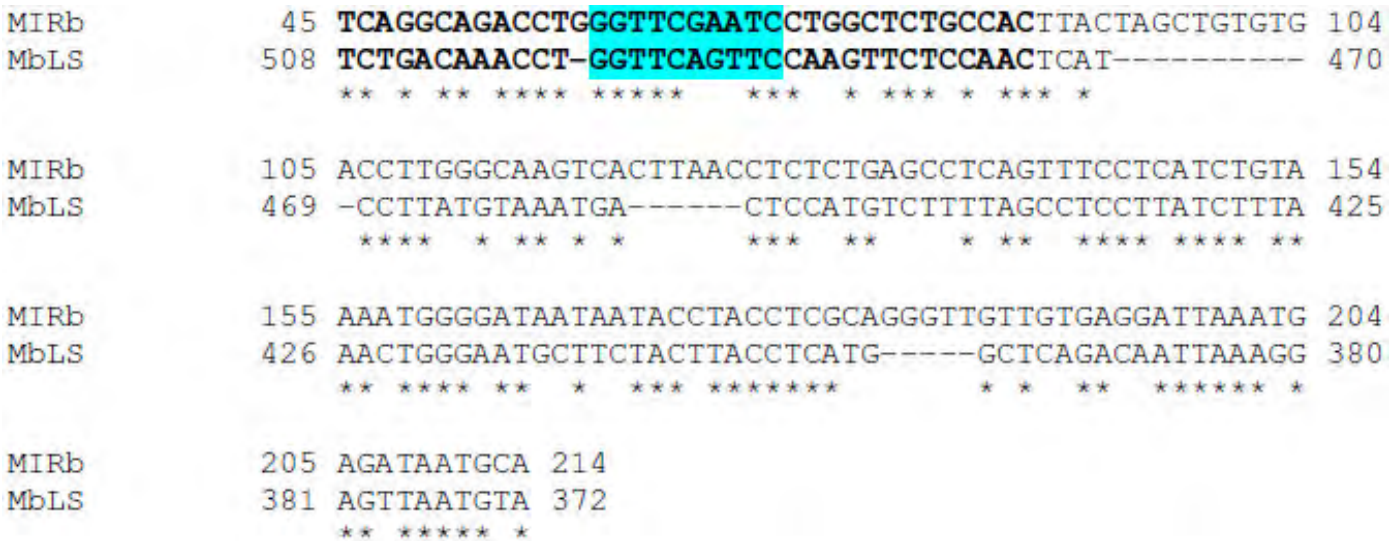

Figure 2. Alignment of the MbLS of Iranian river buffalo with the MIRb sequence. The shaded box shows RNA polymerase III 2nd promoter, bold letters show partial tRNA derived structure of MIRb and common letters show tRNA unrelated region. The nucleotides identical to the MIRb and deletions are shown by stars and bars, respectively.

similarity with Bov-tA2, respectively. Alignment of two BtA2LS, which were shown in Figure 4, revealed that two sequences had 79.5% similarity.

Bov-A2-like sequence (BA2LS)

There was a sequence (BA2LS) similar to Bov-A2 related to a 778 bp RAPD fragment at positions 14-215. Length of Bov-A2 is 269 bp, and has a high similarity (95%) with BA2LS (Figure 5). Bov-A2 is a dimer of Bov-A that was separated by (CACTTT) n repeat; this repeat can be seen in BA2LS.

CHR-2-like sequences

There were two CHR-2-like sequences: CHR-2_BT (CHRbtLS) and CHR-2B (CHRBLS) like sequences. The first one identified in the 1231 bp RAPD band within the positions 841-1057, and the latter is derived from the 994 bp RAPD fragment within the positions 776-992 (Figure 6). Level of similarity between CHRbtLS and CHR-2_BT was 73% and that of CHRBLS and CHR-2B was 79%. In both sequences, RNA polymerase III first and second promoters, tRNA related and unrelated sequences can be seen (Figure 6).

ERVL-B4-like sequence (ERVLB4LS)

There was a LTR-like sequence (ERVLB4LS) which is identified in our 994 bp RAPD amplified fragment at positions 102-518 (Figure 7). ERVLB4LS has 56.5% similarity with a part of ERVL-B4 sequence.

L1_Carn7-like sequence (L1C7LS)

There was a LINE-like sequence (L1C7LS) related to a 778 bp RAPD fragment within the positions 222-776 Figure 8). L1C7LS has 74.5% similarity with a part of

Figure 3. Alignment of BtA2LS sequences of Iranian river buffalo with Bov-tA2 sequence. A and B boxes show RNA polymerase III 1st and 2nd promoters, respectively. tRNA related sequence of Bov-tA2-like sequence is shown by bold letters and tRNA unrelated sequence is shown by common letters, the nucleotides identical to Bov-tA2 and deletions are shown by stars and bars, respectively.

L1_Carn7.

DISCUSSION

Various retrotransposon-like elements were identified after cloning the RAPD fragments from Iranian river buffaloes. All identified sequences were partial sequences of detected retrotransposons in other species. However, the main parts of SINE-like sequences are included within the identified sequences. Hitherto, almost all of the SINEs reported from the genome of mammals and plants, are related to tRNAs (Okada, 1991; Okada and Hamada, 1997); except to human ***Alu*** and mice B1 families that were derived from 7SL RNA. In this study, we identified four SINE-like elements that are related to tRNA. These elements are regarded as pseudogenes for tRNAs and structurally are composed of region homologous to tRNA (Shimamura et al., 1999). In SINE-like elements related to tRNAs, characteristic of SINE structure including tRNA

Figure 4. Alignment of two BtA2LS sequences (BtA2LS_1 and BtA2LS_2) of Iranian river buffalo, A and B boxes show RNA polymerase III 1st and 2nd promoters, respectively. tRNA related sequence of Bov-tA2-like sequence is shown by bold letters and tRNA unrelated sequence is shown by common letters, the nucleotides identical to Bov-tA2 and deletions are shown by stars and bars, respectively.

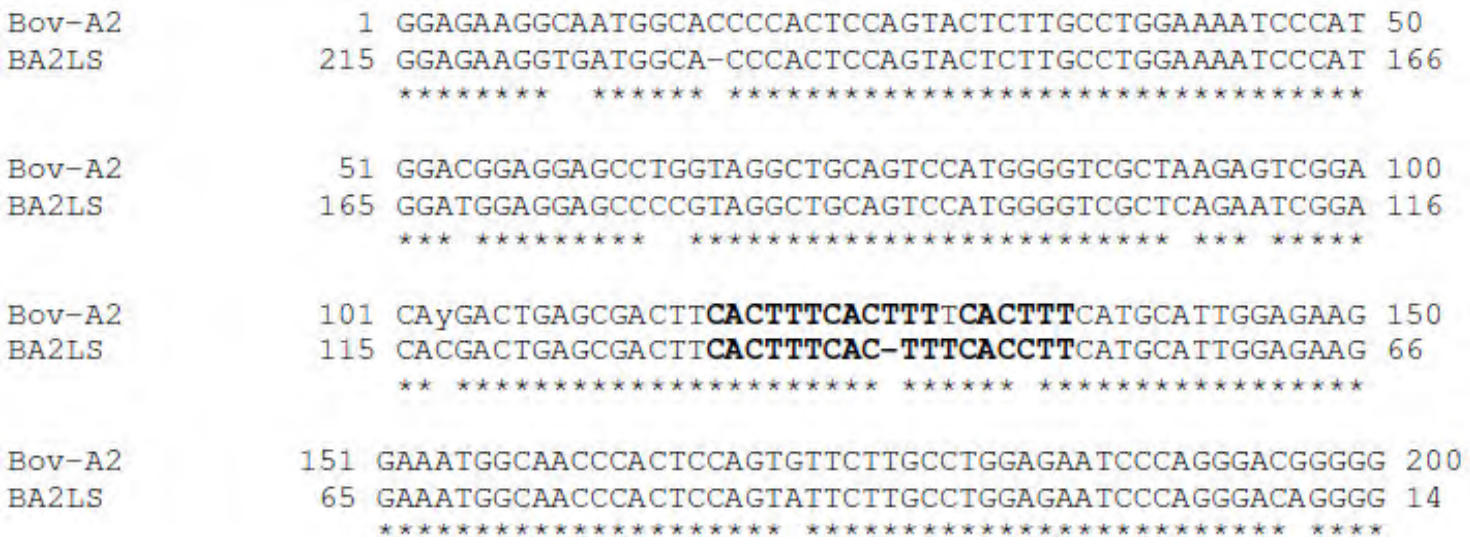

Figure 5. Alignment of BA2LS of Iranian river buffalo with Bov-A2 sequence. The nucleotides identical to the Bov-A2 and deletions are shown by stars and bars, respectively. CACTTT repeat is shown by bold letters.

related and unrelated regions that contained putative promoter motifs recognized by RNA polymerase III (A and B boxes) can be seen. The MbLS that is detected in this study, is partially similar to the sequence of MIRb, except the presence of three gaps due to deletion of 11, 6 and 5 nucleotides at positions 397-402, 448-454 and 469-480 of MbLS, respectively. This is in contrast with the report of Gosso et al. (2007), who suggested that MIRb repeat element is present in human, rhesus (Macaca mullata) and chimpanzee, but not in other mammal lineages. In MbLS, we identified a conserved sequence of GT (or A) TCG (or A) in second promoter of RNA polymerase III (Okada et al., 2003), while other parts of MIRb may be eliminated during evolution process. The fate of a given SINE element will depend on numerous factors in the chromosomal environment and on the accumulation of deleterious mutations that could preclude successful amplification of an element. Furthermore, because SINEs parasitize partner LINEs for access to the RT are necessary for their successful amplification, the death of a LINE automatically dictates the extinction of its corresponding SINEs in the same organism (Okada et al., 2003). Consecutive and conserve BtA2LS sequences, that are identified in this study, are related to promoters boxes of RNA polymerase III and have high homology with Bov-tA2. Bov-tA has been originated by combining a tRNA pseudogene with Bov-A, and Bov-tA2 is two copies of Bov-tA (Sheikh et al., 2002). Both BtA2LS_1 and BtA2LS_2 are partial sequences of Bov-tA2 and other parts of this element

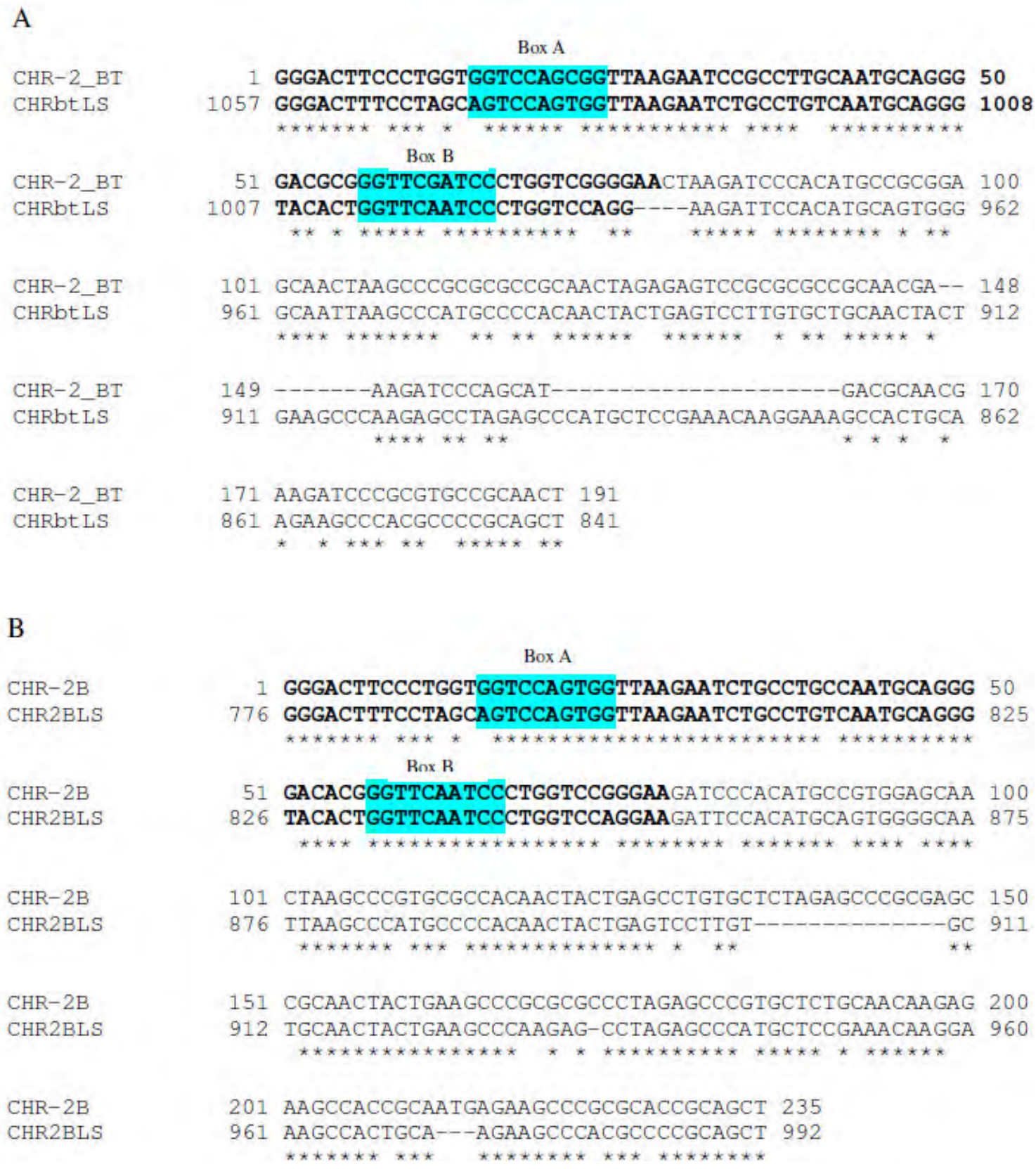

Figure 6. Alignment of CHR-2-like sequences of Iranian river buffalo (CHRbtLS and CHRBLS) with CHR-2 SINE elements [CHR-2_BT (A) and CHR-2B (B)]. A and B boxes show RNA polymerase III 1st and 2nd promoters, respectively. tRNA related sequence of CHR-2 like sequences is shown by bold letters and tRNA unrelated

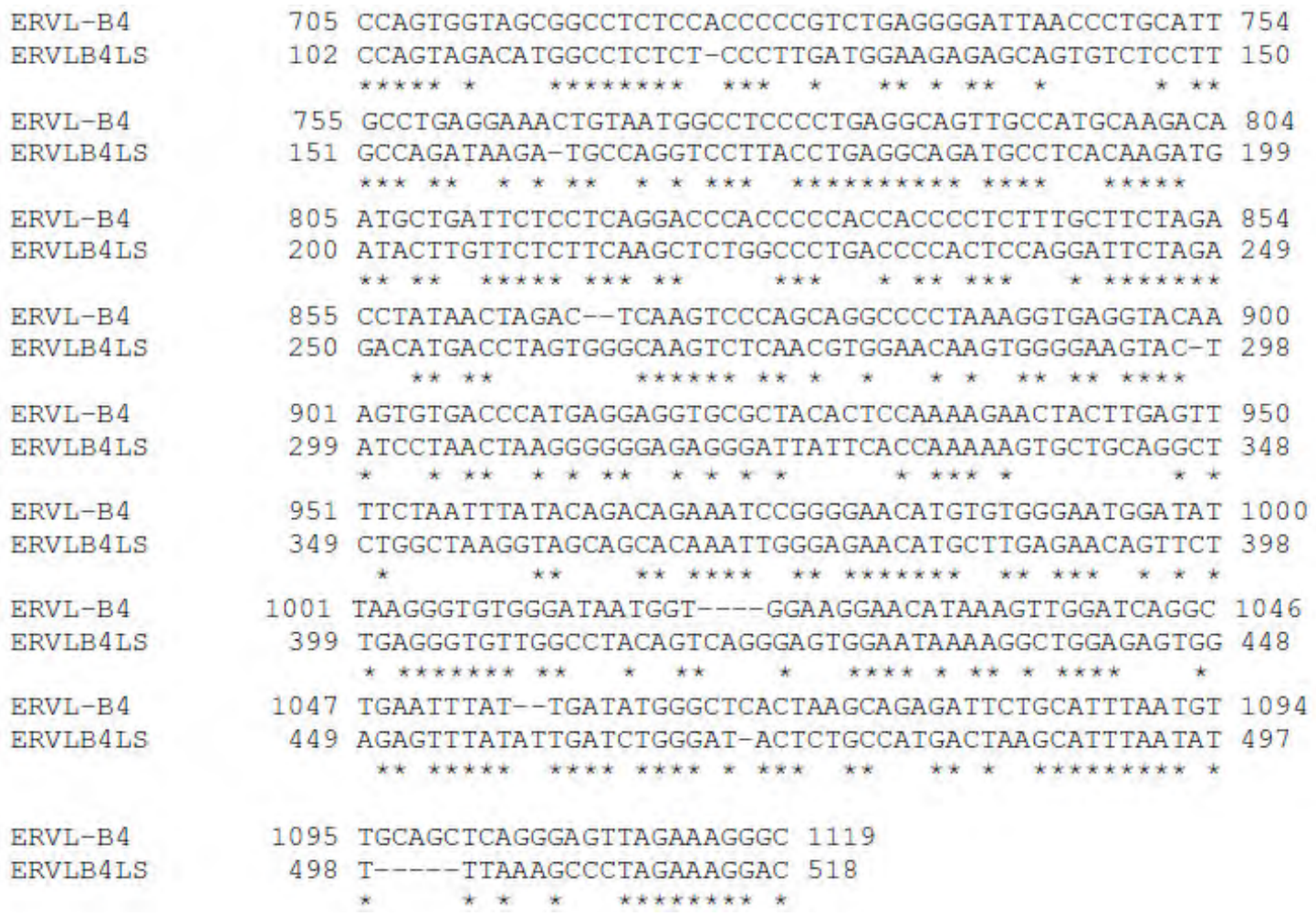

Figure 7. Alignment of a part of ERVL-B4 with ERVLB4LS, the nucleotides identical to the ERVL-B4 and deletions are shown by stars and bars, respectively.

(related to tRNA unrelated region) may be eliminated during evolution. Bov-tA2 has been found in ruminants mainly cattle (Shimamura et al., 1999). It was interesting that BtA2LS sequences were different from each other as they had not 100% similarity; however BtA2LS_2 had a homology with small part of BtA2LS_1. On the other hand, in the present study, three SINE-like elements including MbLS, BtA2LS and CHRbtLS sequences were identified in a 1231 bp RAPD fragment.

A similarity analysis of identified sequences revealed that the BA2LS had a high homology with Bov-A2. The origin of Bov-A has been a mystery, since no promoter for polymerase III typical for SINEs has been identified (Weiner et al., 1986; Rogers, 2005). According to the hypothesis presented by Okada and Hamada (1997), Bov-A is generated by deletion of the central part of the Bov-B LINE, and Bov-A2 has arisen by duplication of Bov-A. The Bov-A2 includes two Bov-A elements connected by a 27 bp linker sequence; comprising hexanucleotide (CACTTT)n repeats (Kaukinen and Varvio, 1992). The comparison of Bov-A2 in cattle, which has been identified by Lenstra et al. (1993), and BA2LS sequence revealed that the (CACTTT)n repeat is present in both elements. Taking into account the high similarity of Bov-A2 with BA2LS (about 200 bp), may raise the conclusion that BA2LS could be a partial sequence of Bov-A2.

In our 778 bp RAPD fragment, BA2LS and L1C7LS it was detected that the later element partially had a similarity with L1_Carn7 LINE; one of the most common carnivorous specific LINEs (Smith et al., 1995). Presence of a sequence with high homology with carnivorous specific LINE is very interesting, because extinction of the mentioned LINE is possible during evolution.

CHR-2_BT has been isolated in the genome of cattle (Shimamura et al., 1999), there was a sequence similar to CHR-2_BT (CHRbtLS), in this sequence conserved regions related to promoters were recognized by RNA polymerase exist, comparison of sequences showed that CHRbtLS had three deletion regions relative to CHR-

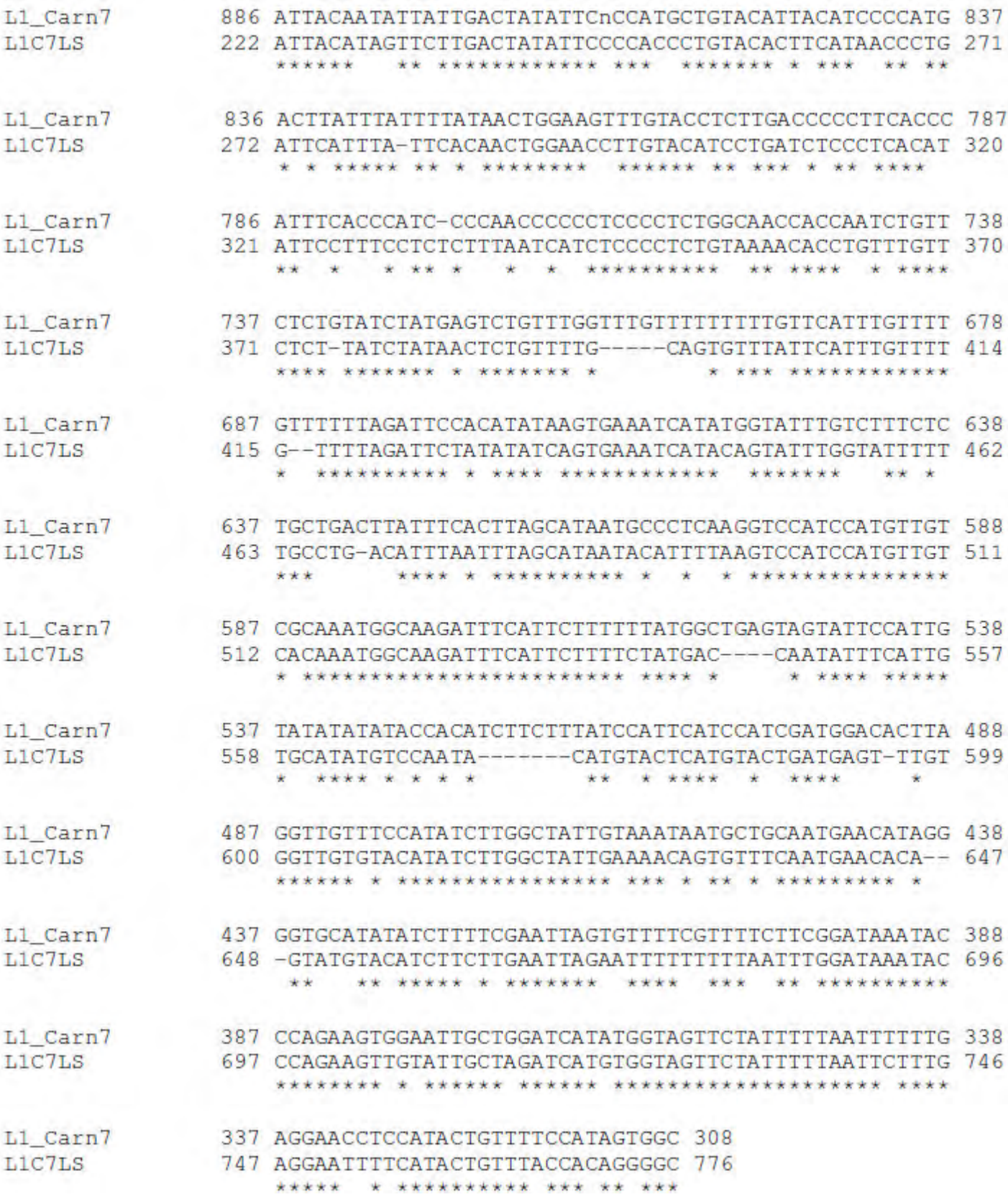

Figure 8. Alignment of L1-Carn7 sequence with L1C7LS identified in Iranian river buffalo, the nucleotides identical to the L1_Carn7 are shown and deletions by stars and bars, respectively.

2_BT sequence. Shimamura et al. (1999) suggested that CHR-2 SINEs were generated in a common ancestor of cetaceans, hippopotamuses, and ruminants and are thus present only in these genomes. CHR-2B can be found in genome of ruminants and the length of this element is 319 bp. In this study, we identified partial sequence of CHR-2B, three deletion region has occurred in CHR2BLS relative to CHR-2B SINE element.

ERVL-B4 is a subfamily of ERV3 from placental mammals (Cohen et al, 2007) with length of 5714. ERVLB4LS had similarity with a small part of ERVL-B4 (565 bp). Alignment shows two nucleotide deletion and a gap in ERVLB4LS and three gaps in ERVL-B4.

Retrotransposon-like elements identified in this study were obtained by cloning and sequencing of RAPD primer (5'-AAAGCTGCGG-3') fragments. We can suggest that this primer is related to retrotransposon sequences. Identification of partial sequences of retrotransposable elements in Iranian river buffalo can be useful for studies of genome evolution with species that have homologue elements and also for other population genetic studies.

The results of this study allow the assignment of these elements to Iranian river buffalo for the first time based on alignment of retrotransposon-like element with reported retrotransposon elements thus extending the river buffalo (***Bubalus bubalis***) physical map

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