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Application of Genetic Techniques to Stock Identification in Lake Trout

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ABSTRACT

Three different types of assays were evaluated to determine their suitability for identification of lake trout stocks. Investigation of these assays was based on previous research involving chromosome polymorphisms in lake trout stocks and a preliminary analysis of the nuclear rRNA cistrons in lake trout. The chromosome research included a study of stock differences in the number and chromosomal location of the nucleolar organizer regions (NORs), which are the chromosomal sites of the rRNA cistrons. Significant differences in the number and chromosomal location of the NORs was found in different lake trout stocks (Phillips and Ihssen, 1985). Since chromosome assays require living cells, we wished to examine whether this genetic difference could be detected by using nucleoli counts from slides made directly from gill tissue or by determining the amount of rDNA per cell. Finally we wished to determine whether there might be stock differences in the size of the rRNA cistrons (rDNA) since a size polymorphism in the repeating unit of these multicopy genes was detected during a survey of different lake trout individuals. For the nucleoli counts we compared individuals from the Marquette stock with the Jenny Lake stocks and for the other assays, individuals from three lake trout stocks were studied: Jenny Lake, Lake Seneca and Gull Island Shoals.

Results showed that the first two assays are not very suitable for stock identification. Although nucleoli counts could distinquish between Jenny Lake and Marquette embryos and 6 month old fingerlings, by 1 year

of age, the differences were of borderline significance. No significant differences were found in concentration of rDNA in the different stocks.

Results from the third assay showed that there are stock differences in the genetic structure of the rRNA cistrons (rDNA) which could be used for stock identification. Significant differences in both the types and frequencies of size variants in the rDNA were found for the three stocks. This means that the rDNA exists in several alternative sizes which vary in the different stocks and it should be possible to construct probes which will be specific for each type. These could be used in a simple dot blot assay for stock identification similar to the DNA assays now being used in diagnostic medicine. An assay utilizing rDNA is especially attractive since these genes are very abundant in the cell and could be detected without the use of a radioactive probe.

INTRODUCTION:

Three different types of assays were evaluated to determine their suitability for identification of lake trout stocks. Investigation of these assays was based on previous research involving chromosome polymorphisms in lake trout stocks and a preliminary analysis of the nuclear rRNA cistrons in lake trout.

In the case of the first two assays, we wished to determine whether the chromosome differences between the stocks could be detected by other methods which would be simplier to apply to population studies. Previous chromosome research had shown that there were stock differences in the number and chromosomal location of the nucleolar organizer regions (NORs), which are the chromosomal sites of the rRNA cistrons (Phillips and Ihssen, 1985). Since chromosome assays require living cells, we wished to examine whether this genetic difference could be detected by using nucleoli counts from slides made directly from gill tissue or by determining the amount of rDNA per cell.

In the case of the third assay, we wished to determine whether there might be stock differences in the size of the ribosomal RNA (rRNA) cistrons. The ribosomal RNAs are an integral part of the ribosome which is the site of protein synthesis in the cell. There are three types of rRNAs in higher organisms: 5.8S, 18S and 28S. The 18S and 28S genes (rDNA) occur as multiple copies of a repeating unit in higher organisms (Figure 1). Salmonid fishes have an unusually large number of these genes (2,000 copies per haploid genome), possibly because the relatively large size of salmonid eggs requires a large volume of ribosomal RNA for development.

Although the structure of the coding region is usually the same in all

of the multiple copies of the rDNA within one individual and within a species, intraspecific and sometimes intraindividual differences occur in the non-transcribed spacer region which result in repeating units of different sizes (reviewed in Gerbi, 1985). Intraindividual differences have been correlated with different chromosomal locations of the rDNA clusters (Arnheim, et al, 1982). Stock specific differences in the size and structure of the repeating unit of the rDNA have been found in a number of different organisms including Drosophilia (Williams et al, 1985), mice (Suzuki et al, 1986), treefrogs (Romano and Vaughn, 1986), and several plants (Dutta et al, 1987). In a preliminary survey of the structure of the rRNA cistons (rDNA) in salmonid fishes (Popodi et al, 1985) we found a size polymorphism in the repeating unit of these multicopy genes in lake trout. We wished to determine if there were stock differences in the size of the rDNA repeating unit which could be utilized for stock identification.

For the first two assays we compared individuals from the Marquette stock with the Jenny Lake stock and for the third assay, individuals from three lake trout stocks were studied: Jenny Lake, Lake Seneca and Gull Island Shoals.

OBJECTIVES:

To evaluate three possible assays for lake trout stock identification: (a) nucleoli counts, (b) concentration of rDNA per cell and (c) rDNA length polymorphisms. These three assays will be considered separately.

MATERIALS AND METHODS:

A. Nucleoli Counts:

Fingerlings from two year classes were obtained from the Jenny Lake, Wyoming and Marquette, Lake Superior stocks. Fish were tested at 6 months and 18 months of age. A small piece of gill tissue from 25 fingerlings from each age group was fixed in Carnoy's fixative. Small pieces of fixed tissue were minced in 45% acetic acid and slides were prepared and stained with the silver staining method for AgNORs and nucleoli of Howell and Black (1980).

Two slides were scored for each individual and 50 cells were scored on each slide. The number of nucleoli bper cell were scored and the average number per cell per fish were determined.

B. Concentration of rDNA per cell:

Liver tissue was obtained from 10 individuals of the Jenny Lake stock and the Marquette stock. The tissue was extracted using SDS and Phenol to obtain DNA, which was precipitated with ethanol and resuspended. To quantitate the amount of ribiosomal DNA present, the DNA concentration was determined spectrophotometrically based on UV absorbance. Samples of known DNA concentration were bound to nitrocellulose filter and cloned rDNA labled with P^{32} from Chinese hamster hybridized to the filter. Comparison with known standards permitted quantitation of the amount present.

C. rDNA length polymorphisms:

Liver tissue was obtained from 12 individual trout from three

different lake trout stocks: Seneca Lake, New York; Gull Island Shoals, Wisconsin; and Jenny Lake, Wyoming. Nuclear fractions were obtained from macerated trout liver as described in Popodi et al (1985). DNA was isolated by SDS phenol extraction of nuclei.

DNA samples were digested with restriction enzymes (Bam and Hind III) previously shown to reveal length polymorphisms in the rDNA repeating unit (Popodi et al, 1985). Restriction digests were performed at 37C for 4 hours using 3ug DNA as recommended by the supplier. Following digestion samples were heated at 65C and separated by electrophoresis through .6% agarose gels for 17 hr at 35V. Hind III-cleaved lambda DNA and Hae III cleaved phi X 174 DNA were run as standard size markers in parallel lanes. DNA was visualized with ethidium bromide and photographed. The gels were treated with .12N HCl and the DNA transferred to nitrocellulose filters as described by Southern (1975).

DNA was transferred to nitrocellulose filters from agarose gels and baked at 80C for 2 hr under vacuum. Filters were prepared for hybridization by washing for 12 hr at 65C in modified Denhardt (1966) solution (3X SSC, 0.1% SDS, 0.02 each of the polyvinyl pyrrolidon yeast tRNA. Hybridizations were performed in 4 SSC, 0.1M TRis-HCl, pH 7.9, 50% formamide, .25ug/ml yeast tRNA at 37C for 24hr. After hybridization, the filters were washed at 65C at 4xSSC. Filters were dried and exposed to X-Omat film (Kodak) at -70C with an intensifying screen (DuPont-Cronex).

To identify polymorphisms in the ribosomal RNA genes of lake trout we used the cloned rDNA from Chinese Hamster as a probe. Since the coding regions of the ribosomal RNA genes are highly conserved between mammals and fishes, a mammalian probe can be used and we had obtained a restriction map of lake trout rDNA previously (Popodi et al, 1985) using

this probe. The DNA was made radioactive by a nick translation reaction (Rigby et al, 1977) incorporating (alpha P^{32}) dCTP. The gels were photographed and the number and position of bands was determined. Frequency of the different types of polymorphisms was scored for each stock and comparisons made.

RESULTS:

A: Nucleoli Counts:

Results of the nucleoli counts are shown in Table 1. The number of nucleoli per cell was significantly different in the two stocks when the fish were tested at 6 months of age, but not when the fish were tested at 18 months of age.

B: Concentration of rDNA per cell:

No significant differences were found between the concentration of rDNA per cell between the two stocks when measured as described above. This method involved applying genomic DNA of the same concentration to gels which were electrophoresed and transferred to nitrocellulose and probed with radioactive cloned rDNA. Bands were of approximately the same intensity from the two stocks.

C: rDNA Length Polymorphisms:

Significant differences were found between the three stocks in the sizes of the rDNA repeating unit (see Table 2) as determined by digestion with the Bam enzyme and probing with P^{32} labeled rDNA.

Previous results (Popodi et al, 1985) showed that the rDNA repeat unit occurred in at least two sizes, approximately 24kb and 26kb in length (see Figure 1). The variable portion was in the non-transcribed spacer (NTS) region and this region was contained within a fragment which varied between 19 and 22kb in length after digestion with the restriction enzyme, Bam. Bam cleaved the repeating unit into three pieces, two smaller pieces found in all fish which are 3kb and .3kb in length and a large piece which varied between 19 and 22kb in length.

When DNA from three different lake trout stocks was digested with the Bam enzyme in the present study, four different sizes of the repeating unit were found. The large fragment after Bam digestion occurred in the following sizes: type A- 21.5-22kb, type B- 19-19.5kb, type C- 15-15.5kb, and type D- 13-13.5kb. These were found either singly or in different combinations in different individuals (See Figure 2), so that there were five different genotypes: A, B, AB, ABC, and ABD.

As shown in Table 2, in the Seneca Lake stock, 70% were of type AB and 30% of type A. In the Gull Island Shoals stock, 88% were of type A, and 12% of type AB. Types B, ABC and ABD were found only in the Jenny Lake stock. In this stock, there were 25% type A, 38% type B, 12% AB, 12.5% ABC and 12.5% ABD.

DISCUSSION:

Previous work had shown that the number of NORs per cell was signficantly different in different lake trout stocks, with the Jenny Lake Wyoming stock having an average of 3.9 NORs per cell, the southern Lake Superior (Gull Island Shoals, Marquette) stocks having an average of 7.8 NORs per cell and the northern Lake Superior (Michipicoten, Lake Killala)

and Lake Huron stocks having an average of 10.8 NORs per cell. Although chromosome preparations must be obtained to determine the number of NORs per cell, we have shown in other research on rainbow trout and coho and chinook salmon the difference in the number of NORs between diploid cells (average of 1.5 NORs/cell) and triploid cells (average of 2.5 NORs/cell) of these species is correlated with the number of nucleoli in cells from gill tissue. Thus nucleoli counts can be used to identify triploid cells in these species (Phillips, Ihssen, and Johnson, 1986). The number of NORs/ cell went down with age in both diploid and triploid cells, but the difference between diploids and triploids at 18 months was still great enough to differentiate the two types of cells. Our results on lake trout stocks show that the intraspecific difference in number of NORs per cell is also reflected in the number of nucleoli per cell in 6 month old fingerlings, but declines with age, so that it cannot be used to distinguish the stocks by 18 months of age. It is known that nucleoli tend to fuse in cells which are not rapidly dividing, and this is the most likely explanation for these results. Since we want a genetic marker which does not vary with the age or physiology of the individual, this assay does not meet our requirements.

The assay involving differences in the genetic structure of the rRNA genes (rDNA) appears very promising for future work for several reasons. First, our preliminary survey has shown that there are several different sizes of the basic repeating unit of the rDNA and that there are differences in the number and different types of the rDNA found in different stocks. These differences are the result of a segment of variable size in the non-transcribed spacer (NTS) region of the rDNA (see figure 1). Currently we are preparing a fine structure restriction map of

this segment in order to determine the location and extent of polymorphism in this region. When we have a good map of this variable region, we should be able to select restriction enzymes which will be best suited for use in population surveys. We are cloning the rDNA from lake trout, so that we can obtain a better restriction map from cloned DNA and prepare specific lake trout rDNA probes for the variable region.

A second advantage to the use of the rRNA gene assay is that these genes are very abundant, at 2,000 copies per cell. This means that in the long term we should be able to use a biotinylated probe instead of a radioactive probe, which would make this assay available to laboratories not certified for radioisotope use. In addition, after specific probes are obtained for the different types of rDNA found in lake trout populations, it should be possible to devise a dot-blot assay for stock identification. In this assay, DNA digested with a specific restriction enzyme is fixed to a filter and probed with a specific probe. This eliminates several steps, including electrophoresis and transfer of the DNA from the gel to the nitrocellulose filter. Such simplified DNA assays are being increasingly used in diagnostic medicine (Finegold, 1986; Rabin and Dattagupta, 1987) and would be very advantageous for population studies.

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Table 1. Frequency of Individuals with Different Numbers of Nucleoli Per Cell in Lake Trout Stocks

Stock	Age	Number of Nucleoli/Cell		Sample Size
		1-2	3-5	
Marquette	6 months	44%	56%	25
	18 months	70%	30%	20
Jenny Lake	6 months	72%	28%	25
	18 months	79%	21%	25

Table 2. rDNA Phenotypes* found in Lake Trout

Type A: one band 21.5-22kb,

Type B: one band 19-19.5kb,

Type AB: two bands- 21.5-22kb and 19-19.5kb,

Type ABC: three bands, 21.5-22kb, 19-19.5kb and 17-17.5kb,

Type ABD: three bands, 21.5-22kb, 19-19.5kb and 15-15.5kb..

**Phenotypes represent variations in the size of the repeating unit of the rDNA as determined by the size of the largest piece obtained after digestion with Bam. Individuals had one, two or three bands of variable sizes as shown.

Table 3. Frequency of rDNA Size Variants* in Lake Trout Stocks

Stock	rDNA Restriction Phenotypes**					Total
	A	B	AB	ABC	ABD	
Seneca Lake	30%	--	70%	--	--	12
Gull Island Shoals	88%	--	12%	--	--	8
Jenny Lake	25%	38%	6%	12%	12%	8

* rDNA size variants were determined by digesting genomic DNA with the restriction enzyme Bam, and Southern blotting with a 28S rDNA probe from Chinese hamster. The largest piece obtained in this digestion is of variable size, labeled A, B, C or D. This piece contains the nontranscribed spacer region (NTS), which is of variable length. Some individuals have more than one large fragment, indicating that the rDNA repeating units in these individuals are present in more than one size class.

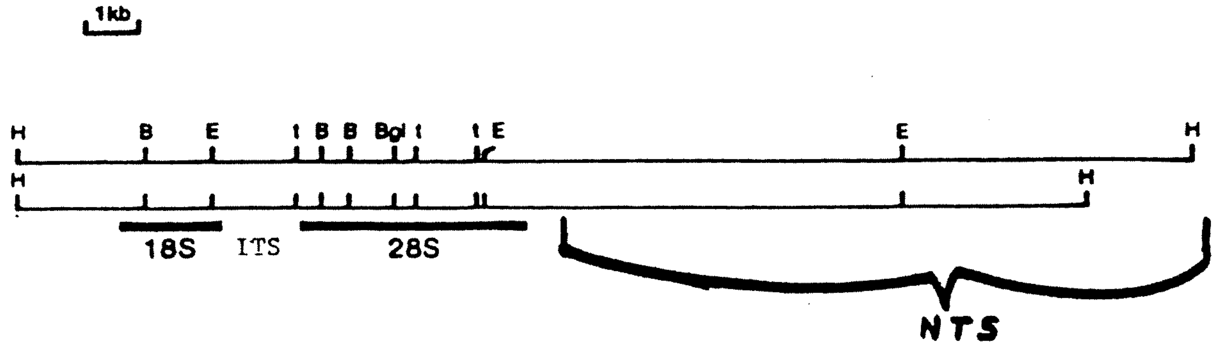


Figure 1: Restriction map of lake trout rDNA showing the repeating unit with the 18S and 28S coding regions, the internal transcribed region (ITS) and the non-transcribed spacer region (NTS) which is of variable length. Note that there are three Bam (B) restriction sites, so that this restriction enzyme would cleave the unit into three pieces, two small pieces of 3kb and .5kb in size and a large piece which varies in size since it contains the NTS which is variable in length. (The 3kb piece includes most of the 18S coding region, the ITS and the left end of the 28S unit; the .5kb piece is entirely within the 28S coding region and the large variable piece includes two thirds of the 28S coding unit, the entire NTS and the left end of the next 18S coding unit.) Two different sizes of the repeating unit are shown here; results described in this paper indicate that the NTS exists in at least four different sizes.

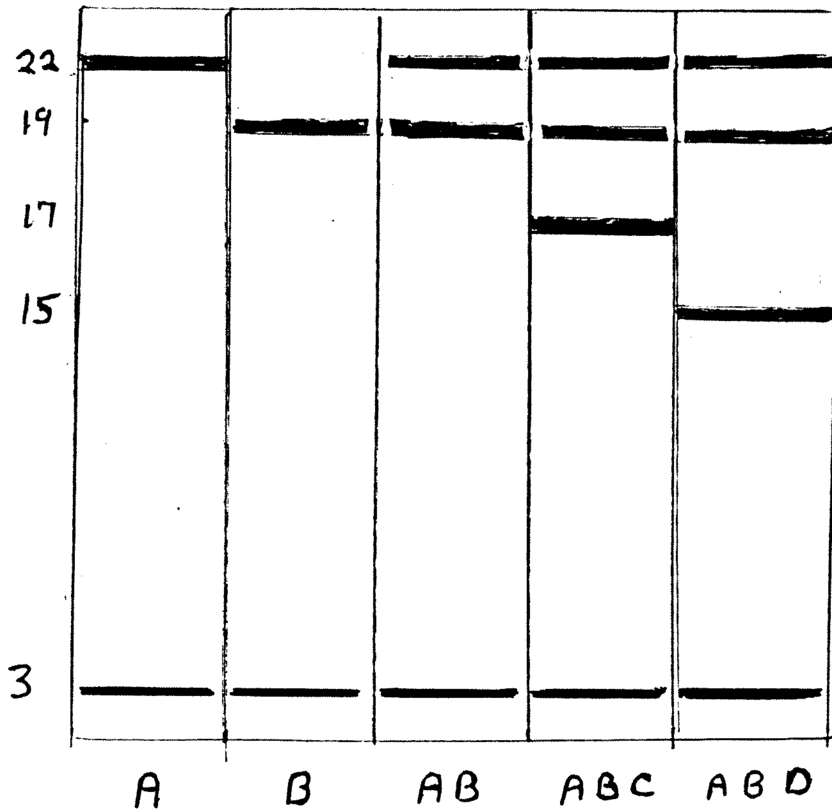


Figure 2: Illustration of the five different restriction phenotypes found after digestion of genomic DNA with Bam and probing with radioactive 28S probe from Chinese Hamster. All of the phenotypes have the 3kb fragment and the .5kb fragment (too small to be detected on these gels) but they vary in the number and sizes of the large fragment (15-21.5kb).

Figure 4. Band patterns obtained when DNA from 3 different lake trout from the Jenny Lake, Marquette, Gull Island Shoal, and Seneca Lake stocks was cut with HindIII and BglII enzymes, electrophoresed in .6% agarose gel and hybridized with a probe to 28S rDNA. These two enzymes each cut only once in the repeating unit. HindIII enzyme cuts in the external transcribed spacer (ETS) and the BglII enzyme cuts in the 28S coding region. The double digest produces a constant 6.5kb band and variable bands which include part of the 28S and the entire spacer region. Each individual has a different pattern of bands.

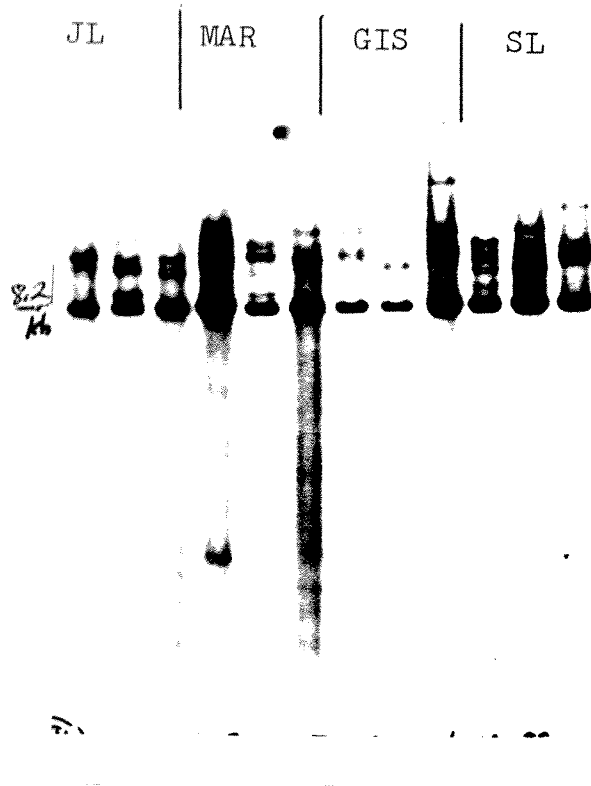
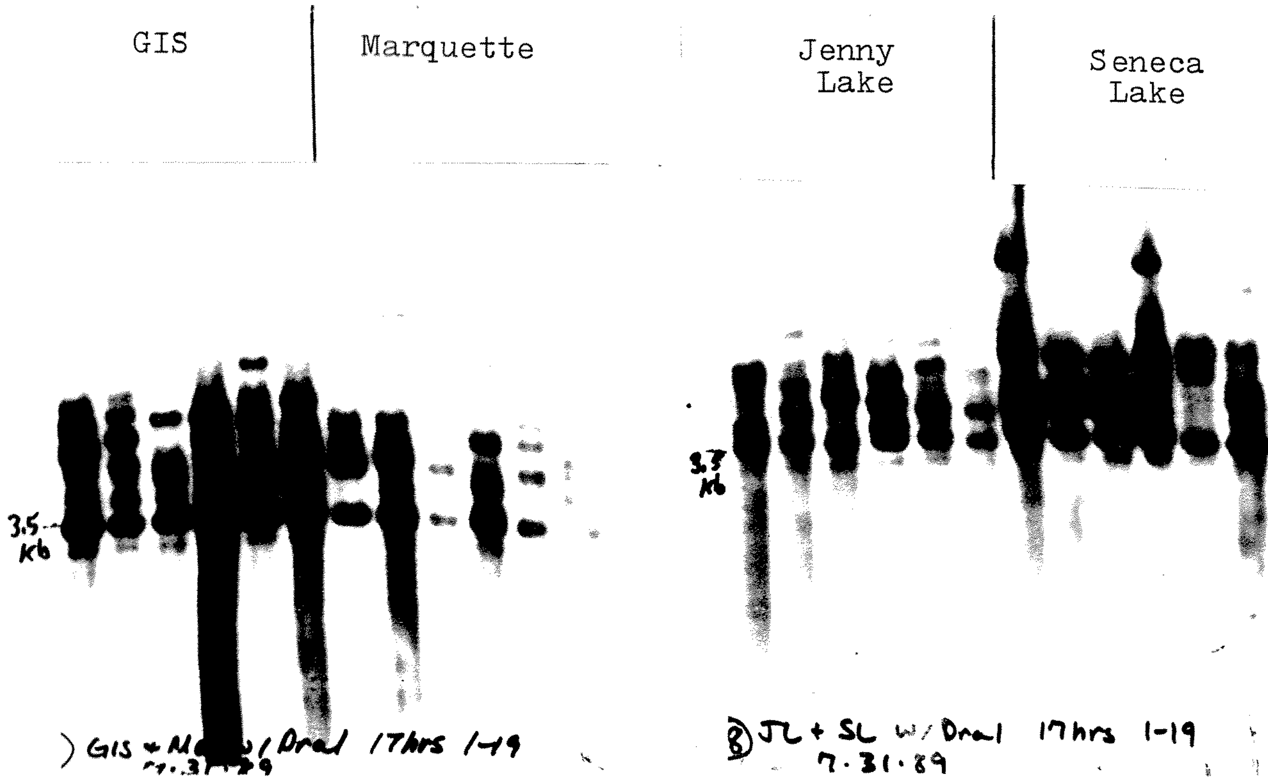


Figure 5. Band patterns obtained when DNA from 3 different lake trout from the Jenny Lake, Marquette, Gull Island Shoal and Seneca Lake stocks was cut with the SspI enzyme, electrophoresed in .6% agarose gel and hybridized with a probe to 28S rDNA. This enzyme cuts once in the external transcribed spacer and once in the 28S coding region to produce a constant band of 8.2kb. This enzyme cuts again at variable places in the spacer region to produce additional bands of 8.8-16kb.



Figures 6 and 7. Band patterns obtained when DNA from 6 different lake trout from the Gull Island Shoal, Marquette, Jenny Lake, and Seneca Lake stocks was cut with the DraI enzyme, electrophoresed in .6% agarose gel and hybridized with a probe to 28S rDNA. This enzyme cuts once in the 18S coding region and once in the 28S coding region to produce a constant band at 3.5kb and again in the spacer region to produce variable bands between 3.7 and 7kb. Although many different patterns are found, stock differences in the number of bands and spacing between the bands was found.

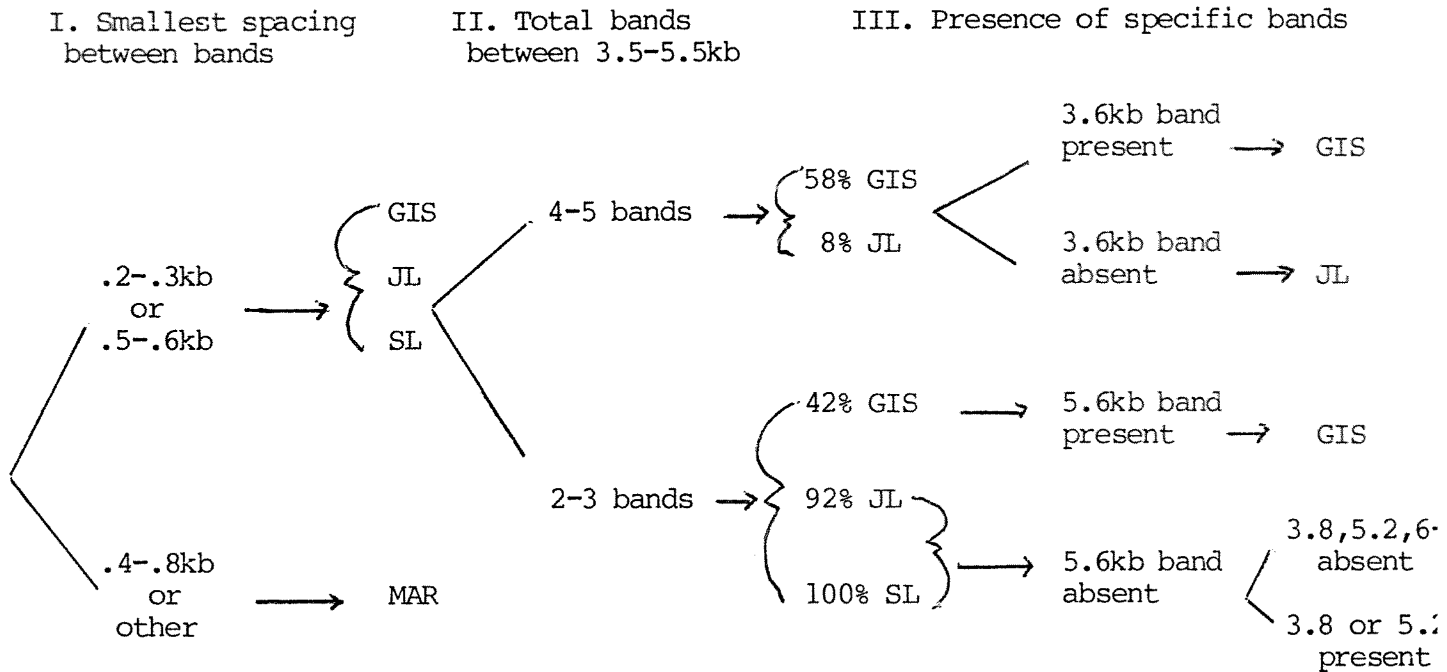


Figure 8. Flow chart showing how the DraI polymorphism can be used to identify the sto lake trout (MAR= Marquette, GIS= Gull Island Shoals, JL= Jenny Lake, SL= Seneca Lake)

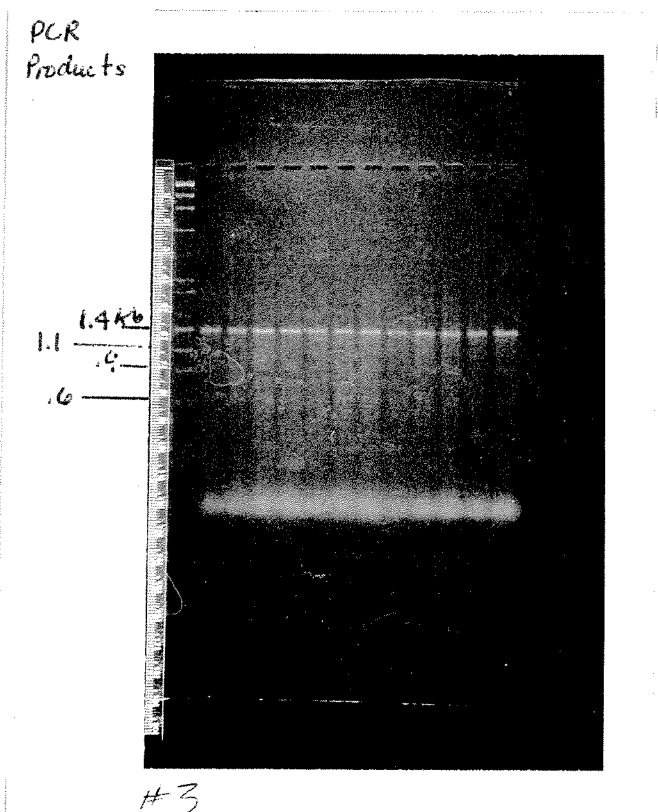


Figure 9. Photograph of a ethidium bromide stained gel of PCR products