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ABSTRACT

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Attempts to rehabilitate Great Lakes lake trout (Salvelinus namaycush) populations rely on stocking programmes based on fish from various hatchery strains. Some natural recruitment is now occuring, but fisheries managers currently have no method of determining which strains are successfully producing offspring. Recent studies of freshwater fishes indicate that base sequence divergence in mitochondrial DNA (mtDNA) may be useful for stock identification, and that known mtDNA restriction fragment polymorphisms may serve as genetic tags for introduced fish. Thus, mtDNA markers could be used to determine which lake trout strains are providing the maternal parents of these naturally recruited offspring. Moreover, it should be possible to produce marked lake trout by selectively breeding female broodstock, such that their offspring have unique mtDNA haplotypes.

Prior attempts to exploit mtDNA variation as a genetic tag have been limited by the need to sacrifice fish in order to purify mtDNA. In this report we describe techniques which permit the mtDNA genotypes of live fish to be determined. These techniques involve probing the mtDNA fragments in total DNA extracts, obtained from blood or small tissue samples, with radioactively labelled mtDNA. Our results show that 0.5 mL blood samples provide a reliable source of DNA and that mtDNA fragments can be readily resolved with a 32P-labelled lake trout mtDNA probe. We demonstrate the usefulness of this method in the analysis of both lake trout broodstocks and natural populations.

Work on broodstock involved determination of mtDNA haplotypes in parent fish and breeding of selected parents. Results confirm that mtDNA is maternally inherited in lake trout and that genetically tagged fingerlings could be produced. The ability to produce mitochondrially marked lake trout and to detect these markers in live fish, makes it possible for fisheries managers to use such fish in stocking and rehabilitation programmes.

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# INTRODUCTION

Great Lakes lake trout (Salvelinus namaycush) populations crashed in the 1940's and 1950's, largely as a result of predation by the sea lamprey (Petromyzon marinus), although overfishing and pollution were also implicated (Eshenroder et al. 1984). In fact, the lake trout was extirpated from Lake Ontario, Lake Erie and Lake Michigan, and only a few natural populations remained in Lake Superior and in Georgian Bay of Lake Huron. Since the 1960's, when more effective control of lamprey populations was achieved using 3-trifluormethyl-4-nitrophenol (TFM), extensive efforts have been made to rehabilitate existing Great Lakes lake trout stocks and to reestablish self-sustaining populations from hatchery stocks.

In most instances, rehabilitation has been achieved by planting a variety of lake trout strains originating from different locations in the Great Lakes. In certain instances, fish from these various strains were differentially fin-clipped to permit comparison of their survival. However, this does not allow for an assessment of their relative reproductive success in the wild. In fact, recruitment has been poor, possibly due to unsuitable or polluted conditions on the spawning reefs, or because inappropriate genetic strains have been used in the plantings (Eshenroder et al. 1984). Even when introduced fish reproduce in the wild (e.g. Jude et al. 1981; Nester and Poe 1984; Marsden et al. 1988), it is currently impossible to ascertain which strains may be successfully reproducing. Thus, a primary difficulty facing the lake trout rehabilitation program

is the lack of an unambiguous "tag" permitting discrimination of the various lake trout brood stocks, or for the discrimination of stocked fish from remnant natural populations.

As artificial tags and fin-clips are only useful for a single generation and thus, are unable to provide information on the recruitment success of stocked fish, efforts have been made to identify intrinsic biological tags. Attempts have been made to discriminate historical lake trout stocks on morphological and behavioural differences (Brown et al. 1981; Goodier 1981), but most of these stocks are now extinct. Moreover, the applicability of morphological and meristic characters for stock identification is complicated by the fact that phenotypic variability in these characters need not have a genetic basis. In fact, meristic variation in fish is very responsive to environmental rearing condition during early ontogeny (see review by Lindsey 1988). As a consequence, efforts have been made to develop genetic tagging systems.

Since the 1970's, much of this work on genetic tags has focussed on electrophoretic studies, because variation in allozyme patterns ordinarily has a simple genetic basis (Ihssen et al. 1981). However, only small gene frequency differences have been detected among lake trout brood stocks even though substantial (50-100) numbers of loci have been surveyed (Dehring et al. 1981; Todd 1981; Phillips and Ihssen 1986; Ihssen et al. 1988). While, such variation provides an indication of population subdivision, it is not dramatic enough to permit the assignment of individual fish to a specific stock on anything more than a probabalistic basis. In response to this lack of

divergence, Ihssen et al. (1981) suggested marking hatchery stocks with otherwise rare alleles, in an attempt to follow the fate of introduced fish; this method has been used with some success in walleye (Ward and Clayton 1975; Schweigert et al. 1977; Murphy et al. 1983). However, allozymically marked fish can only be distinguished from residents for one or two generations at the most, because of recombination and exchange of genetic material between resident and introduced fish.

Efforts to develop better methods of genetic tagging, based on immunological and karyological studies, and ribosomal probing of nuclear DNA digests, are currently in progress. However, in common with protein electrophoresis, these techniques all rely on the examination of the nuclear genome (or products coded for by nuclear DNA). This is unfortunate as such genetic tags will not persist in the face of strain hybridization and recombination.

Recent studies of freshwater fish indicate that base sequence divergence in mitochondrial DNA (mtDNA) may be useful for stock identification (Avise et al. 1984; Wilson et al. 1985, 1987; Bermingham and Avise 1986; Thomas et al. 1986; Grewe 1987; Gyllensten and Wilson 1987; Billington and Hebert 1988; Grewe and Hebert 1988). Furthermore, known mtDNA restriction fragment polymorphisms may serve as genetic tags in introduced fish (Ferris and Berg 1987; Grewe 1987; Billington and Hebert 1988; Grewe and Hebert 1988). The study of mtDNA variation is based on restriction endonuclease digestion of the molecule with the resulting fragments being separated by molecular weight using gel electrophoresis. Variability in fragment patterns is interpreted

as genetic variation at the nucleotide level (see example in Ferris and Berg 1987).

Examination of intraspecific mtDNA variation has several advantages over traditional methods of investigating genetic diversity among conspecific individuals (reviewed by Avise et al. 1987; Ferris and Berg 1987). Animal mtDNA is a circular molecule approximately 17,000 base pairs (bp) in length and is present in high copy number per cell, making it relatively easy to purify. It is expected that mtDNA, with its unisexual haploid mode of inheritance, would give approximately 4 times better resolution than allozymes for deliniating populations than methods based on the nuclear genome, with its bi-sexual diploid mode of inheritance (Takahata and Slatkin 1984; Takahata and Palumbi 1985; Crease et al. 1989). Moreover, the rate of mtDNA nucleotide substitutions among higher vertebrates is approximately 5-10 times that of nuclear DNA (Moritz et al. 1987) further enhancing the resolution capability of mitochondrial DNA studies. Finally, the strict maternal inheritance of mtDNA (Hutchinson et al. 1974; Gyllensten et al. 1985) may prove particularly useful in the context of fish population studies, especially in philopatric species such as walleye (Billington and Hebert 1988) and lake trout (Grewe 1987; Grewe and Hebert 1987, 1988).

Recently, 14 different mitochondrial clones were detected in 180 lake trout representing 12 different broodstocks and natural populations (Grewe 1987; Grewe and Hebert 1987, 1988). Genetic distance analysis showed that these 14 clones belonged to three well differentiated groups (designated A,B,C), suggesting that

the Great Lakes were likely colonized by fish from three different refugia following the Pleistocene glaciation. From a management standpoint, it is significant that the frequencies of these major clonal groups show a clear geographic pattern, with group B dominant in the eastern broodstocks, group A dominant in central Great Lakes broodstocks, and group C in the west (Grewe and Hebert 1988). On the basis of these differences it is very easy to discriminate fish from certain broodstocks. However, for other brood stocks frequencies of mtDNA variants are so similar that discrimination relies on probabalistic techniques similar to those employed for allozymes.

One way of overcoming this problem is to selectively breed adult female lake trout so that each broodstock is fixed for a unique mitochondrial marker (clonal type or haplotype). This can be achieved without excessive inbreeding by crossing large numbers of females that share the same mitochondrial lineage with a large number of males. It should be noted that the clonal type of the males is unimportant for producing mitochondrially marked fish as only the female haplotype marker is passed on to the progeny.

The fixation of different mtDNA clones might initially focus on two or three of the major broodstocks. If hatchery managers resist culling females, then a number of sublines, differing in their mitochondrial phenotype, might be developed from each stock. In this case, all females belonging to a broodstock would be typed and individuals possessing the same mitochondrial haplotypes pooled. The maintenance of such sublines would make

it possible to determine experimentally if the different clonal types show different fitness effects. Currently, such mitochondrial lines are considered neutral, but some recent studies involving Drosophila suggest that this might not always be the case (MacRae and Anderson 1988).

Standard mtDNA analytical protocols represent a primary impediment to the development of such mitochondrially marked lines, as DNA is ordinarily extracted from tissues like gonads, liver, or heart (Gonzalez-Villasenor et al. 1986; Ferris and Berg 1987) and requires sacrifice of the parent fish. The present project aimed to develop methodologies for ascertaining mtDNA genotypes without the need to sacrifice maternal parents. This would provide a basis for producing mitochondrially marked lake trout for use in stocking programmes. This goal has been met by developing a series of mini-tissue protocols, which permit analysis of the mtDNA genotypes of live fish. These mini-tissue techniques all involve the determination of mtDNA patterns in total DNA extracts, obtained from small muscle tissue or blood samples. Total DNA is extracted from these samples, digested with restriction endonucleases and the mtDNA restriction fragment patterns produced are visualized by hybridization with $^{32}P$-labelled mtDNA. A similar approach has been used successfully in meadow voles Microtus pennsylvanicus (Plante et al. 1987) and humans (Kan et al. 1977; Denaro et al. 1981; Johnson et al. 1983).

Using these techniques, adult lake trout broodstock held in hatcheries may be non-lethally typed for diagnostic mtDNA fragment patterns. Then, adults possessing particular or unique

mtDNA fragment patterns may be selectively bred in hatcheries to produce mitochondrially marked lake trout, and these markers verified before the offspring are stocked in natural water bodies. Subsequently, it will be possible to determine the survival of these marked fish over a number of years, or even over many generations, because the markers will be passed on by females to their offspring (due to the maternal inheritance of mtDNA). Moreover, it should be possible to determine which of the stocked lake trout maternal broodstock(s) are successfully producing offspring in the wild.

This report documents several mini-tissue prep techniques for the determination or verification of mtDNA fragment patterns in both hatchery broodstocks and in a lake population of lake trout. Furthermore, it demonstrates that mitochondrially marked lake trout may be readily produced in a hatchery and their mtDNA marker verified. Thus, the stage is set for conducting large scale introductions of genetically marked lake trout in stocking and rehabilitation programmes.

## MATERIALS AND METHODS

During the course of this work, a number of protocols and variations on these protocols were used. Moreover, as new techniques became available through developments in the field of molecular biology, some were adopted for use in this project. To avoid confusion, we report our final recommended procedures, but note and reference other techniques employed in earlier phases of this work.

### Experimental Design

Adult lake trout from two broodstocks were surveyed for their Bam HI mtDNA fragment pattern, using the blood protocol described below. Previous studies have shown that Bam HI is a diagnostic endonuclease for discriminating the major mtDNA clonal groups of lake trout (Grewe 1987; Grewe and Hebert 1987, 1988). Once the adults had been typed, the information was forwarded to Peter Ihssen and Bill Martin (Ministry of Natural Resouces, Maple, Ontario) who then conducted crosses consisting of females exhibiting one mtDNA fragment pattern with males exhibiting a different pattern. It should be noted that the records of the fish used in these crosses were not made available to us until after we had typed the offspring. Hence, there was a "blind" control to this experiment. We collected samples of the offspring approximately 10 weeks after hatching and determined their mtDNA Bam HI fragment patterns after extracting total DNA from (lethal) muscle samples. In this way, we produced lake trout fingerlings with a known mtDNA fragment pattern and confirmed that the fragment pattern of the offspring was

identical to that of the female parent used in the cross. Flow diagrams of the experimental design and of the mini-tissue analysis procedure are presented in Figures 1 and 2, respectively.

In the timeframe of this study, it was not possible to stock marked fish and subsequently, determine that these markers could be detected in a field survey. Therefore, we collected blood samples from 12 Lake Simcoe lake trout and determined their Bam HI mtDNA restriction fragment patterns, to demonstrate that mtDNA markers could be readily determined in field surveys of lake trout.

Sample Collection

(1) Broodstock.

The OMNR fisheries research facility at Maple, Ontario holds individually branded Killala Lake and Lake Manitou lake trout broodstock. Twenty-four fish were sampled from each broodstock (Table 1), on July 21, 1988. Fish were anaesthetized using MS222, rinsed with fresh water to remove excess anaesthetic, then 0.5 ml blood samples were collected as described below (page 18); it took approximately 90 min. to process 24 fish. Blood samples were returned to the laboratory on ice and stored at $4^{o}C$ until processed to extract total DNA as described below. Immediately after the samples were taken, fish were returned to fresh aerated water until they were able to swim unassisted again, then they were returned to their stock tanks. It was noted that bleeding stopped within 30 s. to 2 min. Some fish were kept in a seperate tank for 30 min. and the wound re-examined; only slight swelling and bruising was evident. No mortality was observed in any of

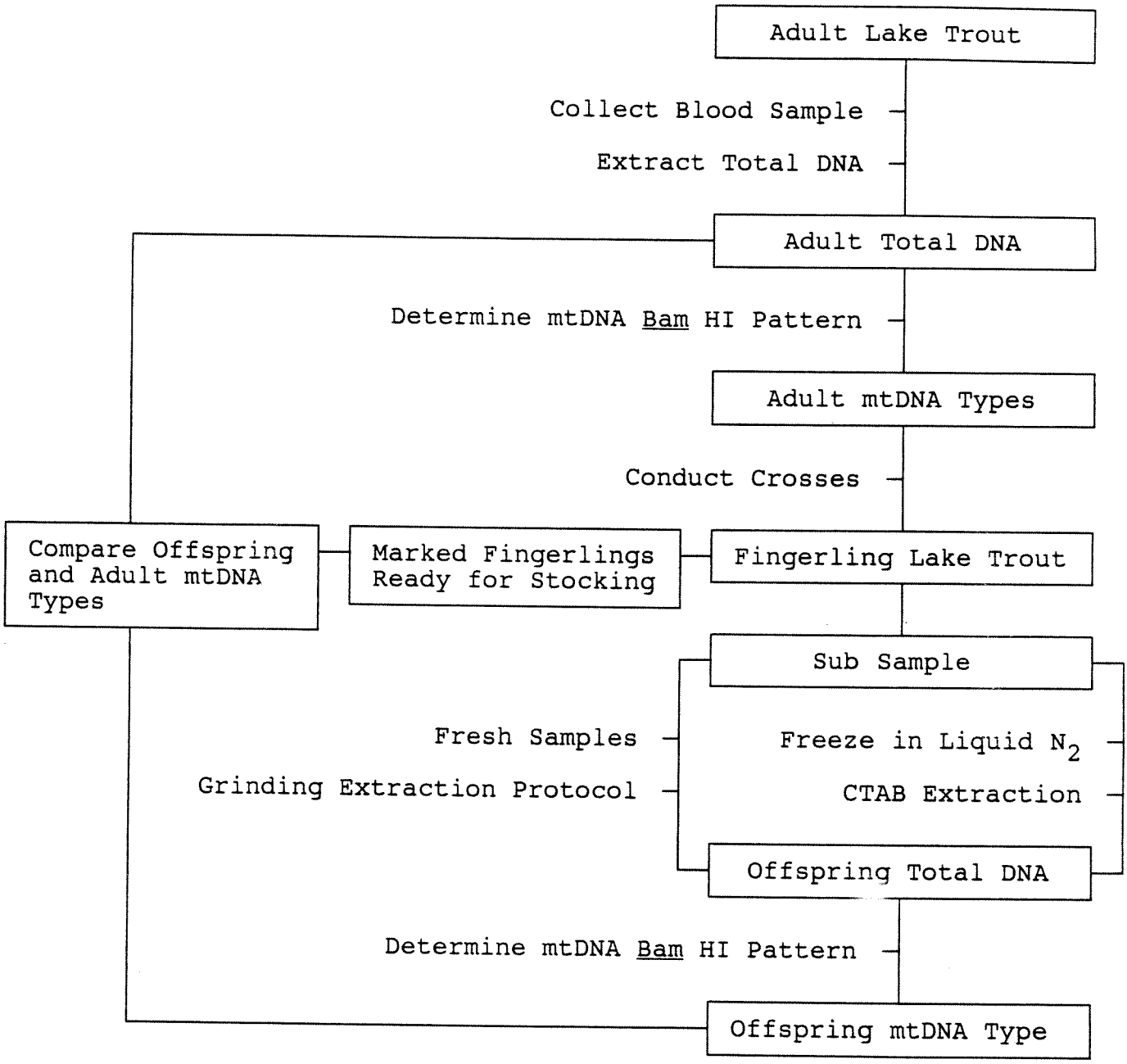


Figure 1: Flow chart detailing major steps in producing mitochondrially marked lake trout fingerlings and verifying the marks.

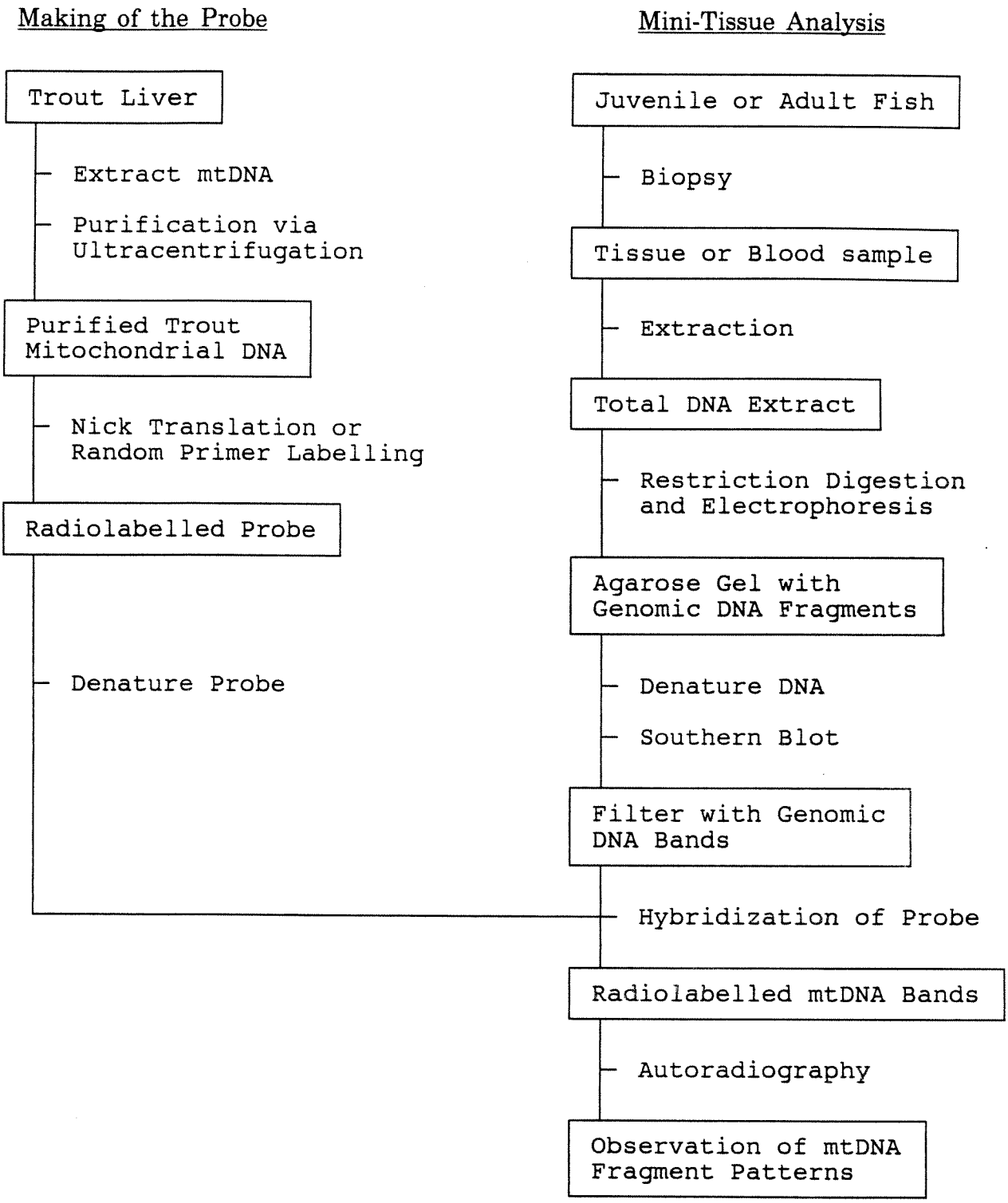


Figure 2: Flow chart detailing the major steps in determination of mtDNA restriction fragment patterns from a small tissue sample.

Table 1. Sample numbers, individual brands and sexes (where known) of OMNR Killala Lake and Lake Manitou lake trout broodstock surveyed for mtDNA Bam HI polymorphisms.

| KILLALA LAKE BROODSTOCK | | | | LAKE MANITOU BROODSTOCK | | | |
|---|---|---|---|---|---|---|---|
| Blood # | Brand # | | Sex | Blood # | Brand # | | Sex |
| 1 | 853 | LMB | F | 25 | 181 | 5RAB-AC | M |
| 2 | 982 | RMB | M | 26 | 162 | 9RAB-AC | F |
| 3 | 77 | LMA | M | 27 | 166 | 5RAB-AC | F |
| 4 | 220 | RMB | M | 28 | 183 | 5RAB-AC | F |
| 5 | 781 | LMA | M | 29 | 177 | 5RAB-UC | M |
| 6 | 207 | LMB | F | 30 | 186 | 9RAB-AC+RV | M |
| 7 | 121 | RMB | F | 31 | 171 | 5RAB-AC | F |
| 8 | 261 | LMB | F | 32 | 169 | 5RAB-UC | M |
| 9 | 309 | LMA | M | 33 | 168 | 5RAB-AC | M |
| 10 | 991 | LMB | F | 34 | 173 | 5RAB-UC | M |
| 11 | 203 | LMA | M | 35 | 109 | 9RAB-AC | F |
| 12 | 564 | LMA | M | 36 | 191 | 5RAB-AC | M |
| 13 | 455 | LMB | M | 37 | 190 | 5RAB-AC | M |
| 14 | 545 | LMA | F | 38 | 172 | 5RAB-AC | M |
| 15 | 131 | LMA | M | 39 | 158 | 5RAB-AC | F |
| 16 | 890 | LMA | F | 40 | 161 | 5RAB-AC | F |
| 17 | 455 | RMB | ND | 41 | 167 | 5RAB-AC | M |
| 18 | 910 | LMB | M | 42 | 187 | 5RAB-UC | M |
| 19 | 867 | LMB | F | 43 | 182 | 5RAB-UC | M |
| 20 | 51 | LMB | F | 44 | 184 | 5RAB-AC | F |
| 21 | 618 | LMB | ND (M) | 45 | 175 | 9RAB-AC+RV | F |
| 22 | 478 | LMA | M | 46 | 188 | 9RAB-AC | M |
| 23 | 368 | LMB | ND | 47 | 179 | 5RAB-AC | M |
| 24 | 43 | LMB | F | 48 | 108 | 5RAB-UC | M |

Note: Sexes were taken from record books. ND - not determined. Where a sex is noted in parentheses, this was determined during spawning.

the 48 fish sampled over a 6 month period (P. Ihssen and W. Martin, OMNR, Maple - pers.comm.).

(2) Offspring.

Crosses were made within the Lake Manitou broodstock on September 27, 1988 and within the Killala Lake broodstock on October 13, 1988. In both cases, three females were used and six males; crosses were allocated lot numbers (Table 2). The eggs had hatched by December 11, 1988 (Lake Manitou) and by December 29, 1988 (Killala Lake), and the offspring were sampled on March 15, 1989. As many (200-300) offspring were available for each cross, we collected eight whole fingerlings from each lot. Four fish were frozen immediately in liquid nitrogen, while the remaining four were returned to the laboratory on ice. Total DNA was extracted from the fresh fish by a grinding method (Billington et al. 1987; Stanton 1988) and from the frozen fish by the CTAB method (Saghai-Maroof et al. 1984).

(3) Field sampling.

Twelve lake trout were surveyed from Lake Simcoe, Ontario (Willow trapnet, site #401) on October 20, 1988. The fish were removed from trapnets and 0.5 mL blood samples were collected by the same procedure employed on the broodstocks; blood samples were collected on the boat and it took approximately 45 min. to process the 12 fish.

Table 2. Adult lake trout from two broodstocks used in crosses to produce marked offspring. Note crosses were done blind, so that the parents used were unknown until we had typed the offspring. mtDNA column refers to the Bam HI restriction fragment pattern, values in parentheses for females of the Lake Manitou stock are based on values determined for offspring assuming maternal inheritance of mtDNA. Lot and tank numbers are from OMNR, Maple Fisheries Laboratory records.

| Lot # | Tank # | Female | mtDNA | Male | mtDNA |
|---|---|---|---|---|---|
| Lake Manitou. | | | | | |
| 30 | L- 4B | 166 | (A) | 188 | ? |
| 31 | L- 20A | 166 | (A) | 191 | ? |
| 32 | L- 20B | 171 | (A) | 173 | ? |
| 33 | L-142A | 171 | (A) | 177 | A |
| 34 | L-142B | 184 | (A) | 108 | ? |
| 35 | L-147A | 184 | (A) | 182 | ? |
| Killala Lake. | | | | | |
| 188 | L-209A | 991 | B | 781 | A |
| 189 | L- 37A | 991 | B | 309 | A |
| 194 | L- 37B | 207 | D | 982 | B |
| 195 | L- 38A | 207 | D | 220 | B |
| 196 | L- 38B | 121 | B | 618 | A |
| 197 | L- 39A | 121 | B | 781 | A |

Collection of blood samples from fish.

The method used to collect lake trout blood was identical to that described by Billington and Hebert (1989) for use with walleye. However, we summarize the procedure here.

Small 100-500 uL (0.1-0.5 mL) blood samples were collected from the sinus venosus (Wingo and Muncy 1984) with a 25 mm X 23 gauge hypodermic needle attached to a 3 mL syringe. The syringe and needle were rinsed with 0.5 M EDTA (pH 8.0) immediately prior to sample collection. The EDTA served a double function: firstly, as an anticoagulant (Hesser 1960); and secondly, as an inhibitor of nonspecific nuclease activity. The blood was immediately transfered to 1 mL of 1X SSC (standard sodium citrate: 150 mM NaCl; 15 mM sodium citrate) in a 1.5 mL microcentrifuge tube. Blood samples could then be stored at $4^{o}C$ for at least a month without loss or degradation of DNA.

Extraction of total DNA from blood.

Total DNA was extracted from blood cells using a protocol modified from Plante et al. (1987). Blood cells were pelleted in a microcentrifuge for 30 s at 16,000 x g, following which the supernatant was discarded. The cells were haemolysed by rapid suspension in 750 uL of sterile double distilled water, then immediately made isotonic by adding 250 uL of 5X SSC. The red cell ghosts and white blood cells were pelleted by centrifugation for 2 min and the supernatant again discarded. The pellets were resuspended in 400 uL of 0.2 M sodium acetate (pH 5.2), lysed by treatment with 15 uL of 20% SDS (sodium dodecyl sulphate) for 5 min at room temperature and incubated for 1-6 h at $65^{o}C$ with 25 uL proteinase K (10 mg $mL^{-1}$).

At the end of this incubation, 1 ml of phenol (saturated with TE) was added, the tubes gently shaken at room temperature for 15 min. and centrifuged for 10 min. in a microcentrifuge. The upper (aqueous) layer was removed and placed in a fresh tube; the lower (phenolic) layer was discarded. Six hundred microlitres of phenol: chloroform: isoamyl alcohol (25:24:1) were added, the tube mixed by shaking and then centrifuged for 5 min. at room temperature. The aqueous phase was again removed and placed in fresh tubes, while the lower phase was discarded. A final extraction with 600 ul of chloroform; isoamyl alcohol (24:1) was performed and, after centrifugation for 2 min. the upper 400 ul removed and placed into fresh tubes, which were sitting on ice. Forty microlitres of 2 M NaCl and 1 ml of 100% ethanol (at -20$^{o}$C) were added to precipitate the DNA. The samples were centrifuged for 5 minutes at 4$^{o}$C, the ethanol decanted off, the pellets rinsed with 70% ethanol (at -20$^{o}$C) and re-spun for 5 minutes at 4$^{o}$C. The ethanol was decanted off, the pellet allowed to air dry for 2 hours at room temperature, resuspended in 100 ul of 1 mM TE (10 mM Tris-HCl, pH 7.6; 1 mM EDTA, pH 8.0) and stored at 4$^{o}$C. Total DNA samples stored in this manner were stable for fish for at least three months (N. Billington, unpublished data) and for the crustacean Daphnia for up to 2 years (Stanton 1988).

Yields of up to 300 ng of total DNA were obtained from the 0.5 mL blood samples. Yields were improved by back-extracting the first phenol phase with 300 uL TE as recommended by Maniatis et al. (1982). Full step-by-step details of this protocol are provided in Appendix III.

Extraction of total DNA from muscle

Grinding protcol.

Samples of fresh muscle tissue (0.1-0.2 g) were placed into 1500 ul micro-centrifuge tubes and homogenised in 300 ul of grinding buffer (10 mM Tris-HCl, pH 7.2; 60 mM NaCl; 5% sucrose; 10 mM EDTA, pH 8.0). Two hundred microlitres of lysis buffer (0.3 M Tris-HCl, pH 7.2; 1.24% SDS; 5% sucrose; 0.1M EDTA, pH 8.0) were added, the mixture was vortexed briefly and incubated at 55$^{o}$C for 10 minutes. Fifty microlitres of proteinase K (10 mg $mL^{-1}$) were then added and the mixture incubated for 1-6 h at 65$^{o}$C.

The resulting slurry was extracted once with phenol, twice with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1) and the DNA recovered by ethanol precipitation (as described above). Once again, pellets were resuspended in 100 uL of 1 mM TE (1 mM Tris-HCl, pH 7.6; 0.1 mM EDTA, pH 8.0) and stored at 4$^{o}$C. Further details of this protocol are provided in Billington et al. (1987) and in Stanton (1988).

CTAB protocol.

In the later stages of this project, we used a modified version of the CTAB protocol (Saghai-Maroof et al. 1984), suggested by Peter Grewe (Cornell University, Ithaca, NY). This technique is valuable in that total DNA can be reliably extracted from frozen tissue. We tested this procedure on samples of frozen liver from four Lake Superior siscowet (Salvelinus namaycush siscowet ) which were provided by Mary Burnham (University of Michigan, Ann Arbor), and also on muscle samples

obtained from frozen specimens of the lake trout crosses.

Twenty milligrams of thawed liver or muscle tissue were placed in a 1500 ul microcentrifuge tube, 100 ul 2X CTAB buffer (see Appendix III for recipe) added and the tissue was ground using a plastic mortar (Mandel, Cat. # 749520). The mortar was washed off with a further 600 ul of 2X CTAB buffer into the tube and 10 ul of proteinase K (10 mg $ml^{-1}$) added. The mixture was vortexed briefly and then incubated at 65°C for 1 hour.

After this incubation period, the slurry was extracted once with 600 ul chloroform-isoamyl alcohol (24:1), vortexed briefly and centrifuged for 15 min. The supernatant was pipetted off into a fresh tube and the sample extracted twice with 600 ul phenol-chloroform-isoamyl alcohol (25:24:1) and once more time with chloroform-isoamyl alcohol (24:1). The DNA was precipitated by adding 600 ul of cold (-20°C) isopropanol and the pellet rinsed twice with 1 ml of 70% cold (-20°C) ethanol. The pellet was dried at room temperature, resuspended in 100-200 ul 1 mM TE and the total DNA solution stored at 4°C. Full step-by-step details of this protocol are provided in Appendix III.

Restriction analysis of mtDNA

A 15 uL aliquot of each total DNA extract (containing approximately 30-45 ng DNA per aliquot) was separately digested with 10 units of the restriction endonuclease Bam HI (GGATCC). Ten units of RNase T1 were added to each sample and the total volume adjusted to 30 uL with 1X reaction buffer. Digests were run overnight (>16 h) using the reaction conditions (buffer and temperature) recommended by the suppliers (Bethesda Research

Laboratories). After a minimum of 12 hours, 5 uL of stop dye buffer (7 M urea; 50% sucrose; 1 mM EDTA; bromophenol blue 0.1%) were added to terminate digestion and act as a loading buffer. The samples were then loaded onto horizontal submarine agarose gels (0.8% in 1X TBE: 89 mM Tris-borate; 2 mM EDTA) and electrophoresed overnight at 20 V. Twenty-five nanograms of phage lambda-DNA digested with Hind III were loaded in one lane of each gel as a size standard.

Southern transfer

After electrophoresis was completed, the DNA was stained with ethidium bromide and photographed under UV illumination. The gel was transfered to a glass dish, the DNA denatured with 1.5 M NaCl and 0.5 M NaOH, and the gel neutralized with 1 M Tris-HCL, pH 8.0 and 1.5 M NaCl. DNA fragments were then immobilized on a nylon membrane (Zeta-Probe, BIORAD) by Southern transfer (Southern 1975; Maniatis et al. 1982), using a 10X SSC transfer buffer for 16 hours at room temperature. In prior experiments, we transfered DNA to nitrocellulose filters (Billington et al. 1987; Billington and Hebert 1987, 1989), however, nylon membranes are more robust and therefore, easier to handle (or to reprobe if necessary), and we recommend their use.

Preparation of $^{32}$P-labelled probe

A lake trout mtDNA probe, with a specific activity of at least $10^8$ disintegrations $min^{-1}$ $ug^{-1}$, was prepared with [$\alpha$-$^{32}$P]dCTP and [$\alpha$-$^{32}$P]dATP by the random oligonucleotide primer method (Feinberg and Vogelstein 1983, 1984). We labelled 25 ng of purified lake trout mtDNA using the protocol and components of the Pharmacia kit (Cat. # 1004 760). However, we substituted 2.5

ul each of the $^{32}$P-labelled dATP and dCTP stocks (Amersham; 111 TBq mmol$^{-1}$, 370 MBq ml$^{-1}$) for the 5 ul of radiolabelled C nucleotide recommended in the Pharmacia protocol. In previous studies (Billington et al. 1987; Billington and Hebert 1987, 1989), we used nick-translation (Rigby et al. 1977; Maniatis et al. 1982) to prepare the probe, but the oligonucleotide primer method requires less DNA and produces a much more intensely labelled probe. The mixture was incubated at 37$^{o}$C for 30 min., after which the reaction was terminated by the addition of 20 ul of stop buffer (0.2 M EDTA, pH 8.0; 1% SDS; 40 mg mL$^{-1}$ blue dextran; 0.1 mg mL$^{-1}$ bromophenol blue). The radiolabelled DNA was separated from unincorporated nucleotides using Sephadex G-50 columns (Maniatis et al. 1982). In order to probe the lambda standard, 25 ng of lambda-DNA were also labelled using the same procedure.

Although lake trout mtDNA has been cloned into the phagemid vector BLUESCRIPT (P. Grewe, Cornell University - pers. comm.), we found it more convenient to use lake trout mtDNA purified by density gradient centrifugation as a hybridization probe, rather than growing up the cloned probe. Purified lake trout mtDNA (source Jenny Lake, or Kiknissi Lake fish), obtained using standard cesium chloride gradient protocols (Billington and Hebert 1988; Grewe and Hebert 1987, 1988), was used to prepare the $^{32}$P-labelled probe in all hybridizations reported here.

Hybridization

The nylon membrane containing the lake trout mtDNA restriction fragments was sealed in a plastic bag; in practice 2-

4 membranes were probed per bag. The membranes were treated for at least 1 hour with a 0.1X SSC: 0.5% SDS solution at 65°C to reverse any DNA annealing that had occured during baking; this step serves to expose more binding sites to the probe. The filter was pre-hybridized with 15-20 mL of hybridization buffer (5X SSC; 7% SDS; 10X Denhardt's solution [0.2% Ficoll 400, 0.2% Polyvinyl pyrrolidone PVP-360, 0.2% Bovine Serum Albumin]; 20 mM $NaH_2PO_4$, pH 7.0) (Angelini et al. 1986) and 750 ul of salmon sperm solution (10 mg $mL^{-1}$ salmon sperm DNA [Sigma D-1626], in 1 mM TE, sheared by sonication, denatured by boiling for 10 min and quenching on ice). The buffer was introduced by cutting the corner of the bag and injecting the solution with a pipette, then resealing the bag. Pre-hybridization was carried out at 50°C for 16-24 h. After this period, the pre-hybridization buffer was replaced with fresh hybridization buffer. The lake trout and lambda probes were denatured by adding 100 ul of 0.2M NaOH and boiling for 10 min, followed by quenching on ice for at least 30 min, then added to a tube containing 700 uL of hybridization buffer. The contents of this tube were gently mixed and injected into a cut corner of the plastic bag. After removing as many air bubbles as possible, the bag was resealed and inverted several times to allow the contents to mix thoroughly. The bag was sealed in a larger plastic bag, then incubated, flat, at 50°C for a minimum of 16 h, during which time it was periodically agitated.

After the incubation period, the liquid contents of the bag were drained into a radioactive waste container and the membranes rinsed in the bag with 50 mL of a 2X SSC: 0.2% SDS solution. The

membranes were removed from the bags and subjected to a series of post-hybridization washes (500 ml per set (bag) of membranes) of increasing stringency as follows: (1) 2X SSC: 0.2% SDS, twice for 15 min. at room temperature; (2) 1X SSC: 0.1% SDS) for 30 min. at 65$^{o}$C; (3) 0.5X SSC: 0.1 % SDS for 30 min. at 65$^{o}$C; (4) 0.1X SSC: 0.1% SDS for 1 h. at 65$^{o}$C. The membrane was then blotted between two pieces of Whatman filter paper to remove excess moisture and wrapped in cling-film wrap. Autoradiographs were prepared by exposing the filter to X-ray film (Fugi-RX), using Dupont Cronex intensifying screens at -70$^{o}$C for 48-96 h.

## RESULTS

Seventeen of the 24 Killala Lake adult lake trout broodstock were readily typed for their Bam HI mtDNA restriction fragment patterns by probing the mtDNA fragments in total DNA digests obtained from blood samples (Tab. 3). Seven of the fish showed the A pattern, nine fish the B pattern and one fish a D pattern. Unfortunately, only one of the Lake Manitou samples could be resolved. This fish (Blood sample 29) showed an A pattern. The A pattern showed up as a single band approximately 17 kbp (kilobase pairs) long and the B pattern consisted of two bands, one at 9.2 kbp and the other at 7.4 kbp. The D pattern was represented by a single band, which was approximately 3 kbp shorter than the A band. The and B Bam HI patterns were identical to those described by Grewe (1987) and Grewe and Hebert (1987, 1988). The D pattern described here was assumed to be that described by Grewe (1987), although it was not possible to confirm the presence of a second band at approximately 4 kbp in our initial blots.

Due to time constraints, it was not possible to repeat the analyses on the Lake Manitou broodstock before they spawned. Thus, crosses made within this stock were of fish with unknown mtDNA type. However, the mtDNA types of all parents used to make crosses within the Killala Lake broodstock were known (Tab. 2).

Results obtained from the analysis of the offspring DNA were more successful. All the individuals examined could be typed. All of the offspring from the Lake Manitou crosses showed an A fragment pattern. Thus, we conclude that each of the three females used in

Table 3. The Bam HI mtDNA fragment patterns of Killala Lake lake trout broodstocks as determined from blood samples by the mini-tissue technique.

| Blood # | mtDNA |
|---|---|
| 1 | |
| 2 | B |
| 3 | |
| 4 | B |
| 5 | |
| 6 | D |
| 7 | B |
| 8 | A |
| 9 | A |
| 10 | B |
| 11 | A |
| 12 | A |
| 13 | |
| 14 | A |
| 15 | B |
| 16 | B |
| 17 | |
| 18 | B |
| 19 | |
| 20 | |
| 21 | A |
| 22 | B |
| 23 | B |
| 24 | |

these crosses had a Bam HI A pattern also (from the maternal inheritance of mtDNA). However, despite us being unable to verify the female mtDNA type in these fish, we have produced offspring from this stock tagged with the Bam HI A pattern.

The Killala Lake fish showed a mixture of the B and D patterns (Fig. 3), with the offspring of the B type females showing the B pattern and the offspring of the D type females producing D type offspring, regardless of the mtDNA patterns of the males used (Tab. 4). All of the above results were obtained using the grinding protocol. We have not yet completed analysis of the fingerling results obtained using the CTAB method.

The Bam HI mtDNA fragment patterns could be resolved for eight of the 12 Lake Simcoe fish examined using the blood sample method; 2 fish showed an A pattern and 6 the B pattern. Both these patterns had been previously detected in Lake Simcoe lake trout (Grewe 1987; Grewe and Hebert 1987, 1988). A comparison of previous data with the current data for Lake Simcoe lake trout and for the two broodstocks is presented (Tab. 5), which shows that there is a reasonable agreement between them, despite the relatively small sample sizes involved.

We have succesfully used the CTAB method to survey mtDNA fragment patterns in Lake Superior siscowet (Fig. 4). The restriction fragment patterns obtained for the endonucleases Bam HI and Hind III were identical in this lake trout sub-species to those observed in lake trout. However, for Bam HI two siscowet (1,2) exhibited the C pattern and 2 (3,4) exhibited the A pattern. Other restriction endonucleases, including 4-base recognition enzymes, have been surveyed on CTAB preparations of

Table 4. Comparison of adult and offspring mtDNA types for Killala Lake lake trout.

| Lot # | Offspr. mtDNA | Female mtDNA | Male mtDNA |
|---|---|---|---|
| 188 | B | B | A |
| 189 | B | B | A |
| 194 | D | D | B |
| 195 | D | D | B |
| 196 | B | B | A |
| 197 | B | B | A |

Figure 3. Autoradiograph of Bam HI mtDNA fragment patterns (B, D) obtained from Killala Lake lake trout fingerlings, from various crosses (defined by lot number - Tab. 2). Fragments were visualized after Southern transfer of restricted and denatured total DNA, obtained by grinding mini-tissue extraction protocol, and hybridization to a radiolabelled lake trout mtDNA probe. Numbers under lot numbers refer to sub-sample numbers. LH refers to lambda DNA standard digested with Hind III. Standard bands (marked .) are 23.1, 9.4, 6.6, 4.4, 2.3, 2.0 kbp, from top to bottom.

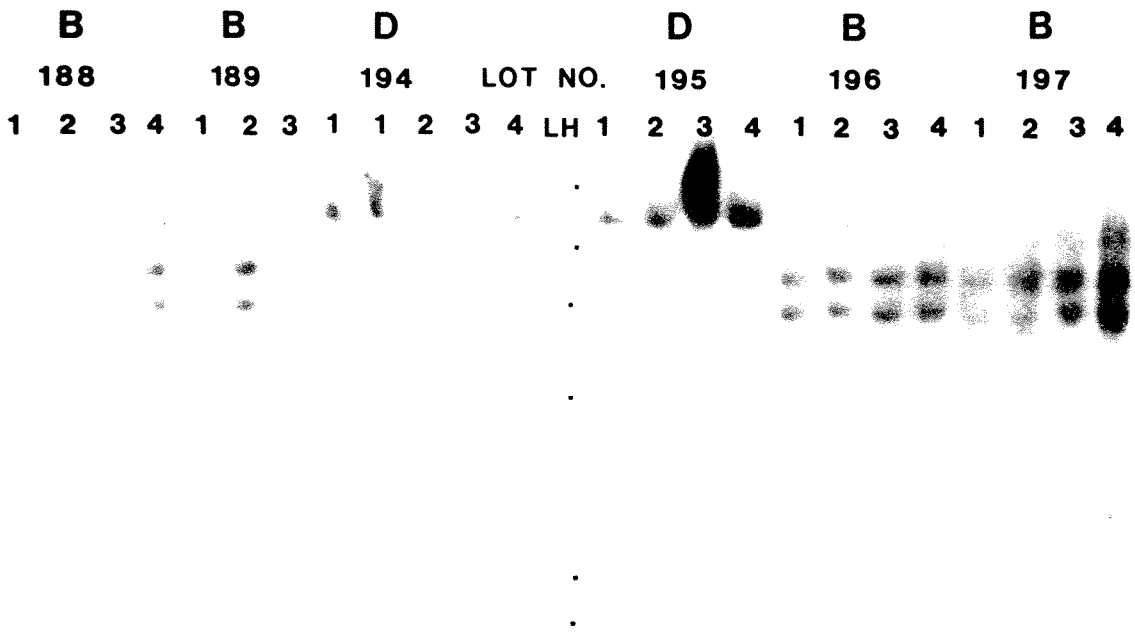

Figure 4. Autoradiograph of mtDNA fragment patterns from four siscowet samples generated with two different restriction enzyme digests. Visualization of the fragments was attained by Southern transfer of restricted, denaturized total DNA, prepared from frozen liver samples by the CTAB protocol, followed by hybridization with a radiolabelled lake trout mtDNA probe. S - size standard of Eco RI/Hind III plus Hind III cut lambda DNA (Billington and Hebert 1988).

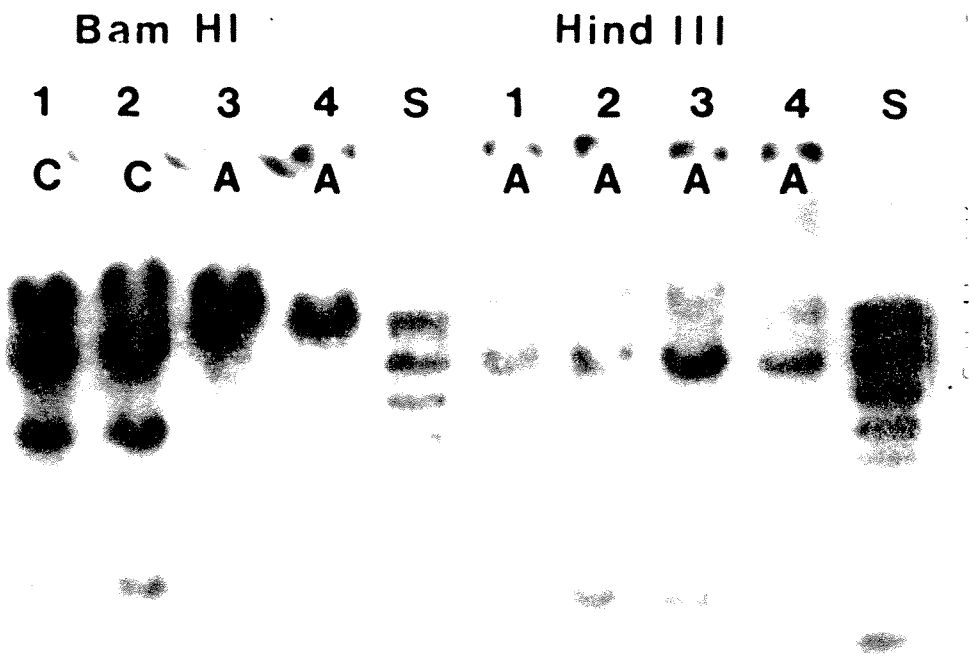

Table 5. Comparison of mtDNA fragment patterns obtained in this study for Lake Simcoe lake trout, and Killala Lake and Lake Manitou broodstocks with results of previous studies (Grewe 1987; Grewe and Hebert 1987, 1988).

| | | PREVIOUS | | PRESENT | |
|---|---|---|---|---|---|
| | | # | (%) | # | (%) |
| LAKE SIMCOE | A | 1 | (10) | 2 | (25) |
| | B | 9 | (90) | 6 | (75) |
| KILLALA LAKE | A | 4 | (36) | 7 | (41) |
| | B | 7 | (64) | 9 | (53) |
| | D | - | ( 0) | 1 | ( 6) |
| LAKE MANITOU | A | 2 | (50) | 4* | - |
| | B | 2 | (50) | - | - |

* Note: 1 male was typed and 3 females inferred from offspring. Percentage not calculated for Lake Manitou in the present study as only 4 of 24 fish could be typed.

these samples and have confirmed the observation that two of the siscowet show a lake trout A type mitochondrial genome and that two possess C type mtDNA (P. Grewe, Cornell University, Ithaca - pers. comm.). The taxonomic relationships of these two species, along with those of other Salvelinus species, based on mtDNA analysis are presented elsewhere (Grewe et al. 1989).

We have also demonstrated that the homology of the lake trout mtDNA probe is such that it will hybridize to mtDNA from other Salvelinus species (bull trout and aurora trout). Moreover, we were able to hybridize the lake trout probe to walleye (Stizostedion vitreum) mtDNA sequences.

## DISCUSSION

Work on natural recruits can provide a basis for determining which lake trout strains are reproducing successfully in the wild. Unique combinations of mtDNA restriction fragment patterns exist in lake trout that could be used as genetic markers in introduced fish (Grewe 1987; Grewe and Hebert 1987, 1988). These mtDNA markers will be passed on by females to their offspring due to the maternal inheritance of mtDNA. By employing a technique to screen fish, female lake trout with distinctive mtDNA fragment patterns could be selectively bred to produce a number of mitochondrially marked lines. Subsequent determination of the mtDNA type of naturally recruited fry will enable fisheries managers to determine which of these female strains are successfully reproducing, and therefore, focus lake trout rehabilitation efforts. Thus, mtDNA markers offer exceptional promise as tools in lake trout rehabilitation and management, both in stock identification and as genetic tags which will persist for many generations.

In order to fully exploit this potential, it is essential that mtDNA markers can be detected in live fish. This crucial step has now been achieved. Restriction fragments of mtDNA were successfully visualized in total DNA extractions, obtained from small (0.5 mL) blood samples collected from live lake trout (adult broodstock and fish from the wild), by hybridization to a $^{32}$P-labelled lake trout mtDNA probe. Furthermore, we have shown that by restricting the mtDNA type of females in certain lake trout broodstocks to one of the diagnostic Bam HI fragment

patterns, it is possible to produce genetically marked fish for use in stocking programmes. The fate of these fish in the wild, and that of their offspring, could be monitored by using a mini-tissue method of mtDNA fragment pattern determination.

In the current study, we produced six sets of lake trout fingerlings with mtDNA markers. This was achieved by using 6 female fish and 12 male fish. Obviously, the numbers of females used to produce marked offspring will have to be greatly increased if such marked fish are to be used in a stocking programme, to prevent loss of genetic variation due to the effects of small population sizes, such as bottlenecks, drift and inbreeding depression (Meffe 1986).

Various tissues were considered as possible nonlethal sources of total DNA. In prior experiments, eggs, fin, muscle and skin tissue were used as sources of total DNA, in addition to blood. No DNA could be extracted from fin or skin tissues, although egg and muscle tissue proved satisfactory sources of total DNA for probing (N. Billington, unpublished data). In the context of sampling live fish, small blood samples may be obtained using a syringe and hypodermic needle from the sinus venosus (Wingo and Muncy 1984), the duct of Cuvier (Lied et al. 1975) or by other routine methods (Hesser 1960). Alternatively, small muscle samples (100-300 mg) may be collected by biopsy (eg. Uthe 1971; McAndrew 1981). We have also compared several different techniques for determining the mtDNA restriction fragment patterns of fish (Tab. 6). Some techniques (e.g. blood sample techniques, muscle biopsy) are more useful for studies on live fish, some (e.g. CTAB) for determining mtDNA fragment

Table 6. Comparison of the features of three techniques for visualizing mtDNA restriction fragment patterns, that have fisheries applications (Modified from Billington and Hebert 1989). The techniques are: (1) visualization of purified mtDNA fragment by end-labelling or ethidium bromide staining (e.g. Ferris and Berg 1987; Billington and Hebert 1988); (2) probing mtDNA fragments in digests of total DNA extracted from muscle tissue samples (current study); (3) probing mtDNA fragments in digests of total DNA extracted from blood samples (Billington and Hebert 1989; current study).

| Method | (1) | (2) | (3) |
|---|---|---|---|
| Tissue requirements: | Liver, heart, gonads | Muscle (liver) | Blood |
| Amount of tissue: | 1-5 g | 0.02-0.10 g | 0.1-0.5 mL |
| Impact on specimen: | usually death | little trauma (biopsy), death (fingerlings) | little trauma |
| Special requirements: | ultracentrifuge | mtDNA probe | mtDNA probe |
| Number of restriction digests: | > 40 | 6-16 | 6-16 |
| Number of samples/run: | 15-30 | 24-400 | 24-400 |
| Laboratory time (6 R.E. analyses): | 350 hours | 150 hours | 150 hours |
| Laboratory costs/sample (ratio): | 7 | 3 | 3 |
| Level of resolution: | 20-17000 bp | 300-17000 bp | 300-17000 bp |
| Scientific interpretation: | Excellent | Intermediate | Intermediate |
| Fisheries applications: | Detection of markers for use in stock identification | Visualization of known markers, lethal and non-lethal surveys frozen tissue (CTAB) | Visualization of known markers, nonlethal surveys |

patterns in frozen samples and some (cesium chloride gradient method) for determining initial mtDNA fragment polymorphisms.

These mini-tissue prep methods allow for sufficient DNA to be extracted such that mtDNA markers for up to 16 diagnostic restriction endonucleases may be easily determined. Thus, they will allow routine surveys to be conducted of mtDNA variation in large numbers of live fish, in order to examine differences between stocks. For example, 14 mitochondrial clones can be resolved in Great Lakes lake trout broodstocks based on their restriction patterns for 10 polymorphic endonucleases (Grewe 1987; Grewe and Hebert 1987, 1988). Therefore, it should be possible to determine the relative proportions of these clones in additional lake trout populations or broodstocks without the need to sacrifice fish. Moreover, mtDNA fragments as small as 300 bp can currently be resolved in lake trout using a similar DNA hybridization method, permitting analysis of fragment patterns generated by 4- and 5-base sequence recognition endonucleases (P. Grewe, Cornell University, - pers. comm.).

Alternatively, fish that are to be used in transfer programs could be screened for mtDNA markers, so that only fish of a known haplotype will be introduced to other water bodies. The fate of these fish in the wild, and that of their offspring, could be monitored by subsequent determination of their mtDNA fragment pattern. Such sampling could easily be performed as part of the annual surveys of fish populations already undertaken by management personnel.

The technique may also be suitable for interspecific comparisons of variation in fish mtDNA. For example, Gonzalez-

Villasenor et al. (1986) used a cloned mtDNA probe from the teleost Fundulus heteroclitus as an interspecies hybridization probe. They were able to hybridize the Fundulus probe to complementary mtDNA fragments obtained from members of six fish families (Centrarchidae, Siaenidae, Salmonidae, Cichlidae, Percichthydae, Ictaluridae). We have demonstrated that the homology in DNA sequences was sufficient to allow lake trout mtDNA to probe other Salvelinus species (S. confluentus, S. fontinalis timagamiensis) and also the percid Stizostedion vitreum.

As most of the described techniques are relatively simple and routine, it should be possible for many fisheries laboratories to perform the total DNA extractions, digests, and Southern blotting with only minimal expenditures on equipment or staff training. Only the hybridization stage with the radioactively labelled $^{32}P$ probes would need to be conducted in a specialized laboratory. We have already demonstrated that selective crosses can be carried out in a small hatchery, and it should be quite feasible to scale up this aspect for use in the major lake trout hatcheries. Thus, the large scale utilization of mitochondrially marked lake trout in rehabilitation programmes should be practical almost immediately with the techniques described here.

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## APPENDIX I.

Co-operative research arising from this project.

(1) In conjunction with Dr. Peter Ihssen and Bill Martin (OMNR, Maple, Ont.), we have produced a series of mitochondrially marked lake trout, which could be used in stocking experiments.

(2) We have provided samples of nuclear DNA from lake trout and other Salvelinus species to Dr. Ruth Phillips (Department of Biological Sciences, University of Wisconsin-Milwaukee), for her studies on ribosomal DNA variation in Salvelinus species.

(3) We have used the CTAB method to examine the mitochondrial genome in Siscowet (Salvelinus namaycush siscowet) for Mary Burnham (Department of Biology, University of Michigan).

(4) In April 1989, we ran a mtDNA workshop at the University of Windsor, where fisheries biologists were instructed in both traditional and rapid isolation methods of DNA analysis. This workshop was attended by participants from the U.S. Fish and Wildlife Service, Bowling Green State University, Fisheries and Oceans Canada, Ontario Ministry of Natural Resources, and the University of Guelph.

(5) We have developed a series of mini-tissue sampling techniques for use by Fisheries and Oceans Canada in their work on arctic fish and mammal species.

APPENDIX II.

Publications and presentations wholly or partially supported by GLFC funding:

Papers:

Billington, N. & Hebert, P.D.N. (1989) A technique for determining mitochondrial DNA markers in blood samples from walleye (Stizostedion vitreum). International Symposium and Educational Workshop on Fish-Marking Techniques, AFS Special Publication - in press.

Grewe, P.M. & Hebert, P.D.N. (1988) Mitochondrial DNA diversity among broodstocks of the lake trout, Salvelinus namaycush. Can. J. Fish. Aquat. Sci. 45: 2114-2122.

Grewe, P.M., Billington, N. & Hebert, P.D.N. (1989) Phylogenetic relationships among Salvelinus species as inferred from mitochondrial DNA divergence. Can. J. Fish. Aquat. Sci. - re-submitted after revision.

Technical Reports:

Billington, N., Crease, T.J., Grewe, P.M., Hebert, P.D.N. & Stanton, D.J. (1987) Development of a small tissue sample technique for the rapid analysis of mitochondrial DNA in walleye and lake trout. Tech. Rep. to Fisheries & Oceans Canada. Jan. 1987. 32 pp.

Billington, N. & Hebert, P.D.N. (1987) Development of a small tissue sample technique for the rapid analysis of mitochondrial DNA in walleye and lake trout. 2nd Tech. Rep. to Fisheries & Oceans Canada. Dec. 1987. 25pp.

Grewe, P.M. & Hebert, P.D.N. (1987) Mitochondrial DNA diversity among brood stock of the lake trout, Salvelinus namaycush. Research Completion Report to Great Lakes Fishery Commission. Feb. 1987. 53 pp.

Theses:

Grewe, P.M. (1987) Divergence of mitochondrial DNA among the sub-family Salmoninae with special reference to the genus Salvelinus. M.Sc. Thesis, University of Windsor, 110 pp.

Conference Presentations:

Billington, N. & Hebert, P.D.N. (1987) Use of mitochondrial DNA (mtDNA) techniques in fish stock management. Paper presented at 30th IAGLR Conference, 12th May 1987, University of Michigan, Ann Arbor, Michigan, U.S.A.

Billington, N. & Hebert, P.D.N. (1988) Mitochondrial DNA markers in walleye (Stizostedion vitreum) and a simple technique for

their determination in live fish. Poster paper presented at the International Symposium and Educational Workshop on Fish-Marking Techniques, 29th & 30th June 1988, University of Washington, Seattle, Washington, U.S.A.

Billington, N. & Hebert, P.D.N. (1988) Selection of unique mitochondrial DNA clones of walleye for use in stocking programs. Paper presented at 50th Mid-West Fish & Wildlife Conference, 6th Dec. 1988, Columbus, Ohio, U.S.A.

Invited Research Seminars:

Billington, N. (1987) Mitochondrial DNA variation in walleye: management implications and the development of a small tissue sample technique. Research Seminar, Freshwater Institute, Winnipeg, Manitoba, Canada, 26th June 1987.

Hebert, P.D.N. (1987) Mitochondrial DNA diversity in Great Lakes fish - management and evolutionary implications. Research Seminar, Great lakes Environmental Research Laboratory, Ann Arbor, Michigan, U.S.A., 14th October 1987.

## APPENDIX III

### Extraction of total DNA from blood cells.

1. Pellet blood cells by centrifugation (microcentrifuge) for 30 s, then remove and discard supernatant. Haemolyse cells by rapid suspension in 750 ul of sterile double distilled water, then immediately make isotonic by adding 250 ul of 5X SSC.

2. Pellet red cell ghosts and white blood cells by centrifugation for 2 min. Remove and discard supernatant.

3. Resuspend pellets in 400 ul of 0.2 M sodium acetate (pH 5.2), then lyse by adding 15 ul of 20% SDS, mix well and allow to stand for 5 min at room temperature.

4. Add proteinase K (25 ul, 10 mg $ml^{-1}$) and incubate for 1-6 h at 65°C.

5. Extract once with phenol, twice with phenol-chloroform-isoamyl alcohol (PCI, 25:24:1) and once with chloroform-isoamyl alcohol (CI, 24:1), retaining the upper (aqueous) layer each time. Use 800 ul of solvent, mix well by shaking and/or gentle vortexing and centrifuge for 2 min. Transfer upper layer to a fresh tube using cut tips.

6. Recover DNA by ethanol precipitation. Add 40 ul of 2 M NaCl, then 1 ml of cold (-20°C) absolute (100%) ethanol. Stand for a minimum of 10 minutes at -70°C, then centrifuge for 10 minutes at 4°C. Place tubes on ice and decant off supernatant, add 1 ml of 70% ethanol and gently rinse pellets. Centrifuge for 5-10 min, then decant off ethanol as above. Allow pellets to dry at room temperature or use a vacuum dessicator.

7. Resuspend pellets in 100 ul of 1 mM TE (pH 7.6) and store at 4°C. Volume of 1 mM TE used to resuspend pellet depends on the amount of DNA extracted and the number of digests etc. that are to be conducted, but range is usually 15-500 ul.

N.B. Full details of phenol extraction and ethanol precipitation procedures and of solutions used (ie SSC, SDS, TE etc.) can be found in Maniatis et al. (1982) or in Billington et al. (1987).

## CTAB mini-prep for isolating DNA from tissue.*

1. Place 20 mg of thawed liver or muscle tissue and buffer in a 1500 ul microcentrifuge tube. Add 100 ul 2X CTAB (see below).

2. Grind up tissue with plastic mortar. Wash mortar off with 600 ul 2X CTAB into tube.

3. Add 10 ul of proteinase K (10 mg $ml^{-1}$), vortex briefly and incubate at 65°C for 1 hour.

4. Extract once with 600 ul chloroform-isoamyl alcohol (24:1), vortex briefly and centrifuge for 15 min.

5. Pipette off supernatant to a fresh tube and extract with 600 ul phenol-chloroform-isoamyl alcohol (25:24:1), vortex and centrifuge for 15 min. Repeat this step once more.

6. Extract one final time with chloroform-isoamyl alcohol (24:1), vortex and centrifuge for 3 min.

7. Decant supernatant to a fresh tube and add 600 ul of cold (-20°C) isopropanol, mix gently and thourougly. A stringy white pellet should form. Let tube sit at -20°C for at least 1 hour, then centrifuge pellet at 4°C for at least 20 min.

8. Pipette off supernatant, add 1 ml of 70% cold (-20°C) ethanol. Mix gently then spin at 4°C for 10 min. Repeat once more to ensure that all salts are removed.

9. Dry pellet, either air dry, or use a vacuum centrifuge for approx. 25 min.

10. Add 100-200 ul TE and mix to resuspend pellet. Let tube sit at 4°C overnight before use. Store at 4°C.

### 2X CTAB buffer (500 ml)

50 ml 1 M Tris-Hcl, pH 8.0
175 ml 4 M NaCl (or 40.9 g)
20 ml 0.5 M EDTA, pH 8.0
10 g CTAB (hexadecyltrimethylammonium bromide) (SIGMA H5882)
1 ml 2-mercaptoethanol
Make up to 500 ml with autoclaved double distilled water.
Store at room temperature.

* - Modified from Saghai-Maroof et al. (1984).

```
Herpes Simplex 1        TTTATATAGACGGTCCTCACGG
Herpes Simplex 2                     A  C
Feline                     C     T   G CT T
Marmoset                 G  CC C         C
Turkey                      C         A  GTTT
Equine                  GCCT TC A     G A

consensus               TTTATATAGACGGTCCTCACGG
                           CC         A  CT T

TK1                           TAGACGGTCCCCACGG
                               C      A  T  G
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```
Herpes Simplex 1        GAGCCGATGACTTACTGGCAGGTG
Herpes Simplex 2
Feline                     G      AAAT GAC G  AT
Marmoset                        G G         GATCT
Turkey                  CC      C     GGTCAAAGTT T
Equine                       T    G G         G ACT

consensus               GAGCCGATGACTTACTGGCAGGTG
                        CC         G G     A  G TCT

TK4                     GAGCCGATGACTTACTGGC
(Complimentary)           A       G G
```

Figure One

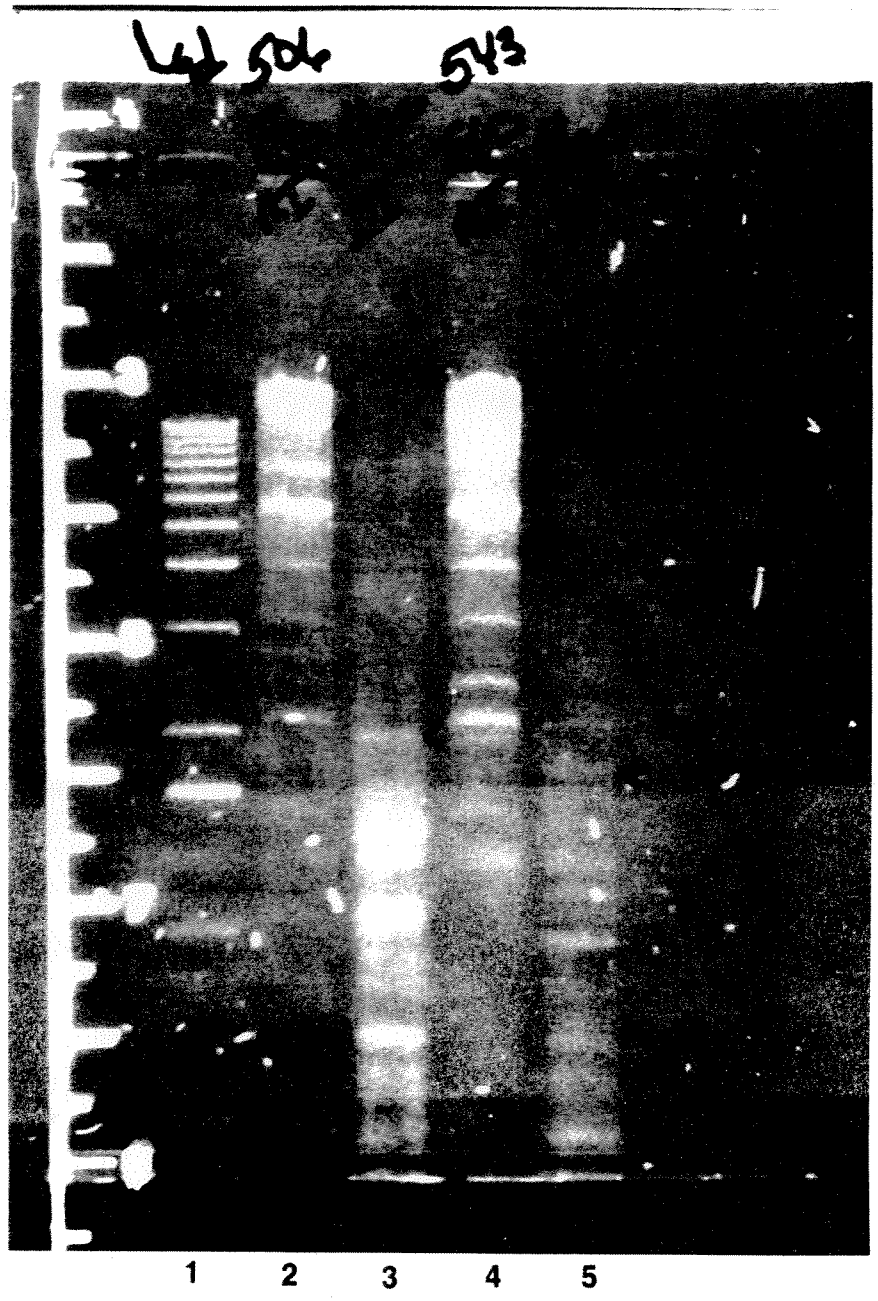


Figure two

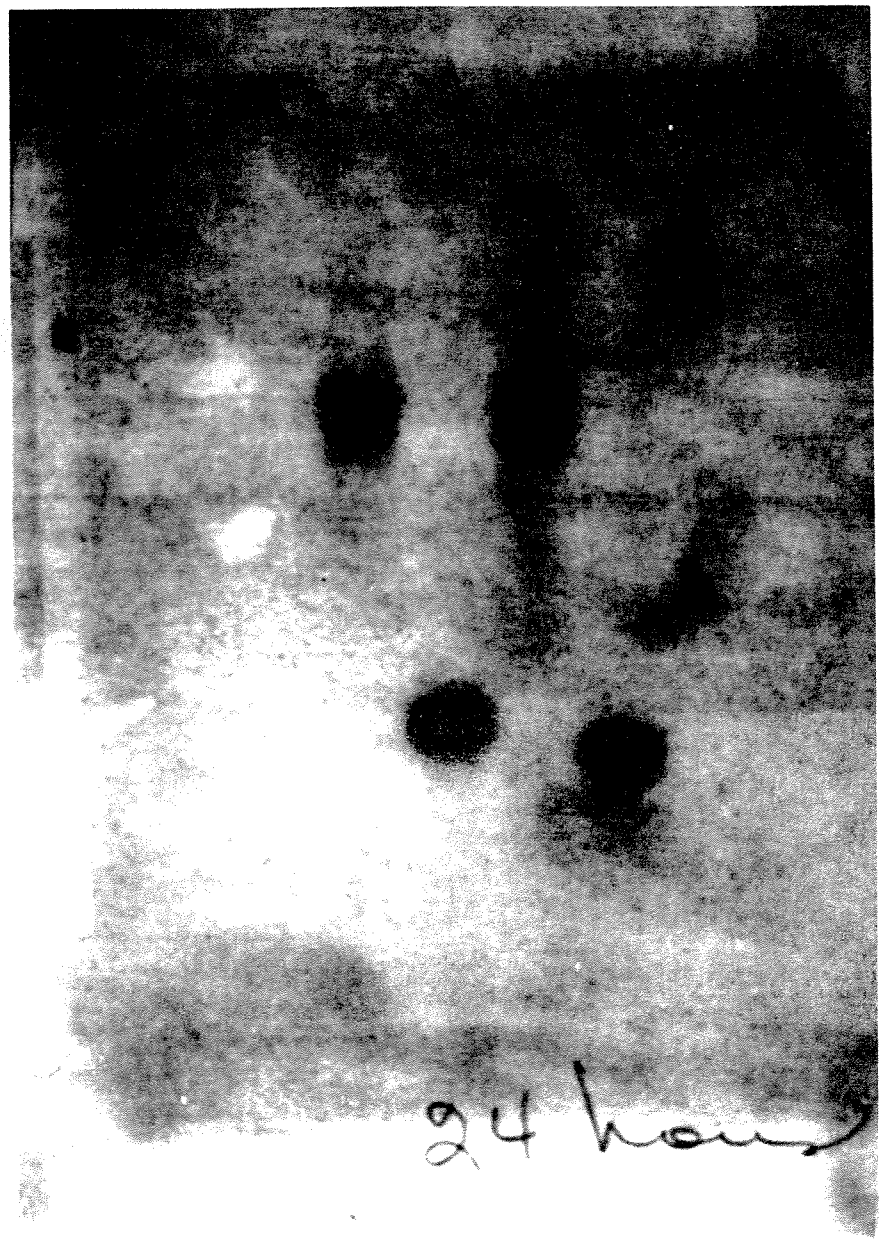


Figure three

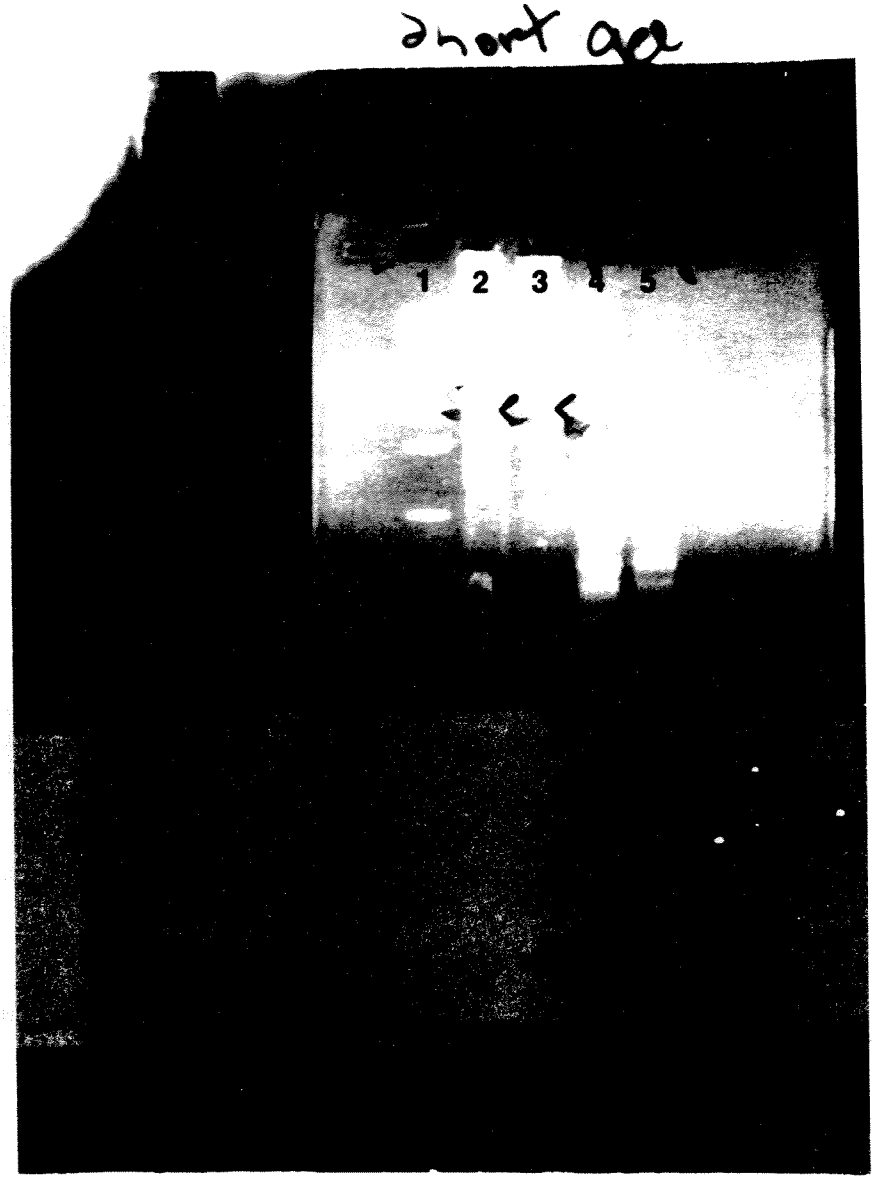


Figure four


Figure five