

An *AluI* fragment isolated from lake trout (*Salvelinus namaycush*), maps to the intergenic spacer region of the rDNA cistron

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Abstract

The relationship between a 217-bp *AluI* fragment (SnAluI-33c) from lake trout (*Salvelinus namaycush*) which hybridizes to the nucleolar organizer regions (NORs) and the ribosomal RNA genes was examined by Southern analysis and comparative hybridization. Restriction enzymes with recognition sites mapped in the lake trout rDNA cistron were used to digest genomic DNA into fragments of predetermined size. Comparison of the hybridization pattern of SnAluI-33c with those of two rDNA-specific probes placed this fragment within the intergenic spacer region of the rDNA cistron, approximately 3 kb upstream (5') of the 18S gene. This finding is consistent with in situ hybridization experiments showing hybridization of this fragment to sites of rDNA [Reed, K.M. and Phillips, R.B., *Cytogenet. Cell Genet.* 70 (1995) 104–107]. Based on cross hybridization and sequence comparisons, homologous sequences are present in other salmonid species.

Keywords: Comparative hybridization; Nucleolar organizer region; Salmonid

1. Introduction

Several families of repetitive DNAs have been isolated and characterized from species of the salmonid genus *Salvelinus*. Recently, Hartley and Davidson (1994a,b) isolated several families of repetitive DNAs from Arctic char (*S. alpinus*) and Reed and Phillips (1995b) isolated and localized similar sequences from lake trout (*S. namaycush*). Two sequences from lake trout showed partial similarity to the *DraI/BstEII* family described from Arctic char. These lake trout sequences were 184 and 217 bp in length and were over 80% identical to sequences isolated from Arctic char (86-bp overlap). One of these lake trout clones (SnAluI-33c) was localized to ribosomal DNA (rDNA) sites in lake trout chromosomes using the technique of fluorescence in situ hybridization (FISH, Reed and Phillips, 1995b).

Sites of the multiple rDNA cistrons (nucleolar orga-

nizer regions, NORs) in lake trout can be divided by size into major and minor NORs (Phillips et al., 1989a; Reed and Phillips, 1995a). The number of NORs can vary among individual lake trout from three to as many as 12 with most variation occurring in number of minor NORs (two to eight per individual). Approximately half of the hybridization sites of the clone SnAluI-33c corresponded to positions of major sites of rDNA (Reed and Phillips, 1995b). However, direct association between several weakly hybridizing sites and number of minor NORs could not be verified. FISH data suggested that the lake trout sequences were either part of the rDNA cistron or a repetitive sequence associated with rDNA. The present study was designed to further investigate these hypotheses by examining the relationship between the *DraI/BstEII*-like sequences of lake trout and the rDNA cistron. In addition, closely related species were examined to investigate the evolutionary conservation of this DNA sequence.

2. Experimental and discussion

2.1. Genomic mapping of the *AluI* fragment, SnAluI-33c

Comparative hybridization techniques were used to examine the relationship between the 217-bp lake trout

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Abbreviations: bp, base pair(s); DIG, digoxigenin; dNTP, deoxy-nucleotide triphosphate(s); ETS, external transcribed spacer; FISH, fluorescence in situ hybridization; NOR, nucleolar organizer region(s); IGS, intergenic spacer; ITS-2, second internal transcribed spacer of the rDNA cistron; kb, kilobase(s) or 1000 bp; PCR, polymerase chain reaction; rDNA, ribosomal DNA.

AluI fragment (SnAluI-33c) and the rDNA cistron. Based on the lake trout rDNA map (Fig. 1), restriction enzymes were chosen that would produce fragments of the rDNA cistron of known size. For example, a *BglII* restriction site is present in the 28S coding region and is the only *BglII* site present in most copies of the rDNA cistron of lake trout. Digestion of lake trout DNA with this enzyme resulted in a broad band in excess of 20 kb when probed with the 28S probe I-19 (Fig. 2A). Minor bands of 2.5 and 1.0 kb were also present indicating presence of additional polymorphic *BglII* sites in or near the 28S gene. The SnAluI-33c probe hybridized to a large *BglII* fragment equal in size to that detected by I-19 (Fig. 2B). Double digestion with *BglII/HindIII* cuts the rDNA cistron into two fragments. One is approximately 7 kb and includes most of the coding regions. The second represents the remainder of the cistron and hybridization of SnAluI-33c to this larger band further indicates similarity of the clone to sequences in the IGS. These hybridization results are consistent with FISH experiments showing hybridization of SnAluI-33c to major NOR sites (Reed and Phillips, 1995b).

Examination with additional enzymes confirmed presence of a sequence homologous to the SnAluI-33c fragment within the IGS. Several lines of evidence place this sequence approximately 3 kb upstream (5') of the 18S gene. Strongest support for this interpretation is the similar pattern of hybridization seen with lake trout clones 14-B and SnAluI-33c (Figs. 2B and C). For example, digestion with *PstI* produced several fragments common to both 14-B and SnAluI-33c but not shared with I-19. However, hybridization patterns of these two probes were not identical. For example, 14-B detected additional fragments including the 12.5-kb band in the *PstI* digestion (Fig. 2C). This weak band corresponds to the region of the rDNA cistron 3' to the 18S *PstI* site (Fig. 1). Location of homologous sequence is further supported by the *PstI/NheI* digest which eliminated the 12.5-kb band and produced a 1.8-kb band unique to 14-B. Hybridization pattern following digestion with *PvuII* also excluded the SnAluI-33c sequence from the 3'-region of the IGS in that it did not hybridize to the ~3-kb fragment detected with I-19.

Similarity of banding patterns produced with the 14-B and SnAluI-33c probes allowed for finer scale mapping of the sequence to the 5'-IGS. Digestion with *PstI*, *DraI*, and *EcoRI* concurred with the *BglII/HindIII* data and excluded the 3'-portion of 14-B (5'-external transcribed spacer (ETS) and 18S coding region). The *PvuII* digestion placed the sequence upstream of the ITS-2 *PvuII* site (between 5.8 and 28S, Fig. 1) and lack of hybridization to the approx. 7-kb *BglII/HindIII* fragment indicated a location 5' to the *HindIII* site. Digestion with *SspI* shows that the sequence lies at or near the *SspI* site upstream from the *HindIII* site. SnAluI-33c and 14-B cross-hybridized to a 7-kb fragment and several

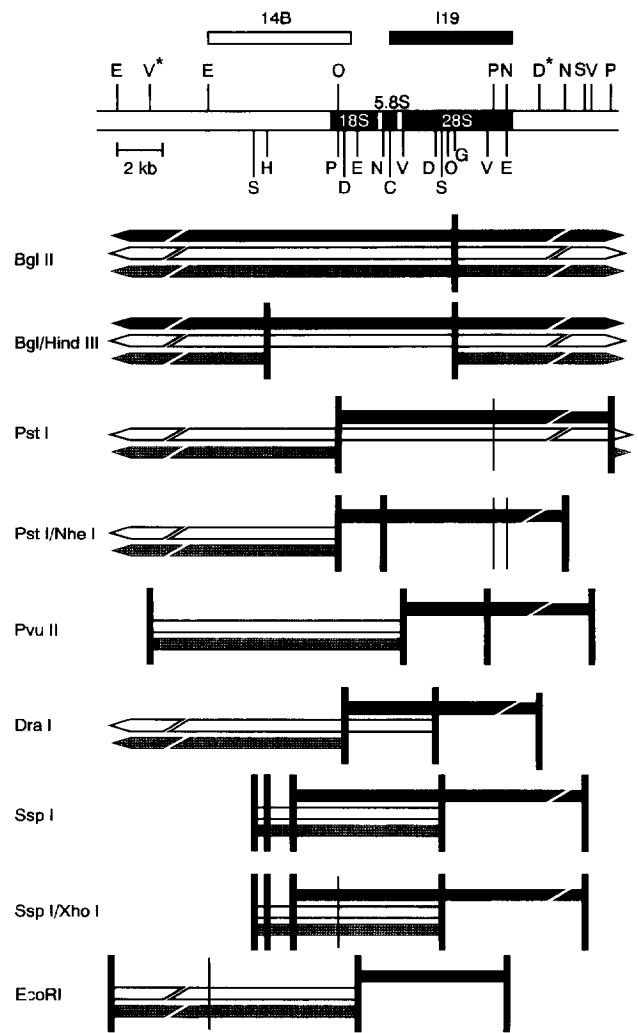


Fig. 1. Restriction map of the ribosomal DNA cistron of lake trout showing the location of restriction sites for the enzymes used in this study (top) and summary of the mapping results for each enzyme combination (bottom). Symbols are as follows: C, *BclI*; D, *DraI*; E, *EcoRI*; G, *BglII*; H, *HindIII*; N, *NheI*; O, *XhoI*; P, *PstI*; S, *SspI*; V, *PvuII*. Sites indicated below the map are invariable in lake trout and conserved in other salmonid fishes. Sites above the map are variable. Variable sites between the *PvuII* and *DraI* sites (asterisks) show intra-individual variation. Length of rDNA cistron of lake trout varies from 20 to 26 kb, resulting in part from variation in copy number of repetitive elements in the 3'-region of the IGS (Popodi et al., 1982; Zhuo et al., 1995). Thus, restriction fragments obtained with *PvuII*, *DraI* and enzymes with flanking sites are variable in length. Two rDNA probes were used in our analysis and the positions of these probes are indicated above the map. I-19 is a *SalI-EcoRI* fragment isolated from the mouse and includes most of the 28S coding region and part of the second internal transcribed spacer (ITS-2). A second clone (14-B) contains a 6-kb *EcoRI* fragment isolated from lake trout (Zhuo et al., 1994). This clone contains approximately 4.2 kb of the 5'-intergenic spacer (IGS) and most of the 18S coding region. Fragments detected by hybridization of the three probes to the rDNA cistron are indicated for each digestion below the map. Solid horizontal bars denote fragments detected with I-19; open bars, 14-B; and shaded bars, SnAluI-33c. Vertical bars correspond to mapped restriction sites, thin vertical bars denote minor sites.

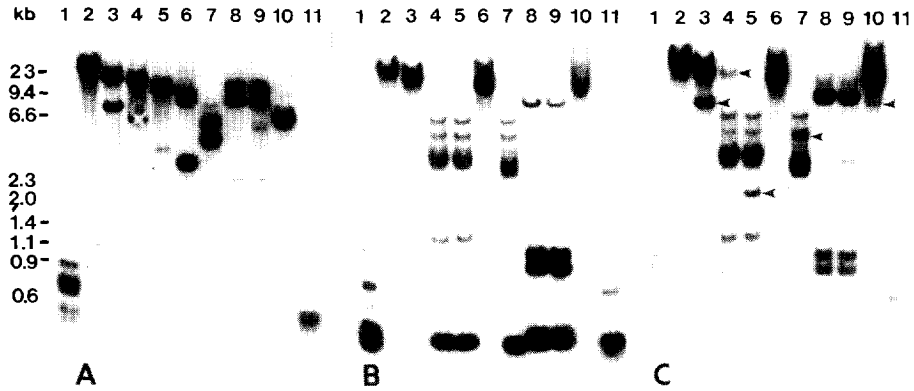


Fig. 2. Southern analysis of lake trout genomic DNA (5 μ g) digested with restriction endonucleases and probed with digoxigenin-labeled probes. (A) I-19 rDNA probe. (B) SnAluI-33c. (C) 14-B, lake trout rDNA-specific probe. Arrowheads in (C) denote bands hybridizing to 14-B that did not hybridize to SnAluI-33c (see text). Lanes in each panel are as follows: (1) *AluI*; (2) *BglII*; (3) *BglII/HindIII*; (4) *PstI*; (5) *PstI/NheI*; (6) *PvuII*; (7) *DraI*; (8) *SspI*; (9) *SspI/XhoI*; (10) *EcoRI*; (11) *HaeIII*. DNA standards are given in kb. **Methods:** DNA was isolated from red blood cells of lake trout, *Salvelinus namaycush* (Green Lake stock). Genomic DNAs (5 μ g) were digested to completion with an excess of enzyme under conditions described by supplier (Promega, Gibco-BRL). Double digests used buffer combinations to maximize efficiency of both enzymes. Digested DNA was size fractionated on horizontal 1% agarose gels and vacuum blotted onto positively charged nylon membranes. Molecular cloning of SnAluI-33 (GenBank accession No. U27093) is described in detail in Reed and Phillips (1995b). The portion of this clone (c) containing the *DraI/BstEII*-like was amplified by PCR using an oligonucleotide primer complementary to flanking vector sequence (Reverse Amplification Primer, US Biochemical) and the insert-specific primer (5'-CTCCATAAATCCTCAGGCC-3'). The reaction contained: 0.25 μ l of purified single-stranded vector DNA, 2.5 μ l 10 \times *Taq* polymerase buffer (Promega), 1.5 μ l 25 mM $MgCl_2$, 70 ng of each primer, 100 μ M each dNTP, 0.5 μ l *Taq* polymerase, and ddH₂O to a final volume of 25 μ l. A total of 30 cycles of 50 s at 94°C, 30 s at 58°C, and 30 s at 72°C were performed in a COY thermal cycler. A portion of the PCR product (300 ng) was random labeled with digoxigenin-11-dUTP (DIG) according to manufacturer's protocol (Boehringer-Mannheim) for use as probe. The two rDNA clones were linearized prior to random labeling. Membranes were probed separately with the DIG-labeled SnAluI-33c insert, I-19 or 14-B clones. Hybridizations were carried out overnight at 63°C. Probe was detected with the chemiluminescent substrate Lumi-Phos 530 according to protocols of the Genius System User's Guide (Boehringer Mannheim). Restriction fragment lengths were determined by use of molecular standards. Duplicate filters were probed to map location of the SnAluI-33c fragment.

smaller fragments (approx. 800, 650, 250 and 200 bp) in both the *SspI* and *SspI/XhoI* digestions. The 800- and 650-bp fragments are suggestive of either multiple copies of the SnAluI-33c sequence bordering the *SspI* site or presence of this sequence within a larger duplicated region containing *SspI* sites. Several enzymes (*AluI*, *PstI*, *DraI*, *SspI*, *HaeIII*) produced monomer-size fragments (approx. 200 bp) that cross hybridized to 14-B and SnAluI-33c. The hybridization signal was stronger for SnAluI-33c and this is likely due to greater overall specificity and higher relative digoxigenin content of the smaller probe.

The complete human rDNA repeat has now been sequenced including the large intergenic spacer (Gonzalez and Sylvester, 1995). Except for segments immediately adjacent to the rDNA coding region in salmonids (Zhuo et al., 1994, 1995), little is known regarding the DNA sequence of the IGS of fishes. Mapping of SnAluI-33c to a portion of the IGS 5' to the 18S gene makes this fragment a useful probe for further studying rDNA cistrons of lake trout and other salmonid species. There is considerable intra- and inter-individual variation in length of the IGS in lake trout (20–26 kb) resulting in part from presence of repetitive elements just 3' to the 28S gene (Zhuo et al., 1995). Presence of these repeats has made mapping of restriction sites in the IGS problematic. With an additional

probe 5' to the 18S coding region it is now possible to extend mapping studies further into the IGS. For example, the SnAluI-33c fragment could be used to isolate larger IGS fragments from the upstream region. Several restriction sites that occur near the *HindIII* site are conserved in salmonids suggesting a region of relative sequence stability (Phillips et al., 1992). Of particular interest in this region is the site of transcriptional initiation. In humans, the initiation site lies 3.5 kb upstream from the 18S (Financsek et al., 1982). The initiation site has not yet been described for any salmonid species but it may occur near the site detected by SnAluI-33c.

2.2. Examination of other species

Hybridization of SnAluI-33c to genomic DNAs of other salmonids found that homologous sequences are present in rDNA cistrons of related species. Digestion with the enzyme combination *BglII/BclI* divides the rDNA cistron of most species into a conserved 2.3-kb fragment representing about one-half of the coding region, and a large fragment representing the remainder of the repeating unit (Fig. 1). These fragments are present as two main bands when probed with I-19 (Fig. 3). Hybridization of SnAluI-33c to similarly digested DNAs showed strong hybridization to IGS

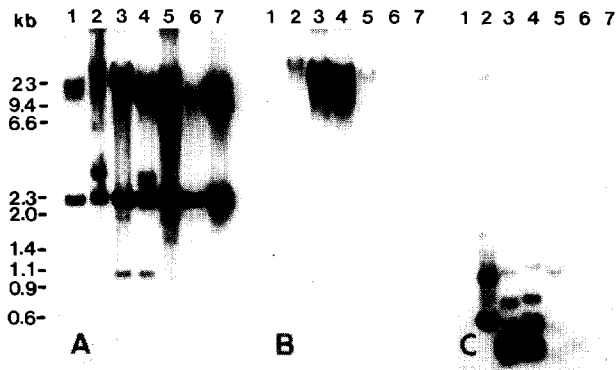


Fig. 3. Southern analysis of genomic DNA (5 μ g) of several salmonids digested with restriction endonucleases and probed with digoxigenin-labeled probes. (A) *Bgl*II/*Bcl*I-digested DNA probed with I-19. (B) *Bgl*II/*Bcl*I-digested DNA probed with SnAluI-33c. (C) *Alu*I-digested DNA probed with SnAluI-33c. Lanes in each panel are as follows: (1) rainbow trout (*Oncorhynchus mykiss*); (2) Atlantic salmon (*Salmo salar*); (3) lake trout (*Salvelinus namaycush*); (4) Arctic char (*S. alpinus*); (5) Japanese huchen (*Hucho perryi*); (6) Danube salmon (*H. h. hucho*); (7) lake herring (*Coregonus artedi*). DNA standards are given in kb. **Methods:** To determine whether sequences homologous to SnAluI-33c existed within genomes of species related to *S. namaycush*, DNA from several other salmonids was obtained from blood, liver, or fin clips of fish from the following localities: *Coregonus artedi*, Lake Superior (Bayfield, WI, USA); *Hucho (h.) hucho*, North Carpathian River (Slovakia); *H. perryi*, Hokkaido Fish Hatchery (Japan); *Oncorhynchus mykiss*, Evergreen Hatchery (Pound, WI, USA); *Salmo salar* (Newfoundland, Canada); *Salvelinus alpinus*, (Labrador stock) Rockwood Hatchery (Winnipeg, Manitoba, Canada). These samples were digested singly with *Alu*I and doubly with *Bcl*I/*Bgl*II. Digested DNA was transferred to nylon filters and probed with I-19 and SnAluI-33c as described in Fig. 2.

fragments of both lake trout and Arctic char (*S. alpinus*). Weak hybridization was seen to Atlantic salmon (*Salmo salar*) and Japanese huchen (*Hucho perryi*). After extended exposure times, a weak signal corresponding to the IGS fragment also was present in rainbow trout (*O. mykiss*), Danube salmon (*Hucho h. hucho*) and lake herring (*Coregonus artedi*). Weaker hybridization is likely due to sequence divergence in the rapidly evolving IGS of these species which have been separated for millions of years. Lack of sequence conservation argues that SnAluI-33c is not directly related to the promoter region.

Additional evidence for sequence variation in the IGS region is the banding pattern produced following digestion with *Alu*I, where each species produced a unique set of bands when probed with SnAluI-33c. Similar to the *Bgl*II/*Bcl*I digests, hybridization to the *Alu*I fragments of rainbow trout and *C. artedi* were weak. Hybridization was stronger to DNA of *H. perryi* than that of *H. h. hucho*. Both lake trout and Arctic char had multiple bands and this may be related to the multiple NORs seen in these species.

Increase in number of rDNA sites and the level of divergence in the IGS among species of *Salvelinus* indicates that this genus would be a good system for

studying evolution of the IGS of lower vertebrates. In lake trout the number of NORs (sites of rDNA) can vary from three to 12 among individuals. Variation in number of NORs within populations is often as great as that found between populations. Multiple NORs are also present in Arctic char (*S. alpinus*) and brook trout (*S. fontinalis*) (Phillips et al., 1988, 1989b). Genetic analyses of lake trout found a correlation between number of rDNA variants and number of NORs of individual fish (Zhuo et al., 1995). IGS length variants were stably inherited indicating their presence at different NORs on nonhomologous chromosomes. Given the level of NOR variation, the SnAluI-33c fragment could be used as a population-level marker within the genus *Salvelinus*.

Evidence of recent NOR transposition in both lake trout and brook trout has been obtained with FISH (Reed and Phillips, 1995a; Phillips and Reed, 1996). In both of these species, some rDNA sites were found to be flanked by copies of the telomeric repeat (TTAGGG)_n. The growing body of evidence suggests that NOR transposition in this genus is ongoing. One hypothesis is that transposition of NORs is mediated by a transposable element inserted in the IGS. The *Alu*I fragment, SnAluI-33c, should be a valuable molecular probe for investigating these and other questions regarding evolution of the rDNA of salmonid fishes.

Hybridization of SnAluI-33c to *Alu*I digests of Atlantic salmon DNA produced two distinct bands of approximately 1 and 0.5 kb. These bands are nearly equal in size to two *Bgl*II fragments characterized from this species (Goodier and Davidson, 1993). FastA searches of GenBank (Pearson and Lipman, 1988) using SnAluI-33c as query sequence retained the Atlantic salmon *Bgl*II element among the group of 40 best scores. Alignment of SnAluI-33c with an Atlantic salmon *Bgl*II sequence (923-bp *Alu*I fragment, GenBank L01505) indicated an area of approximately 70% identity over a 140-bp region of overlap (Fig. 4).

Hartley and Davidson (unpublished results) noted similarity of the *Dra*I/*Bst*EII sequence of Arctic char to a portion of this same *Bgl*II sequence. The SnAluI-33c sequence from lake trout is 81.4% identical (86 bp overlap, Reed and Phillips, 1995b) to a sequence (*Dra*I/*Bst*EII family) isolated from Arctic char. Hartley and Davidson (1994a) found this 172-bp *Dra*I fragment to be present in genomes of all species of *Salvelinus*. Although sequence identity is low, our data suggest that the fragment from lake trout (SnAluI-33c) is related to the *Bgl*II element of Atlantic salmon. Goodier and Davidson (1993) used rDNA primer pairs in PCR assays and found that some of the λ clones containing the *Bgl*II element included portions of the 18S rDNA. This finding and the sequence similarity of SnAluI-33c to the *Bgl*II element support our conclusion that the SnAluI-33c sequence is part of the IGS.

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                                     Hpa II
                                     Hae III
AS      TTTCAAAC- CCATAAATCA GCCAGGCCGG CCCCATTGA CTGGGCCCG TAGTAGGGTG
SnAluI-33c  CT- .....-C CT..... C... ..T...A .....T...
AC      GTAAATTGGT ..G.C.-- CT..... T.C... ..T... C...T.TA.

                                     Dra I
AS      CTGTCAGGGT GGGTATGGAG GTCCCTATAC CCTTTAAAAG T-ATTTAAAA CAGTTATAAA
SnAluI-33c ..CC..TA.C ...C...GT ...TGC.GG- .A.....- .C...TC.T TC.A.T.C..
AC      ..CC..TA.C ...C..T... ..G.....C. ....

AS      ATTCACCCC TG GTAACCCA AATGC-TAC- GGA-CACGTCC CCACCAAAC ATAGAATAT
SnAluI-33c TAGCAG.A.. -.....A. ...A-A...A ..TG...-..A TTCGATTTC.A TAG..GC.C

AS      TTTC
SnAluI-33c .G.AACCAAAA TATACAGGTG CACAGAAGGA GATGAGTACA GACATTTTAA G

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Fig. 4. Alignment of the lake trout sequence SnAluI-33c (GenBank accession No. U27093) to a portion (bases 210–390) of an Atlantic salmon repetitive element (AS, GenBank accession No. L01505, Goodier and Davidson, 1993). Included for comparison are the first 93 bp (5'-reverse complement) of a *Dra*I fragment from Arctic char (AC, GenBank accession No. L00991, Hartley and Davidson, 1994a). '.' indicate bases identical to the first sequence, '-' denote gaps inserted for alignment purposes.

3. Conclusions

- (1) Data support the hypothesis that the *Alu*I fragment, SnAluI-33c, isolated from lake trout is part of the rDNA cistron of this species. SnAluI-33c has characteristics of a repetitive element because it is part of the rDNA cistron, which is present as a tandem repeated unit in the vertebrate genome.
- (2) Mapping of SnAluI-33c indicates that homologous sequences are located in the intergenic spacer region upstream from the 18S coding region (near an *Ssp*I site).
- (3) Based on hybridization results and sequence analysis, sequences homologous to SnAluI-33c are present in rDNA cistrons of related species.

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