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IDENTIFICATION OF LAKE TROUT STOCKS USING NUCLEAR RIBOSOMAL DNA

by

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Identification of Lake Trout Stocks Using Nuclear Ribosomal DNA

Introduction:

The ribosomal DNA of higher organisms is organized in tandem arrays of repeating units, which are clustered at specific chromosomal sites, the nucleolar organizer regions (NORs). Each of the repeating units contains the ribosomal transcription unit and an intergenic spacer (IGS) sequence (reviewed in Long and Dawid, 1980, see Figure 1, p. 14). The three coding regions for the 5.8S, 18S and 28S rRNAs are highly conserved while the spacer regions often show extensive intraspecific variability in both sequence and length (reviewed in Gerbi, 1985).

Population specific or strain specific ribosomal DNA (rDNA) spacer variants have been found in many organisms including *Drosophilia mercatorium* (Williams et al., 1985, *Mus musculus* (Suzuki et al., 1986), the treefrog *Hyla versicolor* (Romano and Vaughn, 1986), the mosquito, *Aedes albopictus* (Black et al, 1988), and rice and wheat (Dutta et al., 1987; Flavell et al., 1986).

Spacer length variation usually results from changes in the copy number of small tandem subrepeats located within the IGS. Often variants can be detected on Southern blots as bands that differ from one another in length by an integral multiple of the subrepeat length. For example, spacer length variants differ by multiples of 180 bp in the garden pea (Polans et al, 1986), and by 240 bp in *Drosophilia melanogaster* (Dover et al, 1982). Structural variations in the IGS may involve the types, lengths, and copy number of the subrepeats and unique regions between blocks of subrepeats (Tautz et al, 1986).

The inheritance of ribosomal DNA has been studied in several organisms including *Xenopus laevis* (Reeder et al, 1976), mouse spp (Arnheim et al, 1982) , *Drosophilia melanogaster* (Boncinelli et al, 1983), wheat (Snape et al, 1985), garden pea (Polans et al, 1986), and humans (Garkavtsev et al, 1988). In each case variation was inherited in a simple Mendelian fashion. In organisms in which the rDNA is found at more than one chromosomal location (multiple NORs) the nonrandom distribution of spacer length variants suggests that genetic exchange between NORs located on nonhomologous chromosomes is limited (Polans, 1986, Arnheim, 1982, Sasaki et al, 1987)

Since previous work showed that variation occurred in the chromosomal location of NORs in different populations of lake trout (Phillips et al, 1989) this study was undertaken to determine if population specific molecular variants of rDNA existed in lake trout.

In previous work, a preliminary restriction map of the rDNA of lake trout was constructed using 5 restriction enzymes (Popodi et al., 1985). In the present study 20 additional enzymes were used, so that a much more detailed map is now available. Two dozen different individuals from 4 lake trout stocks were examined for several polymorphic restriction sites to determine if there were population differences in ribosomal DNA variants.

Material and Methods:

Liver samples were obtained from several wild populations and hatchery stocks of lake trout. Wild populations sampled included Gull Island Shoals, Wisconsin (south shore of Lake Superior), Manitou Lake, Ontario (north shores of Lake Superior and Lake Huron), and Seneca Lake, New York. Hatchery stocks sampled were the Marquette, Michigan (south shore of Lake Superior) stock and the Jenny Lake, Wyoming stock. The Jenny Lake stock resulted from a transplant in 1890 that originated from a now extinct population off Beaver Island in Lake Michigan (Visscher, personal communication).

Genomic DNA was extracted from fish livers by phenol extraction (Popodi et al, 1985). The restriction enzymes used in this study included the following enzymes which recognize 6 base sequences: ApaI, BamHI, BclI, BglII, ClaI, DraI, EcoRI, EcoRV, HindIII, HpaI, KpnI, MluI, NheI, PstI, PvuII, SacI, SalI, ScaI, SspI, SphI, SstI, SstII, XbaI, and XhoI. Restriction enzyme digestions were carried out at 37°C as recommended by the supplier (BRL). Following digestion, samples were separated by electrophoresis for 17 hours at 35 volts in .6% agarose and transferred to a nylon filter (Zeta-bind) as described by Southern (1975). Filters were baked for 2 hr at 80°C. Prehybridization, hybridization, and post-hybridization washes of filters were carried out at 65°C. Filters were hybridized with radioactive probes to rDNA from mouse and Chinese Hamster ovary. Clone I-19 was a 4.2 kb Eco RI-SalI fragment containing most of the 28S rDNA coding region from mouse cells and clone PEB-4 was a 1.9kb Eco RI- SalI fragment containing most of the 18S coding region from Chinese Hamster Ovary cells. Probes were made radioactive using the random primer reaction according to manufacturers instructions (Pharmacia). Filters were dried and exposed to X-Omat film (Kodak) at -70°C with an intensifying screen (DuPont-Cronex). Fragment patterns obtained from different individuals were analyzed and restriction maps drawn.

Results:

Lake Trout Ribosomal DNA Restriction Map

Of the 24 enzymes used to digest lake trout DNA, 18 cut in the ribosomal DNA repeating unit. The current restriction map of lake trout rDNA is shown in figure 2 (p. 15). The sites below the line on the map are those which are constant sites found in all the salmonid fishes examined to date, while sites above the line are sites variable among salmonid fishes. Sites showing intraspecific variability in lake trout are designated with an asterisk. Almost all of these variable sites show both intraindividual and interindividual variation. Thus multiple bands in each individual are seen. In many cases a large number of different individual patterns have been found. Since the ribosomal DNA is present in many copies at a number of different chromosomal sites, these results suggest that different molecular variants are present at different chromosomal sites in lake trout ribosomal DNA.

Variation in the Intergenic Spacer Region

The total size of the repeating unit appears to vary between 20-26kb. Much of this variation is found between repeat units within a single individual. This can be seen by examining patterns obtained from enzymes which cut only once, such as HindIII, BglII, and SphI. Each individual has a specific pattern of bands between 20-26 which is the same with these enzymes, and several different patterns are evident. BamHI gives an similar pattern for each individual except that the size of each of these bands is reduced by about 3kb and two smaller bands are also found. BamHI cuts three times within the coding regions producing two small pieces of .5kb and 3kb, and a third piece varying in size between 15-23kb which contains the entire intergenic spacer region (see Figure 3, p. 16). In preliminary work some stock differences in the frequency of these patterns were found, but it was difficult to size these large fragments accurately.

Since the intergenic (IGS) spacer is quite large and it is difficult to size large fragments accurately, we examined the patterns obtained with a double digest of HindIII and BglII. This double digest cuts the repeating unit into two pieces, a constant piece of 7kb and a variable piece of 13-19kb with most of the spacer region. Comparison of these patterns with those obtained with BamHI shows that some of the larger bands are now resolved into several bands, so that what appeared to be only a few patterns in the length of the spacer region is in reality many different patterns with almost every individual having a different pattern (see Figure 4, p. 17).

Stock Variation in Ribosomal DNA Patterns

Since variation in the total length of the intergenic spacer (IGS) appeared to be too great to be useful for stock identification, several enzymes which cut the IGS in different regions were examined in order to evaluate stock variation in particular regions of the spacer. Enzymes examined included EcoRI and PvuII which cut the IGS 5' to the coding regions and NheI, PstI, SspI, and DraI which cut the IGS 3' to the coding regions. In every case, a large number of individual patterns were found. In the case of NheI and SspI which cut 3kb apart in the IGS, there is a close similarity between the patterns (see figure 5, p. 18, for the SspI pattern). This suggests that these patterns result from variation in subrepeat units between Nhe and the coding region.

Table 1 (p. 9) shows the frequency of bands in different stocks obtained with PstI, which cuts in the IGS starting at about 11kb on the 3' side of the coding region. Some stock differences were found in band freqencies and total number of bands per individual. Double digests with PstI/BglII were done in order to produce smaller fragments which could be sized more exactly (see Table 2, p. 10). However, many additional bands were found with the double digests, so that the patterns with the double digests could not be correlated with the patterns with the single digests.

Table 3 (p. 11) shows frequency of bands in individuals of different stocks obtained with EcoRI which cuts in the IGS starting at about 5.6kb on the 5' side of the coding regions. As with the other enzymes, there were a large number of closely spaced bands with some stock variation in band frequencies.

The DraI enzyme was selected because it cuts in the IGS close to the 28S coding region and produces small bands which can be sized easily without having to do double digestions. Multiple bands were obtained in each individual with this enzyme. These bands include a constant band of 3.4kb spanning part of the 18S and 28S coding regions and variable bands spanning the right end of the 28S coding region and part of the IGS adjacent to it (see Figures 6 and 7, p. 19). Table 4 (p. 12) shows the frequency of bands in individuals of different stocks obtained with DraI. A detailed analysis revealed stock differences in these patterns (see Table 5., p. 13). When the smallest difference in size between adjacent bands (a measure of the size of the variable subrepeat) was computed for each individual, this was .2-.3kb for three of the stocks, but .4kb for the Marquette stock. This latter stock had spacing between bands of .4, .8, 1.2kb etc, while the other stocks had predominately spacing of .2-.3kb, and .5-.6kb etc, indicating a smaller subrepeat size. The Gull Island Shoals stock had more variable bands between 3.4kb and 5.6kb than the other stocks, averaging 4 bands in this region, compared to 2-3 bands for the Marquette and Seneca Lake stocks. The Jenny Lake Stock had fewer total bands than the others. By comparing repeat size, number of bands in a given range and presence or absence of different bands, a flow chart was devised which allowed almost every individual to be sorted into the correct stock (See Figure 8, p. 20).

Discussion:

In this project we used restriction enzymes to study the intraspecific variability in the ribosomal DNA cistron in lake trout in order to determine if any of the variation was stock specific. Stock specific variation was found in the spacer region 3' to the 28S coding region using the DraI enzyme. The variation appears to involve differences in the size and sequence of a small repeating unit. Our next step is to clone and sequence this region so that stock specific probes can be prepared.

Restriction enzymes are useful in determining if length variation occurs in a specific DNA region and give a measure of sequence variation in that region. However, especially for relatively small regions, DNA sequencing is the best way to determine the exact amount of sequence divergence. In the past it has been necessary to carry out a laborious cloning procedure in order to obtain enough material for DNA sequencing. However, recently a new method for direct amplification of specific DNA sequences using the polymerase chain reaction (PCR) has been developed (Saiki et al., 1988). Any sequence can be amplified up to a million times if the sequence of short regions flanking to piece to be amplified are known. The desired sequence is synthesized using short oligonucleotide primers to the flanking sequences. The PCR technology will enable us to quickly clone and sequence specific regions from different individuals.

At the present time we are cloning several regions of the ribosomal DNA cistron from a lake trout gene library. As soon as a clone in the region containing the DraI polymorphism is identified, we will determine the DNA sequence of this region. Once the sequence is known we will be able to amplify the region directly from any individual using the polymerase chain reaction (PCR). This will greatly simplify the use of this polymorphism for surveying large numbers of individuals for stock identification.

Our restriction mapping of the ribosomal RNA cistron in lake trout has shown that it is composed of regions evolving at different rates (See Figure 1, p. 14). No intraspecific variation was found in the 18S or 28S coding regions, the external transcribed spacer containing the promoter region or in the internal transcribed spacer region containing the 5.8S coding region. Although we did not expect to find variation in the coding regions, we might have expected intraspecific variation in the internal transcribed spacer (ITS). Since our study used only restriction enzymes recognizing 6 base sequences (6 base cutters), and the ITS is a short segment of only 1.3 kb, intraspecific variation might be revealed by 4 base cutters or direct sequencing of this region. Since it is known that the sequences in the 18S and 28S regions flanking the ITS are highly conserved in vertebrates, we have designed primers to amplify this region in lake trout. We have now successfully amplified this region in several individuals from each stock using the polymerase chain reaction (PCR) and are examining sequence variation in this region directly (see Figure 9. p. 21).

Although we did not find any intraspecific variation in the coding regions, the internal transcribed spacer or the external transcribed spacer containing the promoter, these regions are especially useful for interspecific comparisons. In recent work (Phillips et al, 1990) we have been able to determine evolutionary relationships between lake trout and other members of the genus Salvelinus which have not been possible with any other genetic technique.

Because of the tremendous intraindividual variation in the IGS, these polymorphisms have great potential for use in DNA fingerprinting in lake trout and other salmonid fishes. In preliminary work we have shown that specific bands are inherited as if different variants are present at different chromosomal sites. If this is confirmed in additional crosses, these markers will have potential for marking specific fish and for genetic linkage studies.

Conclusions:

We have found that by selecting the appropriate region of the ribosomal DNA, genetic markers can be identified which are useful for evaluating phylogenetic relationships between species, for stock identification and for DNA fingerprinting.

Variation suitable for stock identification was found with the DraI restriction enzyme in the intergenic spacer (IGS) region of the ribosomal DNA just adjacent to the 28S coding region. The majority of

fish could be assigned to the correct stock using this polymorphism. The variation involves differences in the size and sequence of a small repeating element, so the next step is to sequence this region so that stock specific probes can be prepared.

In ongoing studies we are using DNA sequencing to examine this region and the internal transcribed region (ITS) for stock specific differences in DNA sequence. We are also developing primers for the polymerase chain reaction (PCR) for amplification ("direct cloning") of different regions of the lake trout rDNA. This will greatly simplify the use of these polymorphisms for surveying large numbers of individuals for stock identification.

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Table 1

Frequency of bands obtained when DNA from lake trout of different stocks was cut with PstI, electrophoresed in agarose gels, transferred to nylon filters, and hybridized with a probe to 28S rDNA.

	Stock			
	Jenny Lake N=12	Seneca Lake N=17	Marquette N=19	Gull Island Shoal N=12
kb band size:				
11.6-11.9	4 (.33)	0 (.00)	0 (.00)	0 (.00)
12.0-12.3	2 (.17)	12 (.70)	8 (.42)	4 (.33)
12.4-12.7	2	0	1	2
12.8-13.1	0	0	0	2
13.2-13.5	0	0	0	0
13.6-13.9	1 (.08)	0 (.00)	1 (.05)	4 (.33)
14.0-14.3	0	0	0	0
14.4-14.7	8 (.75)	15 (.88)	17 (.89)	9 (.75)
14.8-15.1	1	1	0	1
15.2-15.5	0	0	0	0
15.6-15.9	0	0	0	1
16.0-16.3	0	0	0	0
16.4-16.7	0	1	0	0
16.8-17.1	0	0	0	0
17.2-17.5	0	0	0	0
17.6-17.9	0	0	0	0
18.0-18.3	0 (.00)	1 (.06)	4 (.21)	1 (.08)
Above 18.4	0	1	2	1
Total bands:	18	41	33	25
Av # bands per fish:	1.5	2.4	1.7	2.1

(Numbers in parentheses indicate fraction of individuals possessing a given band. These are shown only for cases in which significant stock differences are found)

Table 2

Frequency of bands obtained when DNA from lake trout of different stocks was cut with PstI and BglII, electrophoresed in agarose gels, transferred to nylon filters, and hybridized with a probe to 28S rDNA

	Stock			
	Jenny Lake N=14	Seneca Lake N=15	Marquette N=14	Gull Island Shoal N=13
kb band size:				
6.2-6.4	1	0	0	0
6.5-6.7	2	6	3	2
6.8-7.0	4	1	2	4
7.1-7.1	1	4	0	2
7.4-7.6	7	4	6	4
7.7-7.9	3	4	4	2
8.0-8.2	4	6	0	5
8.3-8.5	3	5	9	6
8.6-8.8	6	4	0	3
8.9-9.1	6	3	8	3
9.2-9.4	0	3	2	1
9.5-9.7	3	3	4	4
9.8-10.0	1	0	2	2
10.1-10.3	7	2	4	1
10.4-10.6	0	2	1	1
10.7-10.9	0	3	3	3
11.0-11.2	1	0	1	0
11.3-11.5	0	0	4	1
11.6-11.8	2	1	1	0
11.9-12.1	0	0	1	1
12.2-12.4	0	0	1	0
12.5-12.7	0	0	4	1
12.8-13.0	0	0	0	0
13.1-13.3	0	2	0	0
13.4-13.6	0	0	0	0
13.7-13.9	0	0	0	0
14.0-14.2	0	1	0	0
Over 15	0	0	2	2
Total bands:	51	55	62	49
Av. total bands per fish:	3.6	3.7	4.4	3.7

*All individuals had a constant band at 3.7-3.9kb in addition to those shown here.

Table 3

Frequency of bands obtained when DNA from lake trout of different stocks was cut with EcoRI, electrophoresed in agarose gels, transferred to nylon filters, and hybridized with a probe to 18S rDNA

	Stock			
	Jenny Lake N=16	Seneca Lake N=17	Marquette N=22	Gull Island Shoal N=12
kb band size:				
5.7-6.0	10	8	13	6
6.1-6.4	6	8	10	5
6.5-6.8	5 (.31)	0 (.00)	1 (.05)	0 (.00)
6.9-7.2	1	0	1	1
7.3-7.6	1	0	0	0
7.7-8.0	0	3	0	2
8.1-8.4	3 (.19)	1 (.06)	7 (.32)	0 (.00)
8.5-8.8	6	4	4	4
8.9-9.2	4	4	8	2
9.3-9.6	2 (.13)	4 (.24)	5 (.23)	6 (.50)
9.7-10.0	4	5	5	3
10.1-10.4	4 (.25)	2 (.12)	0 (.00)	1 (.08)
10.5-10.8	1 (.08)	7 (.41)	7 (.32)	1 (.08)
10.9-11.2	5	3	5	3
11.3-11.6	2	0	0	0
11.7-12.0	2 (.13)	6 (.35)	11 (.50)	5 (.42)
12.1-12.4	1	0	0	1
12.5-12.8	1	2	0	0
12.9-13.2	0	0	0	1
13.3-13.6	0 (.00)	5 (.29)	2 (.09)	0 (.00)
13.7-14.0	0	0	0	0
14.1-14.4	0	1	0	0
14.5-14.8	0	2	1	0
14.9-15.2	0	0	0	1
15.2-15.6	0	3	0	0
15.7 +	0	3	0	4
Total bands:	58	71	80	46
Av. total bands per fish:	3.6	4.1	3.6	3.8

(Numbers in parentheses indicate fraction of individuals possessing a given band. These are shown only for cases in which significant stock differences are found)

Table 4

Frequency of bands obtained when DNA from lake trout of different stocks was cut with DraI, electrophoresed in agarose gels, transferred to nylon filters, and hybridized with a probe to 28S rDNA

	Stock			
	Jenny Lake N=18	Seneca Lake N=17	Marquette N=17	Gull Island Shoal N=15
kb band size:				
3.3-3.4*	18	17	17	15
3.5-3.6	3	3	0	0
3.7-3.8	2 (.11)	4 (.24)	7 (.41)	5 (.33)
3.9-4.0	11 (.61)	8 (.47)	4 (.24)	3 (.33)
4.1-4.2	6	5	7	8
4.3-4.4	2	5	4	5
4.5-4.6	6 (.33)	3 (.18)	8 (.47)	7 (.47)
4.7-4.8	5	5	5	6
4.9-5.0	6	4	7	5
5.0-5.2	4 (.22)	8 (.47)	4 (.24)	5 (.33)
5.3-5.4	4 (.22)	1 (.06)	0 (.00)	4 (.33)
5.5-5.6	2 (.11)	0 (.00)	4 (.24)	5 (.33)
5.7-5.8	1	0	2	1
5.9-6.0	0	0	0	1
6.1-6.2	0 (.00)	5 (.29)	1 (.06)	1 (.07)
6.3-6.4	0	0	3	1
6.5-6.6	0	1	0	0
6.7-6.8	0	0	0	0
6.9-7.0	0	1	0	0
7.1.-7.2	0	0	0	0
7.3-7.4	0	0	1	0
7.5-7.6	0	2	0	0
Above 8kb:	0 (.00)	7 (.41)	2 (.12)	0 (.00)
Total btw 3.4-5.5:	51	46	47	53
Av. total bands per fish:	2.8	2.7	2.9	3.5

*Constant band found in all fish spanning the coding regions, not included in calculations of total bands.

Table 5

Frequency of different banding patterns obtained with DraI

	Stock			
	Jenny Lake	Seneca Lake	Marquette	Gull Island Shoal
I. Minimum Band Spacing:				
.2-.3 or .5-.6	100%	100%	0%	100%
.4 or .8kb	0%	0%	82%	0%
Other:	0%	0%	18%	0%
II. # of bands btw 3.5-5.5kb:				
2-3	92%	100%		47%
4-5	8%	0%		53%
III. Freq. of specific bands in fish with 2-3 bands btw 3.5-5.5kb:				
3.8kb	0%	24%		0%
5.2kb	0%	41%		14%
5.6kb	0%	0%		100%
Above 5.6kb	0%	53%		14%
IV. Freq. of the 5.6kb band in fish with 4-5 bands btw. 3.5-5.5kb:	0%			100%

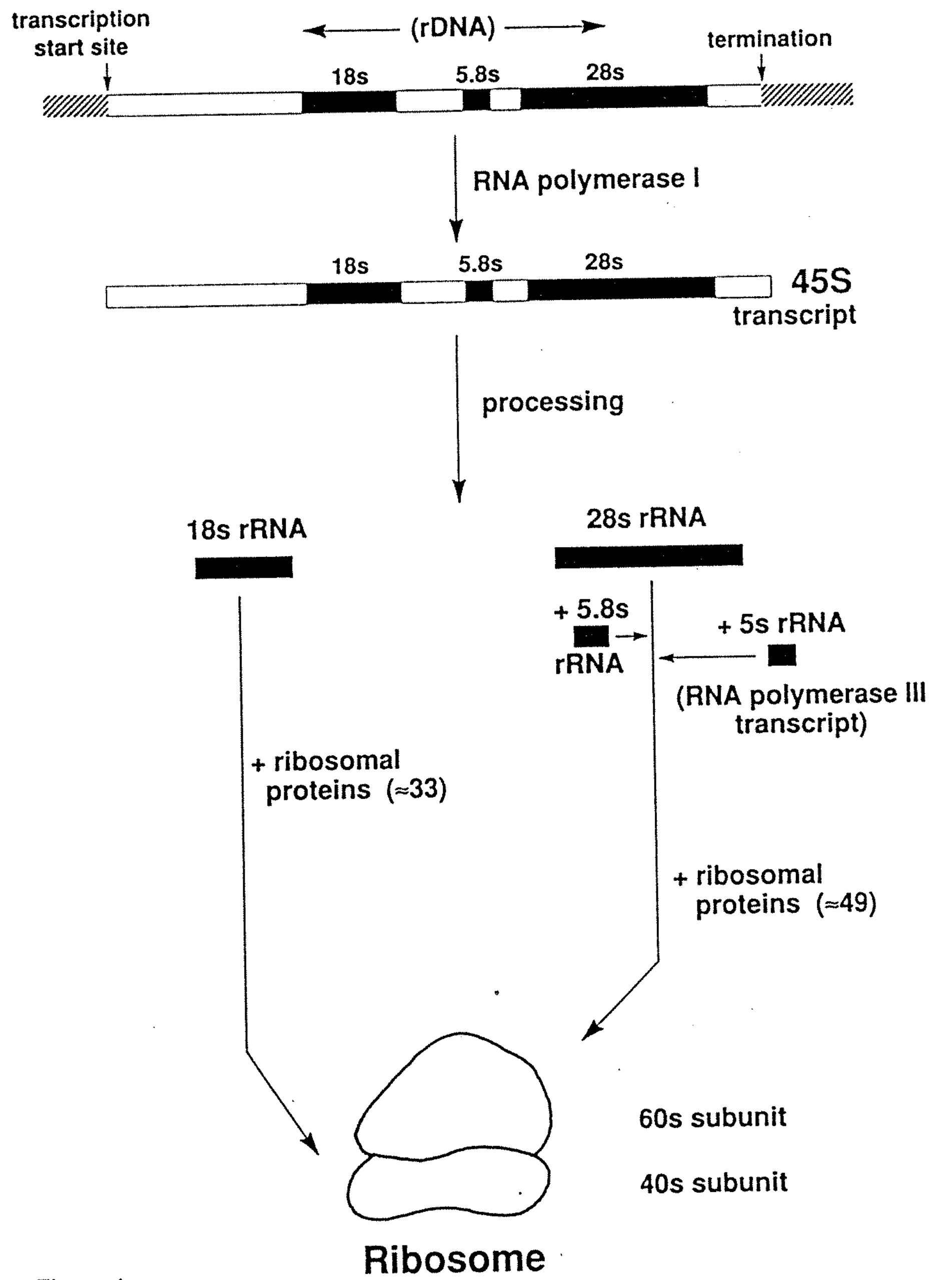
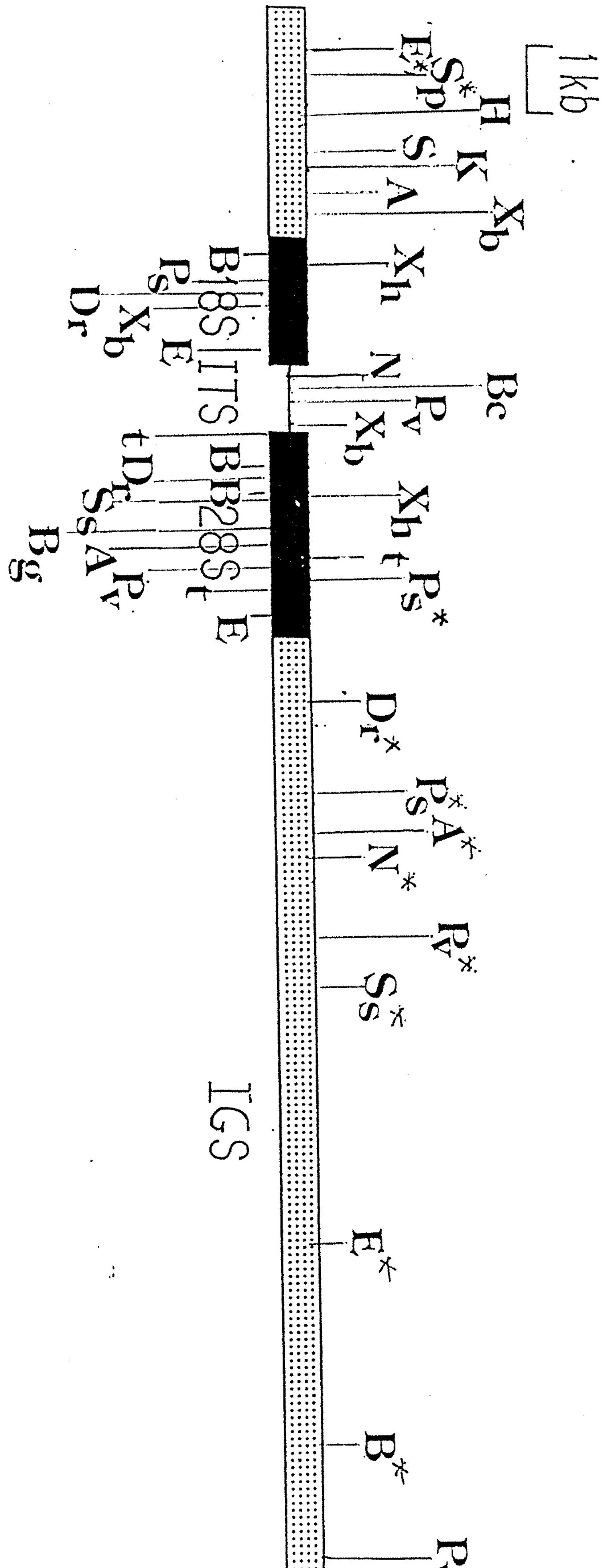


Figure 1


LAKE TROUT
1kb
18S
ITS
28S
IGS