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MITOCHONDRIAL DNA DIVERSITY AMONG BROOD STOCKS OF THE LAKE TROUT SALVELINUS NAMAYCUSH

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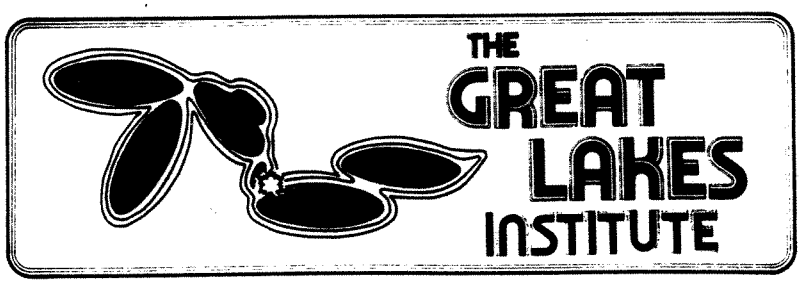

MITOCHONDRIAL DNA DIVERSITY AMONG BROOD STOCKS OF THE LAKE TROUT *SALVELINUS NAMAYCUSH*

GREAT LAKES FISHERY COMMISSION

RESEARCH COMPLETION REPORT

Peter M. Grewe and Paul D. N. Hebert

Department of Biological Sciences,
Great Lakes Institute
University of Windsor
Windsor, Ontario.
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ABSTRACT

Restriction analysis of mitochondrial DNA was used to identify differences among populations of the lake trout Salvelinus namaycush. Mitochondrial DNA was purified from 126 fish representing nine brood stocks along with an additional 47 fish from four natural populations. Seventeen restriction endonucleases were employed to analyse all brood stock fish as well as 17 fish from the British Columbia and Yukon Territory populations. Two of these enzymes (Hind III and Bam HI) were used to examine the 30 fish from two additional natural populations (Lake Superior and Manitoba) along with additional brood stock samples from the Manitou and Seneca strains.

The mitochondrial genome of the lake trout was found to be 16,800±200 base pairs in length. A single heteroplasmic individual was discovered in the Manitou strain. It contained two genomes, the less frequent of which was shorter by 20 base pairs than the other.

The seventeen restriction enzymes resolved thirteen mitochondrial clones which fell into three major groups. These clonal groups, which can be identified by their BamHI restriction phenotypes, have a specific distribution: a western lakes group; a central lakes group; and an eastern Great Lakes group.

Seven mitochondrial clones were unique to a particular stock. In addition there were dramatic shifts in the relative proportions of the six remaining mitochondrial clones among the brood stocks. These results indicate that mt-DNA markers have great potential for the identification and management of lake trout strains.

INTRODUCTION

The lake trout fishery in the Great Lakes collapsed in the mid-1940's as a result of the sea lamprey, pollution, and overfishing (Eshenroder et. al., 1984). Efforts to re-establish self-sustaining populations from hatchery stocks have recently intensified following control of the lamprey. In most cases, plantings include a variety of brood stocks that originated from different locations in the Great Lakes (Pycha and King, 1985; G.L.F.C. internal rep., 1986). Members of the various stocks can be differentially fin clipped to permit comparison of their survival success, but this does not allow asessment of their reproductive capability in the wild. Assessment of reproductive success requires the existence of genetic markers between brood stocks which will ideally persist over many generations.

During the 1970's work on stock identification focussed on electrophoretic studies because variation in allozyme patterns ordinarily has a simple genetic basis. Recent studies which have surveyed as many as 40-50 loci have been more successful (B. May and C. Krueger, pers. comm.) in revealing genetic differences among stocks than earlier studies which were based on too few loci. Yet even comprehensive allozyme surveys loci have failed to reveal evidence of significant genetic variation within some species. For instance, perch populations in Lake Michigan (Leary and Brook, 1982), ciscoes in Lake Superior (Todd, 1981) and lake trout in Lake Superior (T. Todd, pers. comm.) were found to be nearly monomorphic at the loci examined. Even in the most successful cases, differences among populations co-occurring in a specific lake basin have been restricted to fairly minor shifts

in gene frequency. Such variation provides an indication of population structuring, but is not dramatic enough to permit assignment of individual fish to a specific stock on anything more than a probabilistic basis. Allozyme markers have proven more useful in certain other contexts, for example, studying the fate of introduced fish or the incidence of introgression. Yet, even in these cases certain limitations exist. Allozymically marked fish can be distinguished from residents for only one generation and then only if a clear gene substitution exists. Hybridization between the introduced and native stocks and segregation of variants make it difficult or impossible to determine the fate of the introduced fish in later generations.

It is now generally accepted that, when one is dealing with either conspecific or closely related species, restriction analysis of mitochondrial DNA provides more detailed information on phylogenetic relationships than do allozyme studies (Avise et al., 1979a,b; Ferris et al. 1981; Lansman et. al., 1983; Avise et. al., 1984; Avise and Saunders, 1984; Avise et. al., 1986). There are two major reasons for this. Firstly, mitochondrial studies permit examination of variation at the nucleotide level. By contrast, allozyme studies involve detection of variation in charge characteristics of gene products, and thus nucleotide subtitutions which do not alter charge (app. 2/3) are overlooked. Secondly, the resolution capability of mitochondrial DNA studies are enhanced by the fact that mitochondrial DNA evolves at ten times the rate (Brown et al., 1979) of the nuclear genome.

In the context of stock identification in fish,

mitochondrial DNA has the additional advantage of being a maternally inherited asexual genome (Hutchinson et al., 1974; Francisco et al., 1980; Lansmen et al., 1981). This has an important effect on the anticipated level of genetic divergence between stocks. The extent of genetic divergence in any segment of the genome is determined by the interaction between processes which promote divergence, and the process of gene exchange which reduces divergence. When populations are fairly large and selection pressures weak (as is the case for allozyme variants), the amount of gene exchange is critical in determining the extent of divergence. For nuclear genes this exchange can be accomplished by the movement of individuals of either sex. In the case where females are philopatric and males mate randomly, no divergence in the nuclear genome will occur. However, this same pattern of gene exchange permits the divergence of the mitochondrial genome because it is maternally inherited.

In summary, there are two main reasons to expect more clearcut divergence among stocks of fish in the mitochondrial rather than the nuclear genome - the more rapid rate of evolution of mitochondrial DNA and the failure of male exchange to reduce genetic divergence among stocks.

Within the past two years it has become evident that studies of mitochondrial DNA diversity offer exceptional promise in the discrimination of fish stocks in a manner useful to fisheries management. Specifically the studies carried out so far (eg. Avise et al., 1984; Avise and Saunders, 1984; ; Berg and Ferris, 1984; Wilson et al., 1985; Avise et. al., 1986; Billington and Hebert, 1986; Thomas et al., 1986;) have demonstrated the

occurence of abundant mitochondrial DNA variation in a variety of fish species.

This project aimed to examine the value of mitochondrial restriction fragment data in defining brood stocks of the lake trout. The study concentrated on nine brood stocks used in restocking programs in both Canada and the United States. The lake trout is a philopatric species (Swanson, 1974) and thus has a population structure favouring the development of genetically isolated stocks. Yet, attempts to distinguish these stocks on morphological and allozymic criteria have met with limited success. Natural reproduction is now occuring in Lake Ontario (Krueger, pers. comm.), Lake Huron (R. Eshenroder, pers. comm.), and Lake Michigan (J. Epifano, pers. comm.) in areas where lake trout were planted. It is essential to identify these natural recruits in order to determine which strains are reproducing successfully in the wild.

MATERIALS AND METHODS

The lake trout strains analyzed in this study were obtained from hatchery brood stock, with the exception of the wild caught Simcoe and Lewis Lake fish (Table 1). The geographic origin of each strain is shown in Figure 1. In addition to the hatchery strains, a limited survey of mitochondrial diversity was also carried out on lake trout from four natural populations (Figure 1) - Hare Island (Lake Superior), Atlin Lake (British Columbia), Lake LaBerge (Yukon Territory), and North Knife Lake (Manitoba).

Mitochondrial DNA (mt DNA) was routinely extracted from liver tissue, but immature ovaries and mature testes proved to be good sources as well. Fresh tissue was used exclusively as yields of mt DNA from frozen tissue were substantially reduced (pers. obs.). In some cases, fish were transported to the laboratory and kept alive in 1400 litre aquaria, with their livers dissected just prior to extraction of the mt DNA. Liver tissue, however, may be kept on ice for at least 4 days (up to 10 days at $0^{o}C$) without a noticeable decline in mt DNA yield (pers. obs.). Most of the liver samples used in this study (taken in conjunction with disease culls) were, therefore, sent via courier to the laboratory and processed within 4 days of dissection.

Mitochondrial DNA was extracted and purified according to the protocol outlined in Appendix I. Yields of mtDNA were approximately 1500-2000 ng per 3-5 g of liver tissue - enough DNA to perform approximately 300 restriction digests.

Seventeen restriction endonucleases (Table 2) were employed to analyse all 126 brood stock and the 17 fish from Atlin Lake

and Lake LaBerge. Additional fish from the Manitou (n=12) and Seneca (n=13) strains, along with the Hare Island and North Knife Lake fish, were analysed using only Bam HI and Hind III.

Two microlitres (5-10 ng.) of each sample were digested with approximately 1 unit of a particular restriction endonuclease in a total volume of 15 uL using the buffer system and incubation temperatures specified by the supplier (Bethesda Research Laboratories). Samples were digested for one and a half hours and then end-labelled with ^{32}P radio-labelled nucleotides, using the fill-in reaction of the large (Klenow) fragment of DNA polymerase I (Maniatis, 1982). Unincorporated nucleotides were then removed and the samples dried (see Appendix I for details of sample preparation). The samples were then reconstituted in a buffer (8% sucrose and 0.05% bromophenol blue in TBE), split into 7.5 uL aliquots, and simultaneously electrophoresed utilizing agarose and polyacrylamide gels in a TBE (89mM Tris, 89mM Boric Acid, 2mM EDTA pH 8.0) buffer system. DNA fragments ranging in size from 20,000 to approximately 500 base pairs were resolved on 1.2% agarose gels, while fragments of between 1000 and 26 base pairs were resolved on 4% acrylamide gels (38:2 acrylamide : bis-acrylamide).

Upon completion of electrophoresis, gels were dried onto a filter paper backing (3MM), and exposed overnight to X-ray (Fuji-RX) film. Restriction fragments were visualized as sharp black bands on these autoradiographs. Fragment sizes were estimated from the autoradiographs utilizing the program DNAGEL (Kieser, 1982; modified by P. Grewe) run on an Apple II plus computer in conjunction with a HIPAD (model DT-11A, Houston Instruments)

digitizing pad. Restriction fragments of Lambda and pBR322 were used as size standards (Appendix 2) in these analyses.

Homologies of restriction sites were confirmed by conducting appropriate double digests and a preliminary restriction site map of the lake trout mitochondrial genome was determined. The lengths of restriction fragments greater than 11 kb were difficult to estimate accurately from the high percentage agarose gels used. Therefore, lengths of these fragments were obtained from double digests which cut them into smaller pieces that had low errors associated with their size determination. The size of the lake trout mitochondrial genome was then estimated by comparing the sums of restriction fragments produced by the thirteen 6-base enzyme digests.

Restriction patterns obtained for each endonuclease were assigned a letter (A,B,C...etc.) in the order of their discovery and the mitochondrial genotype of each fish was described by a set of 17 letters. Each unique 17 letter combination described the "phenotype" of a specific mitochondrial clone and these were subdivided into three groups on the basis of their Ava I and Bam HI restriction patterns. For example, a clone was labelled "A" if the AvaI or BamHI "A" pattern was present. It was labelled "B" if the BamHI "B" pattern was present and "C" if the BamHI "C" pattern was present. Justification for the recognition of these three groups is provided later.

The effective number of clones in each stock was calculated using the inverse of Simpson's Index (Simpson, 1949; Parker 1979). The effective number of clones and actual number of clones

detected in each brood stock were separately regressed against sample size using the General Linear Models Procedure of SAS.

The maximum likelihood estimates of the number of nucleotide differences per site ($\underline{d}$) were calculated using the method and programs of Nei et al. (1985). This entails comparing the number of restriction sites m_x and m_y (for mt DNA molecules x and y, respectively) with m_{xy}, the number of shared restriction sites. It was impossible to determine m_{xy} between the restriction endonucleases Hinf I, Hpa II, and Taq I due to the complexity of their patterns (Fig. 2c-e). These results were therefore excluded from this analysis. The values of $\underline{d}$ for the 5-base enzyme Nci I and the 6-base enzymes were, however, used to construct UPGMA phylogenetic trees after the method of Nei et al., 1985. Arctic charr mtDNA, isolated from fish taken in the Tarsuk Arm (northwestern Baffin Island), was used to root the 6-base UPGMA dendrogram.

A preliminary phenetic tree utilizing all restriction endonuclease patterns was constructed by parsimony. Clones with the fewest restriction site changes between them were connected together, assuming that parallel site gains and losses were extremely rare or did not occur. For example, to obtain the Bam HI "C" restriction type from the Bam HI "A" restriction type, the Bam HI "B" type was required as an intermediate (see Figure 5). Reanalysis of the parsimony data is in progress using Swofford's (1985) phylogenetic analysis package.

RESULTS

Lake Trout Mitochondrial Genome

The lake trout mitochondrial genome is approximately 16,800±200 base pairs in length (concensus length obtained from thirteen 6-base restriction digests). The mitochondrial genomes of all fish examined were identical in length with one exception. One fish from the Lake Manitou strain was heteroplasmic, containing two mitochondrial genomes of different size (confirmed through double digests). The smaller (by 20 base pairs) genome appeared less intense (app. 1/10) on autoradiographs and was only detected with digests utilizing either Hind III or Hinf I. These digests cut the two genomes into fragments which migrated to positions on the gels affording resolution of the smaller heteromorphic fragment. The larger genome was labelled the A9 clone. Due to the errors involved in fragment length determination, it was not clear which genome was identical in size to the typical lake trout mitochondrial genome. However, both long and short "A9" genomes were characterized by a Hind III site gain. This discovery of heteroplasmy did not hamper any subsequent analysis, but it may prove useful as a diagnostic character of the Manitou strain. No other heteroplasmic individuals were observed among the remaining 142 fish surveyed for the entire 17 restriction enzymes.

Restriction patterns

Seven restriction endonucleases (Bcl I, Eco RI, Pst I, Pvu II, Sal I, Xba I, and Xho I) were monomorphic, i.e. they produced identical restriction patterns for all fish surveyed (Fig 2a). The ten remaining restriction endonucleases revealed

"polymorphic" patterns (Fig 2a,b,c,d,e) and permitted the resolution of thirteen mitochondrial clones (Table 3) within the group of 126 brood stock fish examined. Eight of these clones were resolved by 6-base restriction endonucleases, but the balance could only be recognized by utilizing four- and five-base restriction endonucleases. A preliminary restriction site map (6-base enzyme data only) has been included (Figure 3) detailing the positions of the variable Bam HI sites relative to some of the invariant sites.

Phylogeny of Mitochondrial DNA Types

Two approaches were used to examine the phylogenetic relationships between the 13 mitochondrial clones : genetic distance analysis after Nei et al. (1985); and phenetic analysis by parsimony. Genetic distance analysis, based on 6-base enzymes (Figure 4b), showed that the thirteen clones fell into three major groups (A,B,and C), with groups "B" and "C" being more closely related to each other than either was to group "A". The average genetic distance between members of groups "B" and "C" was .0138, while the average distances between members of "A" and the "B" and "C" groups were .0238 and .0245 respectively. The five-base enzyme data (Figure 4a) supported the distinctiveness of group "A", but suggested that "B" and "C" were more closely related.

The parsimony analysis shows the differences between the mitochondrial clones and the mutational steps required to move from one clone to the next. These results extend those gained from the distance analysis. For example to move from "A" to "B"

required a gain of a Bamd HI site, while moving from "A" to "C" required the gain of both this site and an additional Bam HI site (Figure 5). The parsimony analysis in combining all restriction site data (Figure 6) clearly revealed that group "B" is intermediate to groups "A" and "C". It was separated from members of group "A" by a minimum of 6 restriction site differences and from members of group "C" by at least 4 site differences. Thus, a minimum of 10 mutational steps were required to move from the "A" type to the "C" type.

Clonal Distribution

Clone group "A" was the most diverse of the three groups and included 9 different clones, while groups "B" and "C" were represented by one and three clones respectively. Clones A1, A2, B1, and C1 dominated (>13 fish per clone) the clonal assemblage with approximately 90 percent of the fish belonging to one of these clones (Table 3). The remaining nine clones were "rare", each being represented by fewer than 5 fish. In fact seven of these nine clones were represented by only a single fish and were unique to a particular strain (Table 4). These clones were found in the Green Lake (A5), Lewis Lake (A4), Manitou (A8,A9), Marquette (A7,C3), and Seneca Lake (A6) strains.

The overall distribution of the mitochondrial clones among the hatchery strains, as reported in Table 4, is depicted graphically in Figure 7 which shows that each strain has a unique clonal assemblage. The Clearwater Lake and Marquette strains contained the same three common clones (A1,A2, and C1), but the C1 clone was much more common in Clearwater Lake. The Lewis Lake strain also contained the A1 and A2 clones, but the B1 and C2

clones were present, while the C1 clone was absent. The Green Lake strain also contained the A1 clone, but there was high proportion of the otherwise rare A3 clone, and the B1 and "C" clones were absent. All fish from the Big Bay strain were found to possess the A1 clone, while the Killala Lake strain contained clones A1 and B1 with the latter making up the majority of this stock. Both the Simcoe and Seneca Lake strains contained high proportions (>90%) of the B1 clone, but the two stocks possessed different group "A" clones. The Manitou strain contained a high proportion of the B1 clone (50%), together with two unique clones (A8 and A9) at high frequency.

Three clones were found in the Lake LaBerge and Atlin Lake samples, two of which were identical to mitochondrial clones found in the Great Lakes. Specifically, the B1 (14 fish) and C1 (2 fish) clones were identified, together with a single new clone belonging to group "B". This clone (B2) differed from the B1 clone by the loss of a Taq I site.

Fish from the two other natural populations (Hare Island and the North Knife Lake) were only analysed with Hind III and Bam HI to permit the assignment of these fish to one of the three major clonal groups. Both populations contained all three clonal groups with "A" composing 33% and 44% in the Hare Island and the North Knife Lake samples respectively. Group "B" clones composed 19% and 22% of the populations, while group "C" made up 48% of the Hare Island and 33% of the North Knife Lake samples.

The distribution of the "A","B", and "C" clonal groups exhibited a geographical pattern (Fig. 8). Group "C" clones

predominated in the northwestern section of the Great Lakes and were also common in Manitoba. They were also present but rare in the Atlin Lake sample. Group "B" clones showed a somewhat disjunct distribution, being predominant in both the southeastern Great Lakes and the Atlin Lake and Lake LaBerge samples. Group "A" clones predominated in the central Great Lakes region and were found in every strain, with the exception of the samples from British Columbia and the Yukon Territory.

Clonal Diversity

Analysis of the effective number of clones per strain (Table 4) indicated that the Manitou, Lewis Lake, and Marquette strains showed the highest level of mitochondrial diversity with approximately 3.0 clones per stock. The Green Lake, Killala, and Clearwater Lake strains each contained 2.0 clones, while the Big Bay, Simcoe, and Seneca strains each contained approximately 1.0 clone. The effective number of clones for the natural populations of Lake LaBerge, Atlin Lake, Hare Island, and North Knife Lake were 1.0, 1.6, 2.7, and 2.8 respectively. Regression analysis, of all populations sampled, indicated that there was a significant direct relationship between the effective number of clones and number of fish analyzed ($p<.01$, $r^2= 0.54$). An even stronger direct relationship was indicated between the actual number of clones detected and sample size ($p<.001$, $r^2= 0.70$).

DISCUSSION

The mitochondrial genome in vertebrates ranges from 15-20 kilobase pairs in length (Gray, 1981; Brown, 1983) with that of most fish, including the lake trout, falling between 16.5 and 17.5 kilobases in length (Table 6). The lengths of the mitochondrial molecules were invariant in all lake trout examined with the exception of a single fish from the Manitou stock. This fish was also heteroplasmic, containing two populations of mitochondrial molecules of different size. Heteroplasmy is generally uncommon, but it has been reported in holstein cows (Hausworth et. al. 1984),), Cnemidophorus lizards (Densmore et. al., 1985), frogs (Monnerot et. al., 1984; Bermingham et. al., 1986), and in two fish species - the bowfin (Bermingham et. al., 1986) and the white perch (R. Chapman, pers. comm.). Such length heterogeneity is thought to arise from insertions/deletions near the D-loop region which occur during replication of the mitochondrial DNA molecule (Brown, 1983). Birky (1982) has suggested that heteroplasmy is ordinarily confined to germ cell lines, and becomes evident in somatic tissue only when more than one mt DNA molecule becomes established in the somatic cell line during differentiation of the embryo. Heteroplasmy was obviously not confined to the germ cell line in the Manitou fish as liver tissue was used for extraction. Both size variants in this fish could be identified by an extra Hind III site which should allow easy recognition of its siblings in the Manitou population and further study of this case of somatic heteroplasmy.

Our survey of mitochondrial diversity revealed 13 mitochondrial clones among the brood stock fish and one

additional clone in the Atlin Lake sample from British Columbia. The analysis of mitochondrial diversity within strains of the lake trout using Parker's (1974) "effective number of clones" showed that the Marquette, Lewis, and Manitou strains were the most diverse. However, as sample size varied among stocks and was shown to have a significant effect on the level of mitochondrial clonal diversity, these apparant differences among stocks must be interpreted with caution. Thus, the high diversity seen in the Marquette strain is likely, at least in part, a result of the large number of fish sampled (n=38). However, it is noteworthy that the Marquette strain originated from fish taken from three different locations in Lake Superior (Krueger et. al., 1983). Similarily, fish stocked into Lewis Lake were reared from eggs, obtained from several different reefs in Lake Michigan, and the original population was supplemented with fish of unknown origin. It is uncertain whether these later fish made a significant contribution to the Lewis Lake population (Vischer, 1983), but certainly opportunities existed for the inclusion of a large amount of mitochondrial diversity in this stock. The Manitou strain also seems to have a high amount of mitochondrial diversity, but, as only four fish were examined, more analyses are required before drawing further conclusions. The Green Lake strain contained approximately two mitochondrial clones despite the fact that it has been held in hatcheries for at least three generations (Krueger et. al., 1983) and sujected to several transfers with high mortality (R. Eschenroeder, pers. comm.). It is interesting to note that the three brood stocks (Big Bay,

Simcoe, Seneca) with the lowest mitochondrial diverstiy (ie. one effective mitochondrial clone) were each established from single small collections of females. For example, only 17 females founded the Seneca brood stock, while the Big Bay and Simcoe strains came from small original populations (P. Ihssen, pers. comm.). Our limited work on natural populations suggests that they have levels of mt DNA diversity similar to those found in the most diverse of the hatchery strains examined.

Analysis of the extent of mitochondrial diversity is a complex issue for the number of clones detected depends upon one's sampling intensity on the mitochondrial genome and upon the number of individuals examined. Clearly, two important questions need to be answered in order to estimate the amount of mitochondrial DNA variation in any local population or brood stock. Firstly, how many individuals must be analyzed to detect all the clones present in a specific stock? Secondly, what proportion of the mitochondrial genome needs to be surveyed in order to resolve all mitochondrial variants? Currently, we are developing a method to carry out such analysis which employs a combinatorial approach to study the relationship between the number of clones detected and both sample size and genome sampling intensity. At this point it is obvious that the present survey has not been extensive enough to detect all mitochondrial variants, but sufficient to make it clear that there are a small number of well differentiated mitochondrial lineages in the lake trout, whose frequencies vary among brood stocks and natural populations.

The analysis of genetic similarity among the thirteen

mitochondrial genotypes indicated that they could be divided into three groups with clone groups "B" and "C" more closely related to each other than to "A". Based on the accepted rate of vertebrate mt DNA evolution (Brown, 1979), the "B" and "C" lineages separated from the "A" lineage approximately 500,000 years ago, while the "B" and "C" lineages separated from each other approximately 270,000 years ago. The arctic charr lineage separated from the lake trout about 16.7 million years ago.

Our results suggest that lake trout populations in the Great Lakes derive from three distinct lineages or from separate glacial refugia which contained unique clonal groups. It is significant that clone group "A", which is most common in the Great Lakes, was present in the Manitoba sample and yet was absent from the British Columbia and Yukon Territory populations. However, the presence of clones B1 and C1 in both the Great Lakes and the Yukon Territory, suggests an exchange of fish between these two areas in recent times (during the last 15,000 years). An alternative explanation would require the mitochondrial genome of these fish to have remain unchanged throughout the Wisconsin glaciation (100,000 years). This seems unlikely in view of the rapid evolution of vertebrate mitochondrial DNA (Brown, 1979). More evidence is required from natural populations, particularily from northern Canada along post-glacial dispersion routes (Lindsey, 1964; Bailey and Smith, 1981; Black, 1983), before further conclusions can be drawn. However, it is interesting to note that the geographical distributions of the "A", "B", and "C" mitochondrial clonal groups show some similarities with patterns

of gene frequency divergence at allozyme loci (P. Ihssen, pers. comm.) and chromosome banding patterns (Phillips and Ihssen, 1986) observed among lake trout stocks.

In summary, the present study has shown that lake trout populations contain large amounts of mitochondrial DNA diversity. The level of mitochondrial diversity in some brood stocks appears lower than that in natural populations, but other stocks have maintained a normal level of diversity. In each brood stock there are a small number of common clones and a few individuals with closely similar genomes, which have apparently arisen as mutational derivatives. The frequencies of the common clone groups vary among brood stocks. In part this variation is likely a consequence of founder effect during brood stock establishment, but congruence in the mitochondrial characteristics of brood stocks from specific sectors of the Great Lakes suggests that the variation among strains also reflects historic distributional patterns of mitochondrial variants. The mitochondrial variants are separable into three groups which likely differentiated from one another in glacial refuges and subsequently colonized the Great Lakes. The dominant clone group (A) was probably carried into the lakes by fish which spent the Pleistocene south of the ice sheets. The other two groups (B and C) apparently represent groups which spent the Pleistocene in eastern or western refuge(s). The congruence of mitochondrial genomes between fish from the Great Lakes and the Yukon Territory suggests that gene flow among lake trout populations has been more extensive than previously thought.

MANAGEMENT IMPLICATIONS

Present

The mitochondrial clones identified in the present survey should provide a good indication of the mitochondrial characteristics of progeny produced by each brood stock. While there may be some selection against unfit nuclear genotypes in a population, the mitochondrial genome does not experience this same degree of selection (Brown, 1983). Thus progeny stocked or produced in the wild should possess the original clonal proportions of their parental stock even in the event of strong selection/elimination of certain nuclear genotypes. Mitochondrial markers will exist for many generations unaffected by recombination and be passed on to all offspring of that brood stock.

It is possible to identify the 9 brood stocks in the present study based on variation in the relative frequencies of dominant clones and also by the appearance of unique or rare clones. A simple dichotomous key is presented in Appendix 3 as an aid to stock discrimination based on the mitochondrial genome. The use of allozyme or other data sets should complement the mitochondrial DNA data and aid in further confirming identifications.

Future

When initiating new brood stock lines, all females should be typed (non-destructively) for their mitochondrial phenotype and marked for future identification. Progeny could be monitored (non-destructively) on a yearly basis to study bottlenecking in hatcheries. It should also be possible, utilizing rapid

techniques, to type whole brood stocks and subdivide the females into groups possessing specific mitochondrial clones. The genetic variability in the nuclear genome of these groups could be maintained by utilizing a large number of genetically diverse males for fertilization. Mitochondrial markers for each existing brood stock could be produced in a similar manner. With the fixation of a different mitochondrial clone in each stock, identification of naturally produced progeny would then be straightforward. For example, it would be simple to restrict the mitochondrial base of the Green Lake strain to the rare A3 clone, thus creating a mitochondrially marked Green Lake brood stock.

To supplement the existing mitochondrial diversity it should be possible to manipulate the mitochondrial genome through insertion of novel DNA sequences into the non-coding (D-loop) region of the molecule. With this technology, each hatchery strain could be given its own "genetic tag". Such a tag would be passed on to offspring of the brood stock and would provide a simple diagnostic character for many generations.

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TABLE 1. Lake trout strains used in this study.

Strain	(abrev.)	no.	year class	origin of fish and date obtained
Manitou	(Man)	4	80'	- Maple Research Station, Ontario, May 85.
Big Bay	(Big)	5	80'	- Maple Research Station, Ontario, May 85.
Simcoe	(Sim)	10	unknown	- collected from ice-fishermen on Lake Simcoe and identified as Simcoe stock by personnel at Sibbald Point assessment unit, Feb. 86.
Killala	(Kil)	11	83'	- Hatchery, Ontario, Aug. 85.
Seneca	(Sen)	15	78'	- Allegheny N.F.H., Nov. 84.
Green Lake	(GL)	9	75'	- Jordan River N.F.H., Oct. 84.
	(GLA)	6	75'	- Jordan River N.F.H., Oct. 85.
Lewis Lake	(Lew)	8	82'	- Jackson N.F.H., Aug. 85.
	(Lou)	9	unknown	- wild caught fish from Lewis Lake Sept. 85.
Clearwater Lake Manitoba	(Cma)	11	82'	- Great Lakes Fish. Comm., June 85.
Marquette (domestic)	(Sup)	10	75'	- Jordan River N.F.H., Nov. 84.
		8	81'	- Jordan River N.F.H., Nov. 84.
	(JR)	20	77'	- Jordan River N.F.H., Nov. 85.
Hare Island	(Har)	21	unknown	- collected on the Hare Island reef in Lake Superior by M.N.R personnel in Thunder Bay, Ontario, Aug. 86.
Atlin Lake	(Lin)	13	unknown	- collected from Atlin Lake, British Columbia by P. Etherton, April 86.
Lake LaBerge	(Lab)	4	unknown	- collected from Lake LaBerge, Yukon Territory by P. Etherton, April 86.
North Knife Lake	(Nor)	9	unknown	- collected from North Knife Lake, Manitoba, August 86.

6-BASE ENZYMES

Ava I	C'TCGA,G* C'CCGG,G** C'TCGG,G C'CCGA,G	Bst EII	G'GTGAC,C G'GTAAC,C G'GTTAC,C G'GTCAC,C
Bam HI	G'GATC,C	Pvu II	C,CATG'G
Bcl I	T'GATC,A	Sal I	G'TCGA,C
Eco RI	G'AATT,C	Sma I	CCC'GGG**
Hind III	A'AGCT,T	Xba I	T'CTAG,A
Nco I	C'CATG,G	Xho I	C'TCGA,G*
Pst I	C,TGCA'G		

5-BASE ENZYMES

Hinf I	G'AGT,C G'AAT,C G'ATT,C G'ACT,C
Nci I	CC'C,GG CC'G,GG

4-BASE ENZYMES

Hpa II	C'CG,G
Taq I	T'CG,A

TABLE 2. Restriction enzymes used and their recognition sites.

*,** - Ava I recognizes the sequences also reconized by Xho I and Sma I respectively. Results of the latter two enzymes were therefore used only to define genetic markers and ignored for genetic distance analysis.

CLONE	no. obs.	Ava I	Bam HI	Bst EII	Hind III	Nco I	Sma I**	Hinf I	Nci I	Hpa II	Taq I
A1	40	A	A	A	A	A	A	A	A	A	A
A2	18	A	A	A	A	A	A	D	A	A	D
A3	5	A	A	A	A	A	A	A	C	C	A
A4	1	A	A	B	A	A	A	A	A	A	A
A5	1	A	D	A	A	A	A	D	A	A	D
A6	1	A	A	A	A	A	A	A	D	A	A
A7	1	A	A	A	A	A	A	D	A	A	A
A8	1	A	A	A	A	A	A	D	A	A	E
A9	1	A	A	A	B*	A	A	D*	A	A	D
B1	41	B	B	A	A	A	A	B	B	B	B
C1	13	C	C	A	A	A	A	C	B	B	C
C2	2	D	C	A	A	A	B	C	B	B	C
C3	1	C	C	A	A	B	A	C	B	B	C
TOTAL	126										

TABLE 3. Restriction phenotypes of the thirteen clones resolved by the ten "polymorphic" restriction enzymes, and their abundances among the nine brood stocks.

* - an extra heteromorphic fragment of shorter (app. 20 base pairs) length appears in the restriction patterns of these enzymes.

**- a single restriction site gain is responsible for the Ava I and Sma I polymorphisms of the C2 clone as both enzymes recognize the same sequence, CCCGGG (see Table 2).

Brood Stock	no. fish examined	clones present (no. found)	effective number of clones**	unique clones
Manitou	4	A8 (1) A9 (1) B1 (2)	2.7	A8 (1) A9 (1)
Big Bay	5	A1 (5)	1.0	
Simcoe	10	A3 (1) B1 (9)	1.2	
Killala Lake	11	A1 (4) B1 (7)	1.9	
Seneca Lake	15	A6 (1) B1 (14)	1.1	A6 (1)
Green Lake(GL)	9	A1 (6) A3 (3)	1.9*	
(GLA)	6	A1 (4) A3 (1) A5 (1)		A5 (1)
Lewis Lake (Lew)	8	A1 (1) A2 (2) B1 (5)	2.8*	
(Lou)	9	A1 (1) A2 (2) A4 (1) B1 (4) C2 (1)		A4 (1)
Clearwater Lake Man.	11	A1 (3) A2 (1) C1 (7)	2.1	
Marquette (dom.)		A1 A2 A7 C1 C2 C3	3.1*	
75' (Sup)	10	(7) (3)		C3 (1)
81' (Sup)	8	(3) (3) (1) (1)		
75' (JR)	20	(7) (9) (1) (2) (1)		A7 (1)
Atlin Lake (Lin)	13	B1 (10) B2 (1) C1 (2)	1.6	B2 (1)
Lake LaBerge (Lab)	4	B1 (4)	1.0	

TABLE 4. Clonal diversity among brood stocks of the lake trout.

*- all fish from stock pooled for this estimate.
**- inverse of Simpson's index.

(a)

	A	C	D	B
A	20	--	--	--
C	19	19	--	--
D	19	18	19	--
B	19	18	18	20

(b)

	A1	A4	A5	A9	B1	C1	C2	C3	CHARR
A1	39	--	--	--	--	--	--	--	--
A4	39	40	--	--	--	--	--	--	--
A5	39	39	40	--	--	--	--	--	--
A9	39	39	39	40	--	--	--	--	--
B1	39	39	39	39	41	--	--	--	--
C1	39	39	39	39	41	43	--	--	--
C2	39	39	39	39	41	43	44	--	--
C3	39	38	39	38	40	42	42	42	--
CHARR	31	31	32	31	31	31	31	31	35

TABLE 5. Data used to compute Nei's d for (a) Nci I and (b) 6-base restriction endonucleases. Values on the diagonal are number of restriction sites per clone. Other numbers refer to the number of restriction sites in common between various clones.

TABLE 6. Length of the mitochondrial DNA molecule in fish species based on published data and results from this study.

SPECIES	SIZE (bp)	REFERENCE
Lepomis macrochirus	16,200	Avise et al. (1984)
Katsuwonus pelamis	16,900	Graves et al. (1984)
Oncorhynchus tshawytscha	16,670	Berg & Ferris (1984)
Salmo gairdneri	16,670	
Salmo trutta	16,670	
Salvelinus fontinalis	16,670	
Scorpaena guttata	19,500 ± 300	Beckwitt & Petruska (1985)
Sebastes atrovirens	17,300 ± 400	
Sebastes caurinus	17,400 ± 400	
Sebastes melanostomus	17,200 ± 400	
Sebastes mystinus	16,900 ± 400	
Scomber japonicus	17,200 ± 400	
Salmo salar	16,700	Birt et al. (1986)
Oncorhynchus kisutch	16,500 ± 500	Thomas et al. (1986)
Oncorhynchus tschawytscha	16,500 ± 500	
Oncorhynchus nerka	16,500 ± 500	
Oncorhynchus garbuscha	16,500 ± 500	
Oncorhynchus keta	16,500 ± 500	
Salmo gairdneri	16,500 ± 500	
Stizostedion vitreum	16,833 ± 233	Billington and Hebert (1986)
Stizostedion vitreum	18,475 ± 300	
Stizostedion canadense	16,702 ± 259	
Stizostedion lucioperca	16,736 ± 277	
Salvelinus namaycush	16,800 ± 200	Present study

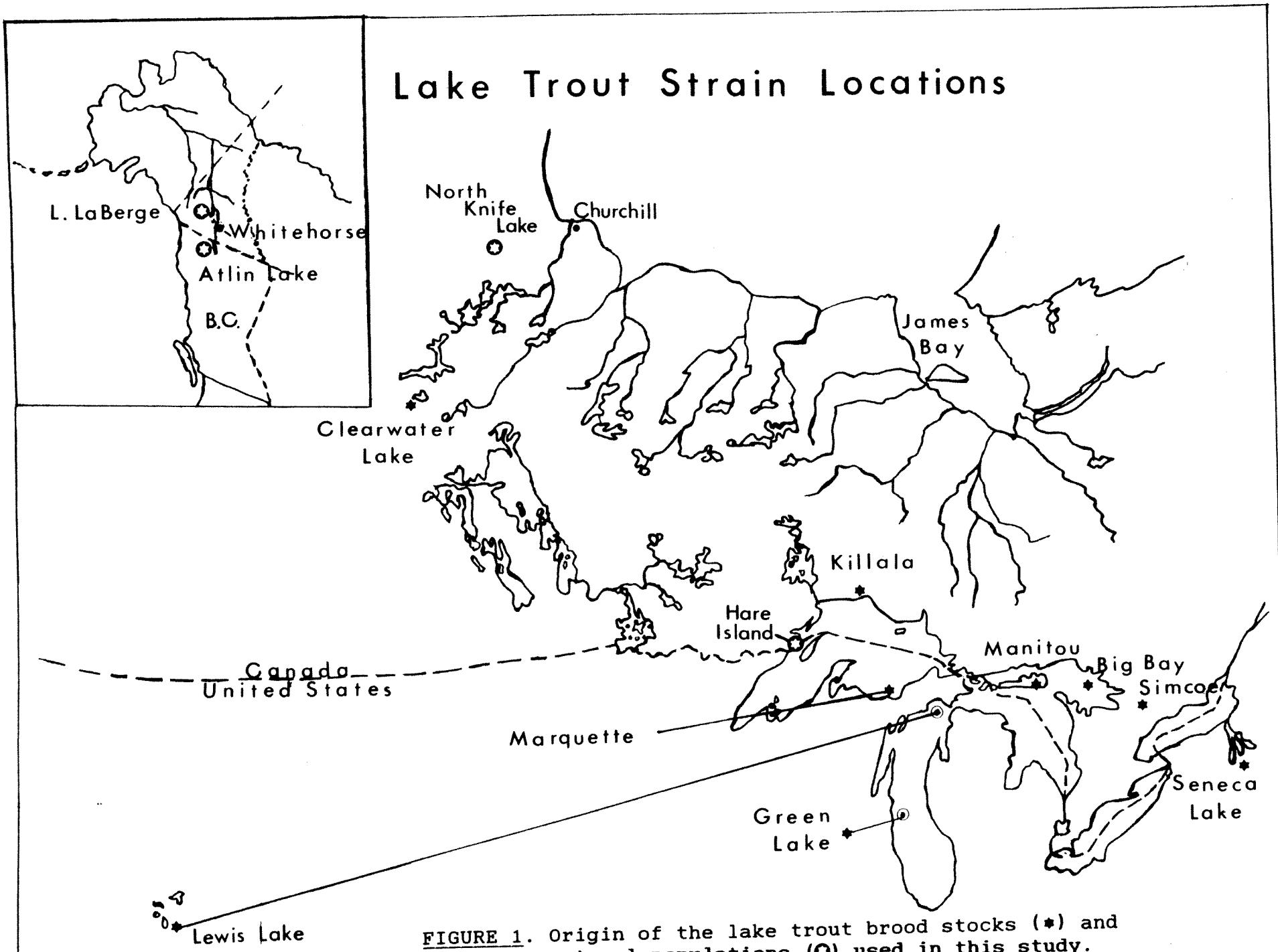


FIGURE 1. Origin of the lake trout brood stocks (*) and natural populations (O) used in this study.

FIGURE 2. Restriction fragment patterns observed for the 17 endonucleases employed in this study. Standard lanes of both agarose and acrylamide gels typically contained a Hind III and Hind III/Eco RI digests of Lambda along with a HpaII digest of pBR322. However, only the pertinent standard lengths are labelled for convenience.

Six-Base Enzymes

(a) Bcl I, Bst EII, Eco RI, Nco I, Pst I, Pvu II, Sal I, Sma I, Xba I, and Xho I patterns.
(b) Ava I, Bam HI, and Hind III patterns.

Note: fragments below the dashed line were resolved utilizing the 4.0% acrylamide gels.

Five-Base Enzymes

(c) Hinf I and Nci I patterns.

Four-Base Enzymes

(d) Hpa II and Taq I patterns.

Autoradiographs of Hinf I Patterns

(e) 1.2% agarose gels of HinfI restriction digests.
(f) 4.0% acrylamide gels of samples visualized in (e).

*- an extra heteromorphic fragment appears in these digests of the Lake Manitou fish, Man2.

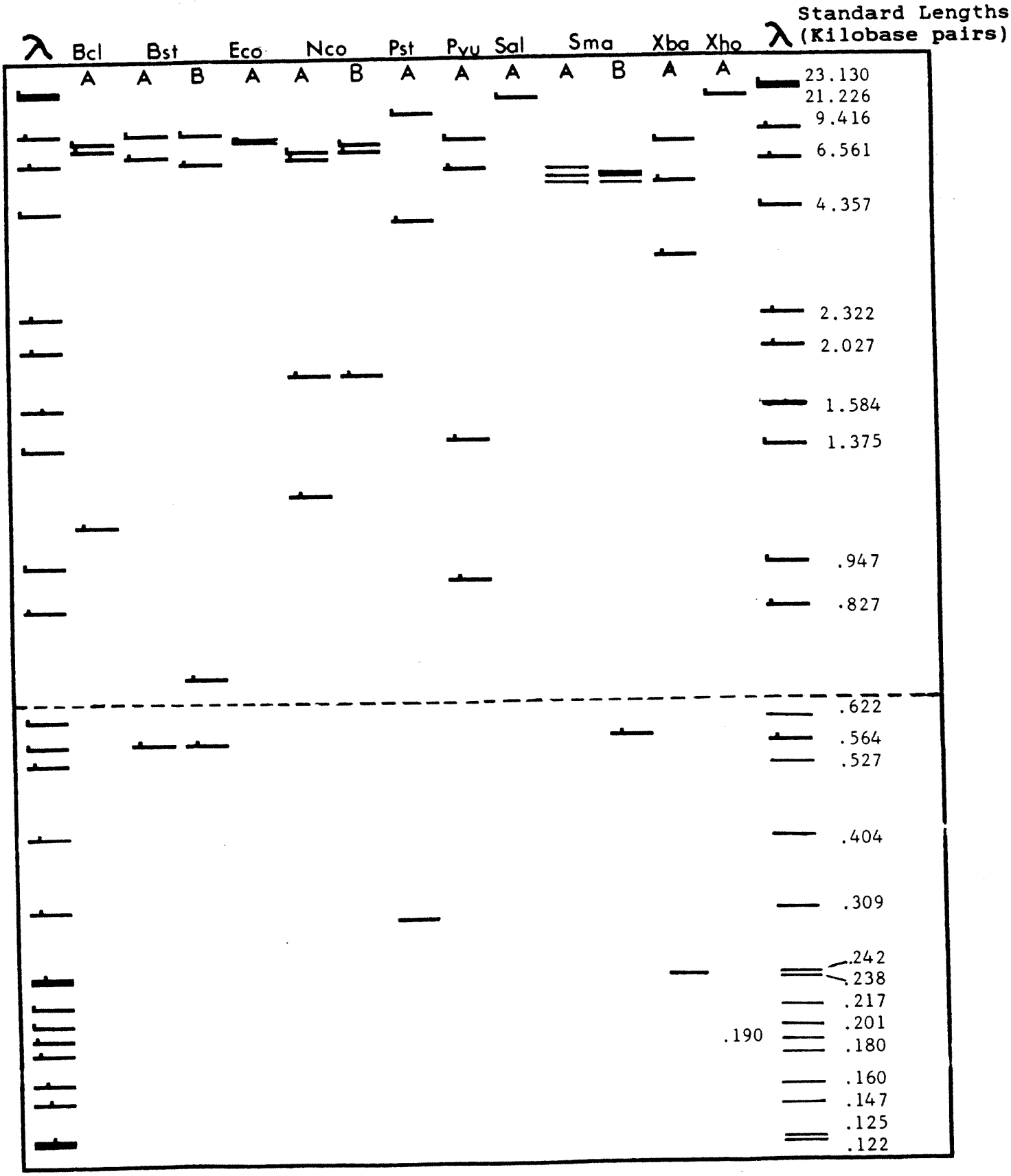

λ
Bcl
Bst
Eco
Nco
Pst
Pvu
Sal
Sma
Xba
Xho
λ
Standard Lengths
(Kilobase pairs)
A
A
B
A
A
B
A
A
A
A
B
A
A
23.130
21.226
9.416
6.561
4.357
2.322
2.027
1.584
1.375
.947
.827
.622
.564
.527
.404
.309
.242
.238
.217
.201
.190
.180
.160
.147
.125
.122

FIGURE 2. (a)

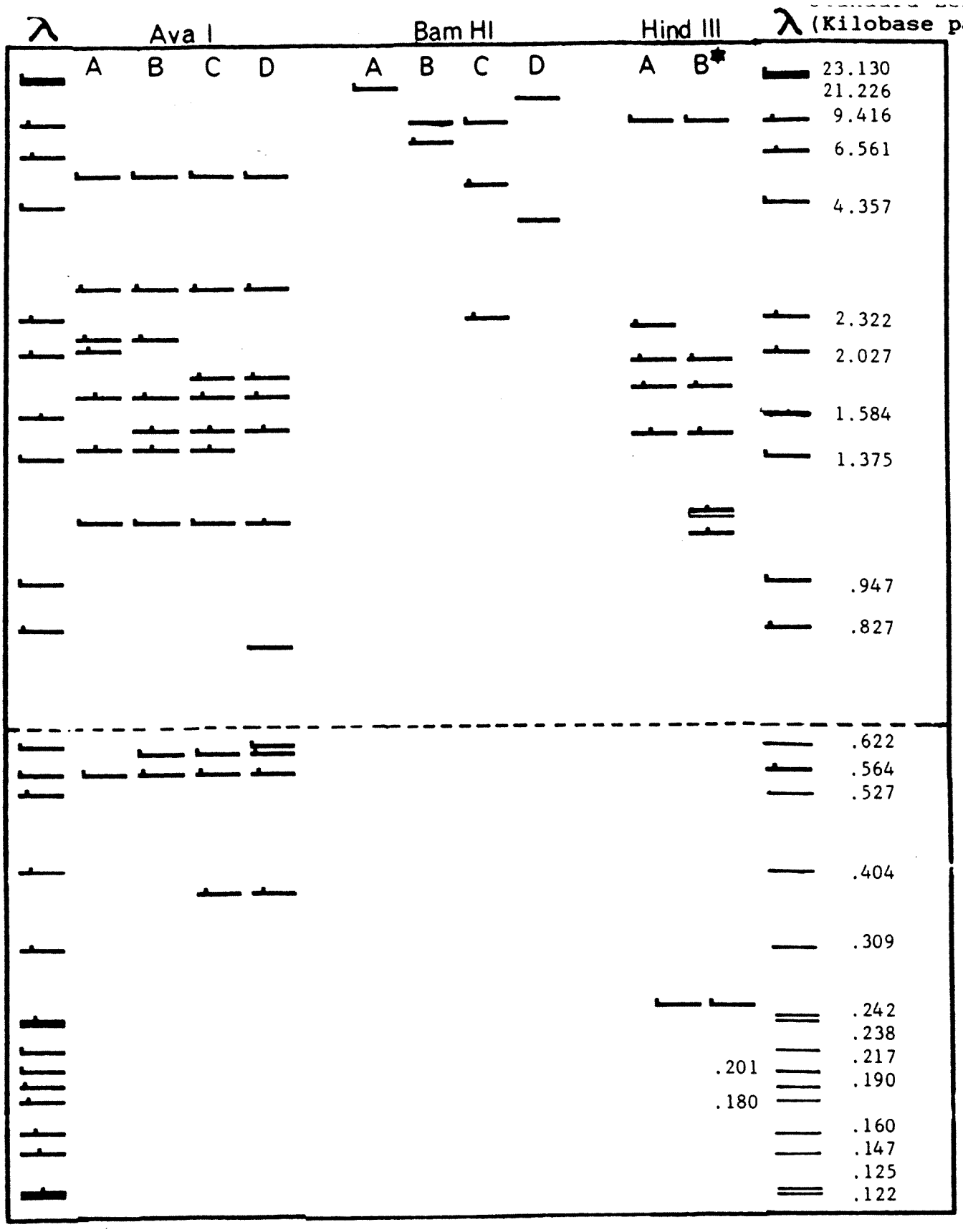

λ
Ava I
A
B
C
D
Bam HI
A
B
C
D
Hind III
A
B*
λ (Kilobase pairs)
23.130
21.226
9.416
6.561
4.357
2.322
2.027
1.584
1.375
.947
.827
.622
.564
.527
.404
.309
.242
.238
.217
.201
.190
.180
.160
.147
.125
.122

FIGURE 2. (b)

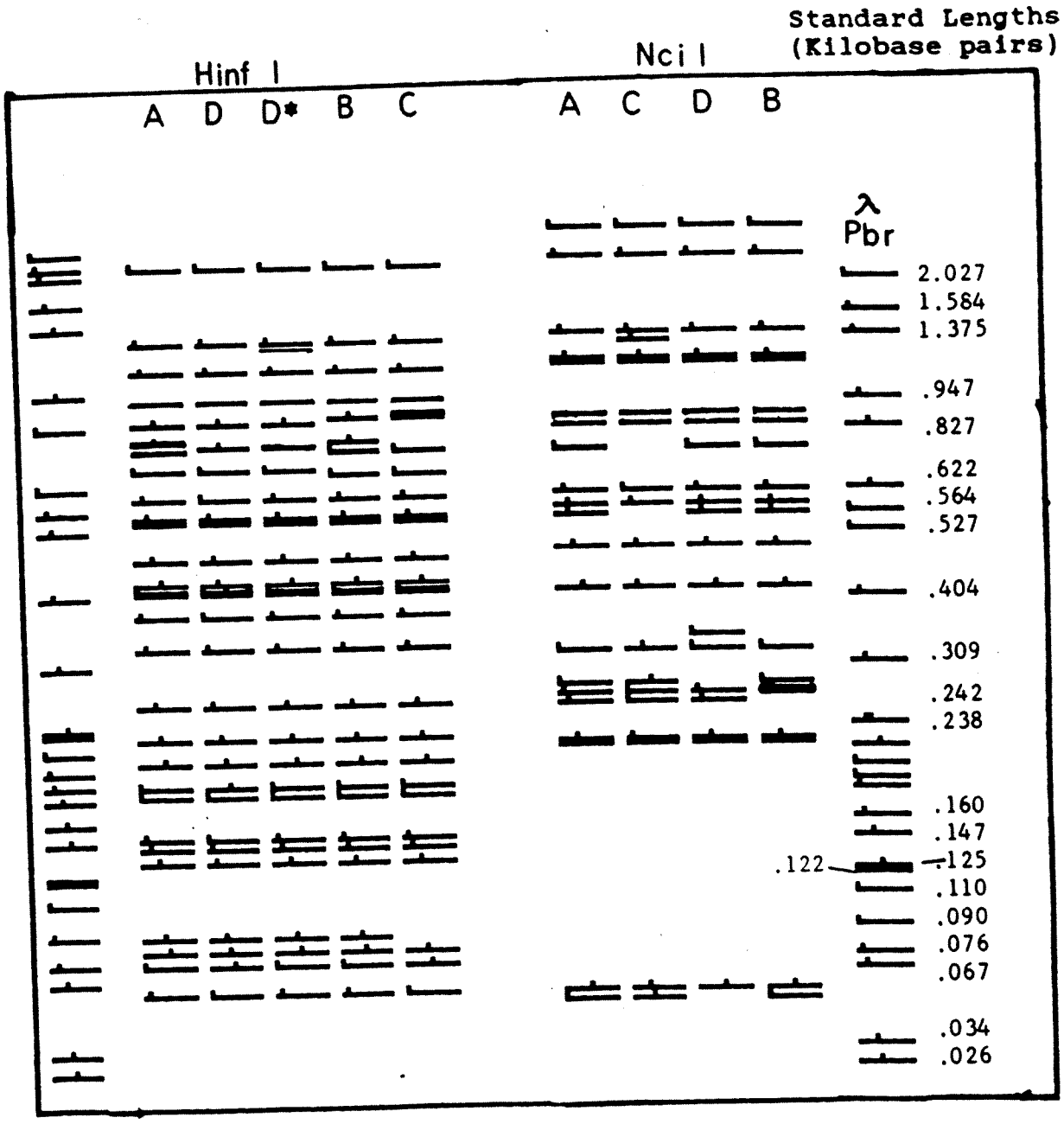
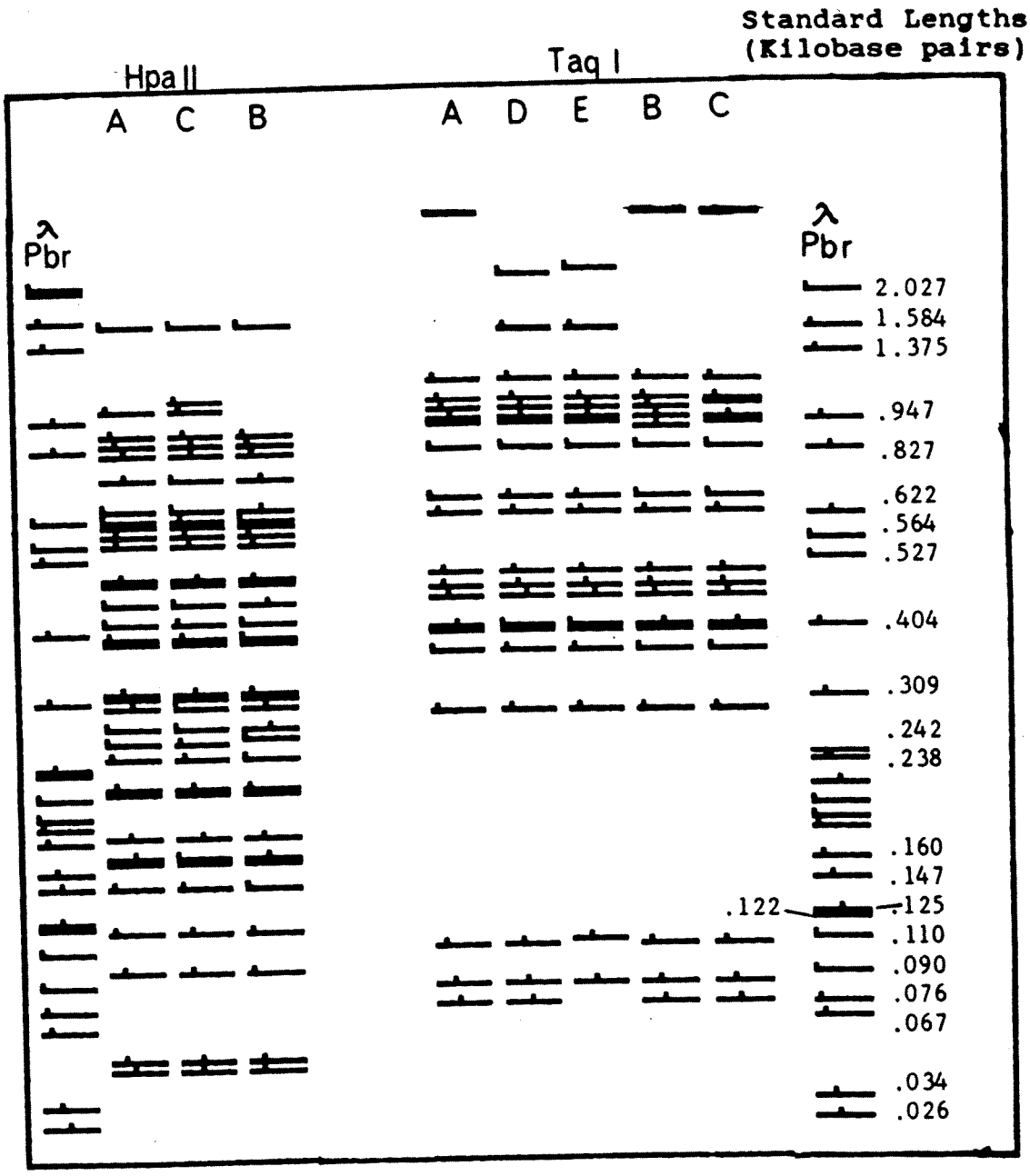

Standard Lengths
(Kilobase pairs)
Hinf I
A D D* B C
Nci I
A C D B
λ
Pbr
2.027
1.584
1.375
.947
.827
.622
.564
.527
.404
.309
.242
.238
.160
.147
.122
.125
.110
.090
.076
.067
.034
.026

FIGURE 2. c)


Standard Lengths
(Kilobase pairs)
Hpa II
Taq I
A C B
A D E B C
λ Pbr
λ Pbr
2.027
1.584
1.375
.947
.827
.622
.564
.527
.404
.309
.242
.238
.160
.147
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.110
.090
.076
.067
.034
.026

FIGURE 2. d)

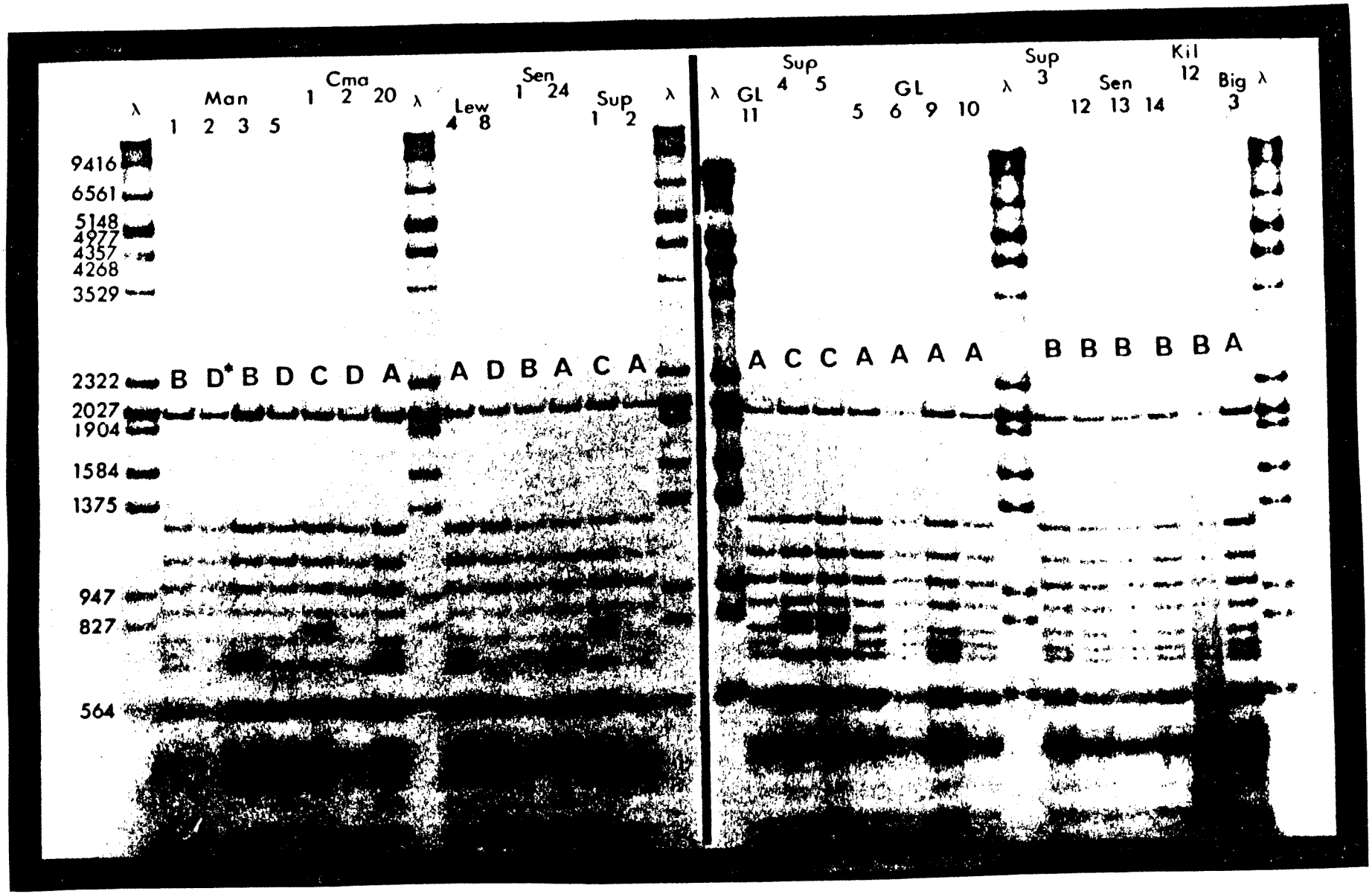

λ
Man 1 2 3 5
Cma 1 2 20
λ
Lew 4 8
Sen 1 24
Sup 1 2
λ
λ GL 11
Sup 4 5
GL 5 6 9 10
λ
Sup 3
Sen 12 13 14
Kil 12
Big 3
λ
9416
6561
5148
4977
4357
4268
3529
2322
2027
1904
1584
1375
947
827
564
B D* B D C D A A D B A C A
A C C A A A A B B B B B A

FIGURE 2. (e)

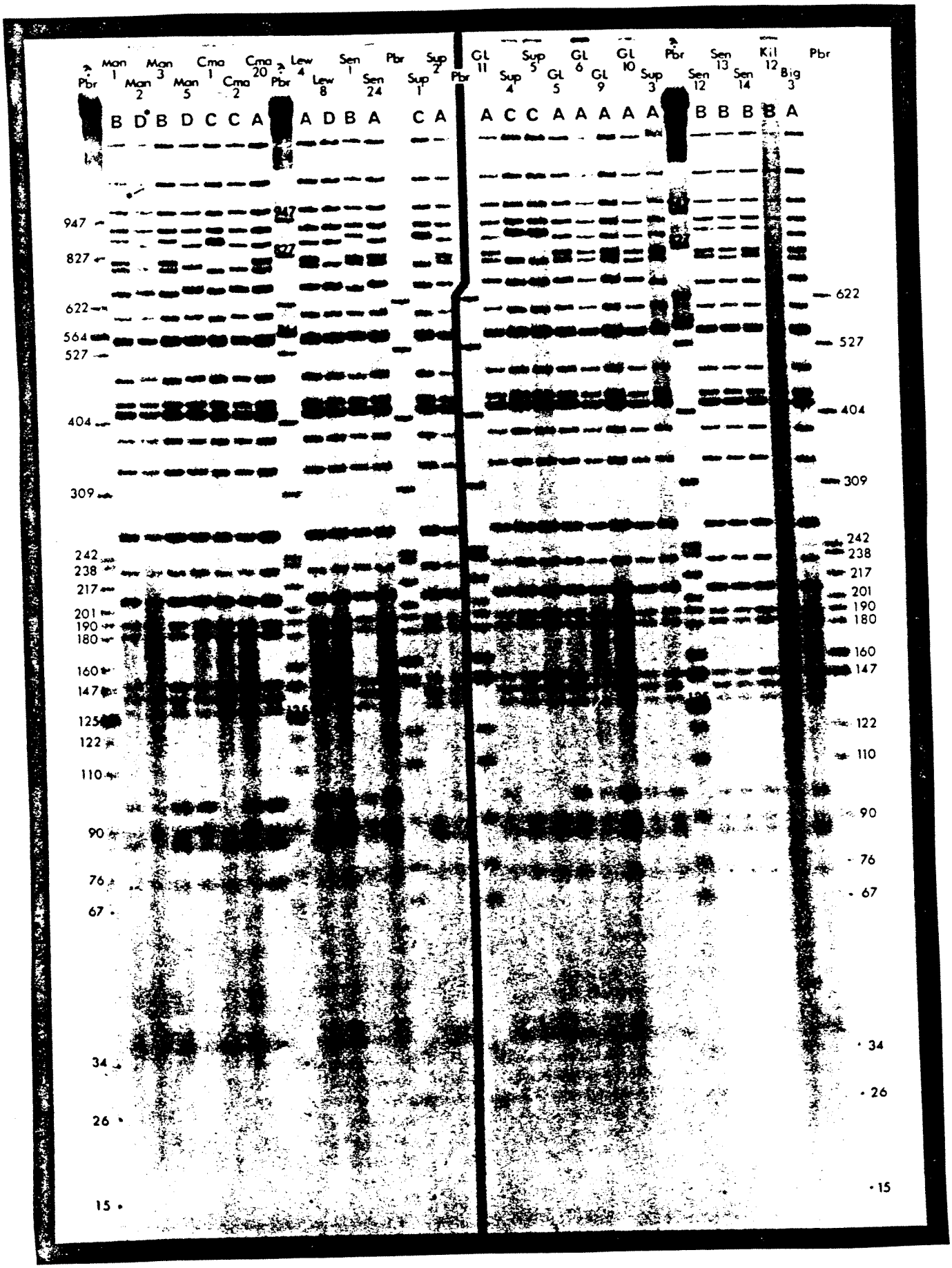

Pbr
Man 1
Man 2
Man 3
Man 5
Cma 1
Cma 2
Cma 20
Pbr
Lew 4
Lew 8
Sen 1
Sen 24
Pbr
Sup 2
Sup 1
Pbr
Gl 11
Sup 4
Sup 5
Gl 5
Gl 6
Gl 9
Gl 10
Sup 3
Pbr
Sen 12
Sen 13
Sen 14
Kil 12
Big 3
Pbr
B D B D C C A A D B A C A
A C C A A A A A B B B B A
947
827
622
564
527
404
309
242
238
217
201
190
180
160
147
125
122
110
90
76
67
34
26
15

FIGURE 2. (f)

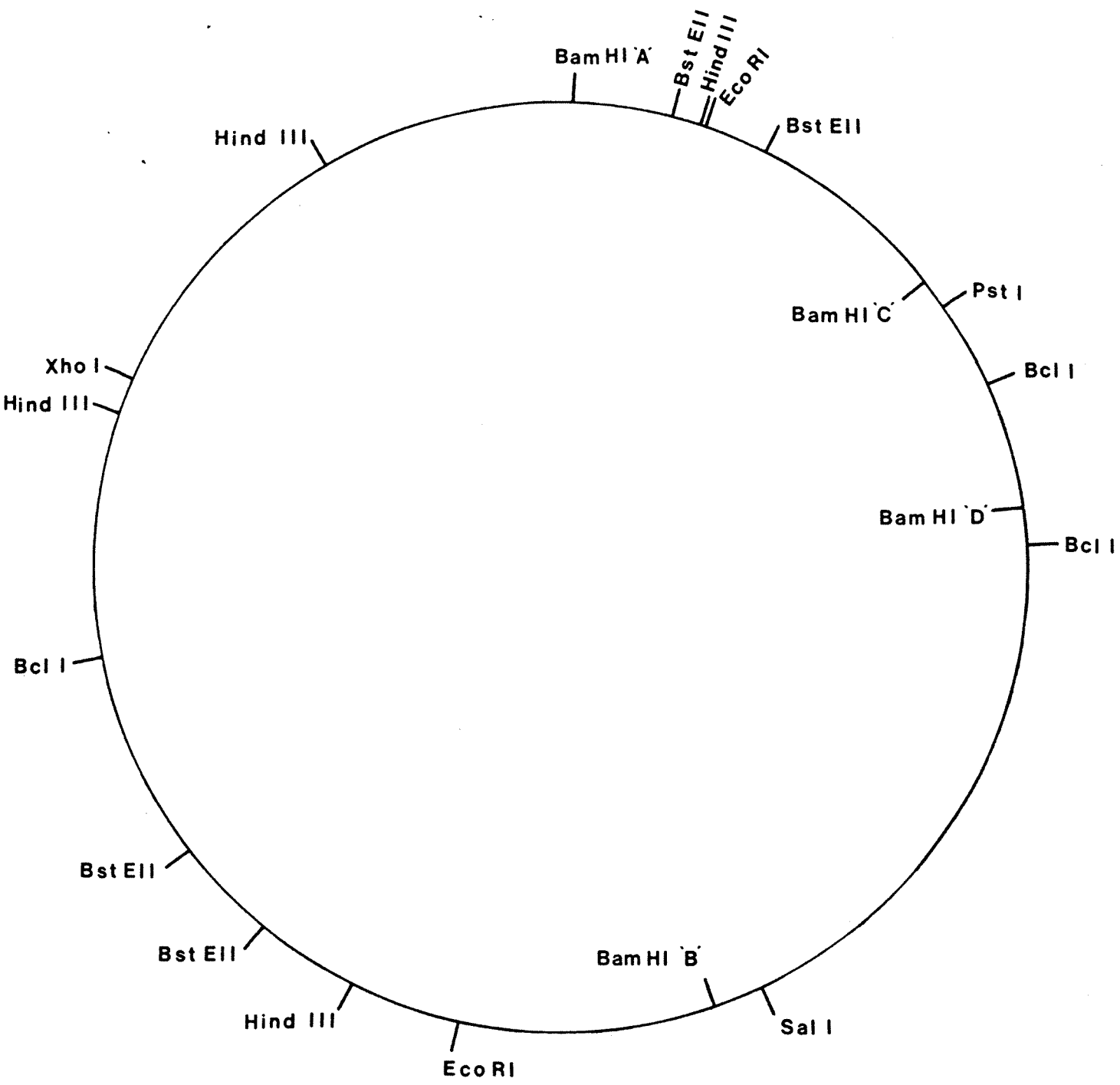


FIGURE 3. Preliminary map of the Lake Trout mitochondrial genome. Variable sites are labeled inside the circle.

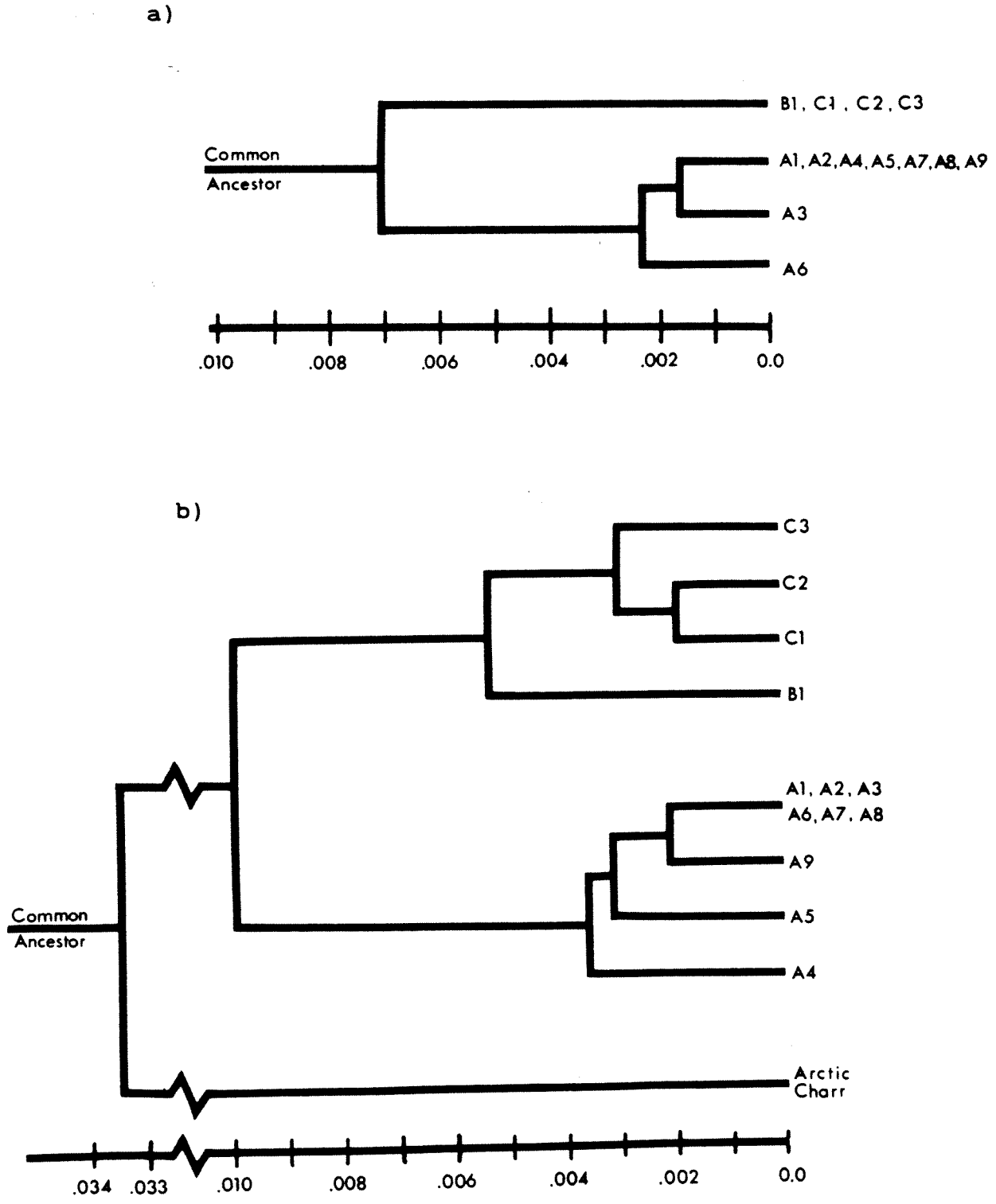
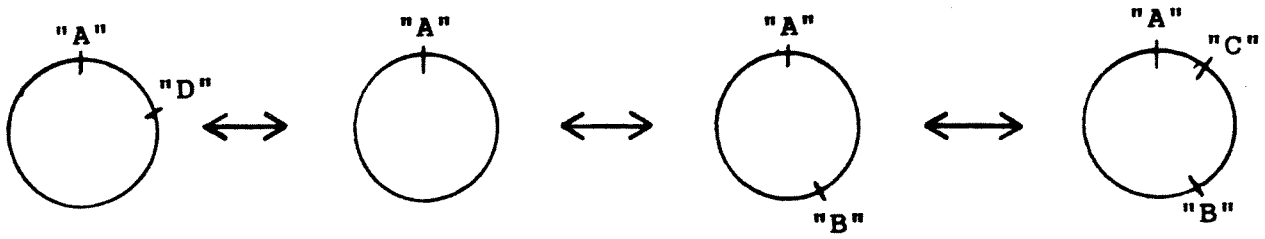


FIGURE 4. UPGMA generated phenograms based on the maximum likelihood estimate of the number of nucleotide differences/site (d) constructed for (a) NciI and (b) the 6 base enzyme site data. UPGMA clustering by the method and programs of Nei et al. (1985).



"D" type "A" type "B" type "C" type

FIGURE 5. Changes occuring in the lake trout mtDNA molecules in order to move from the BamHI "D" type to the "C" type.

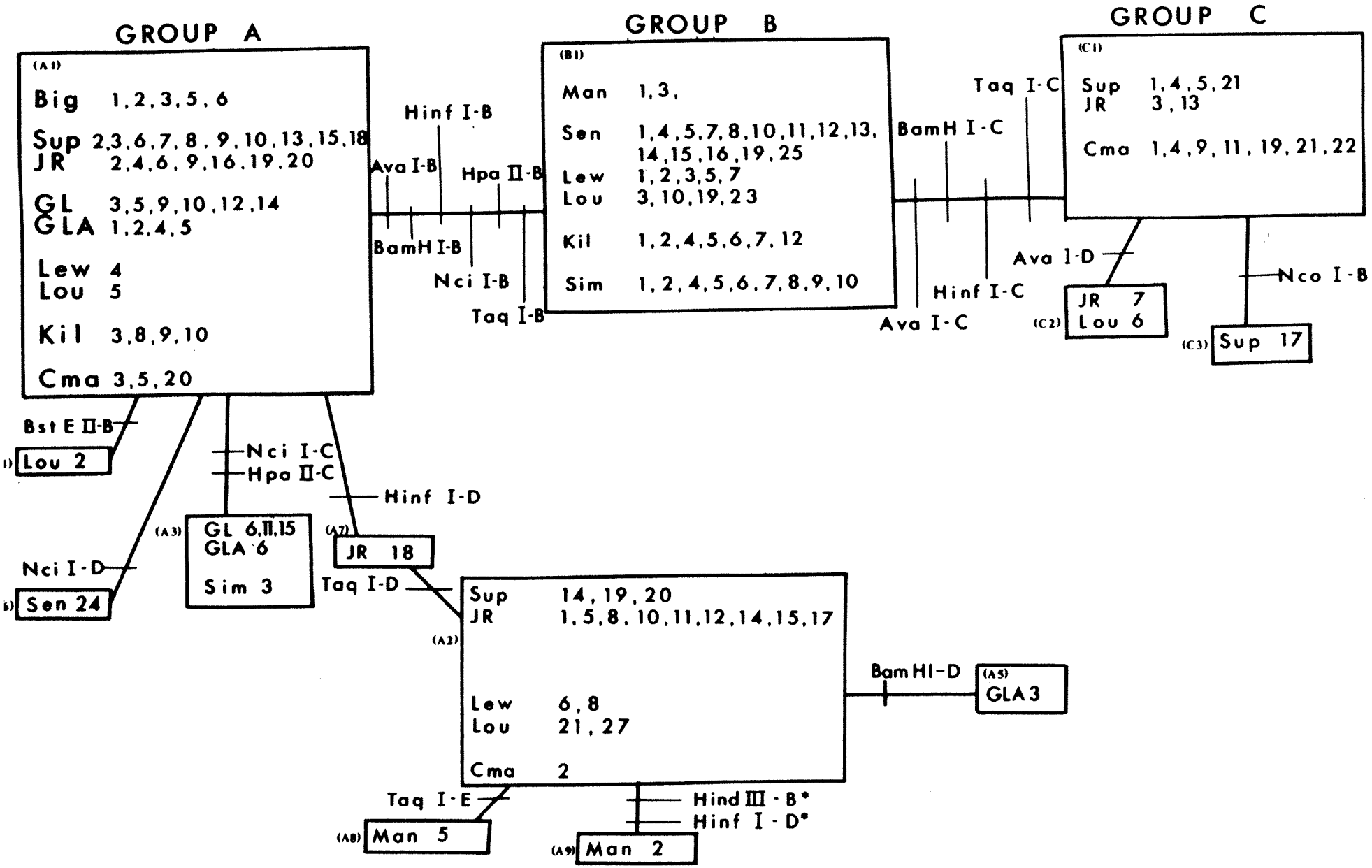


FIGURE 6. Phenogram produced by simple parsimony analysis outlined in the methods section.

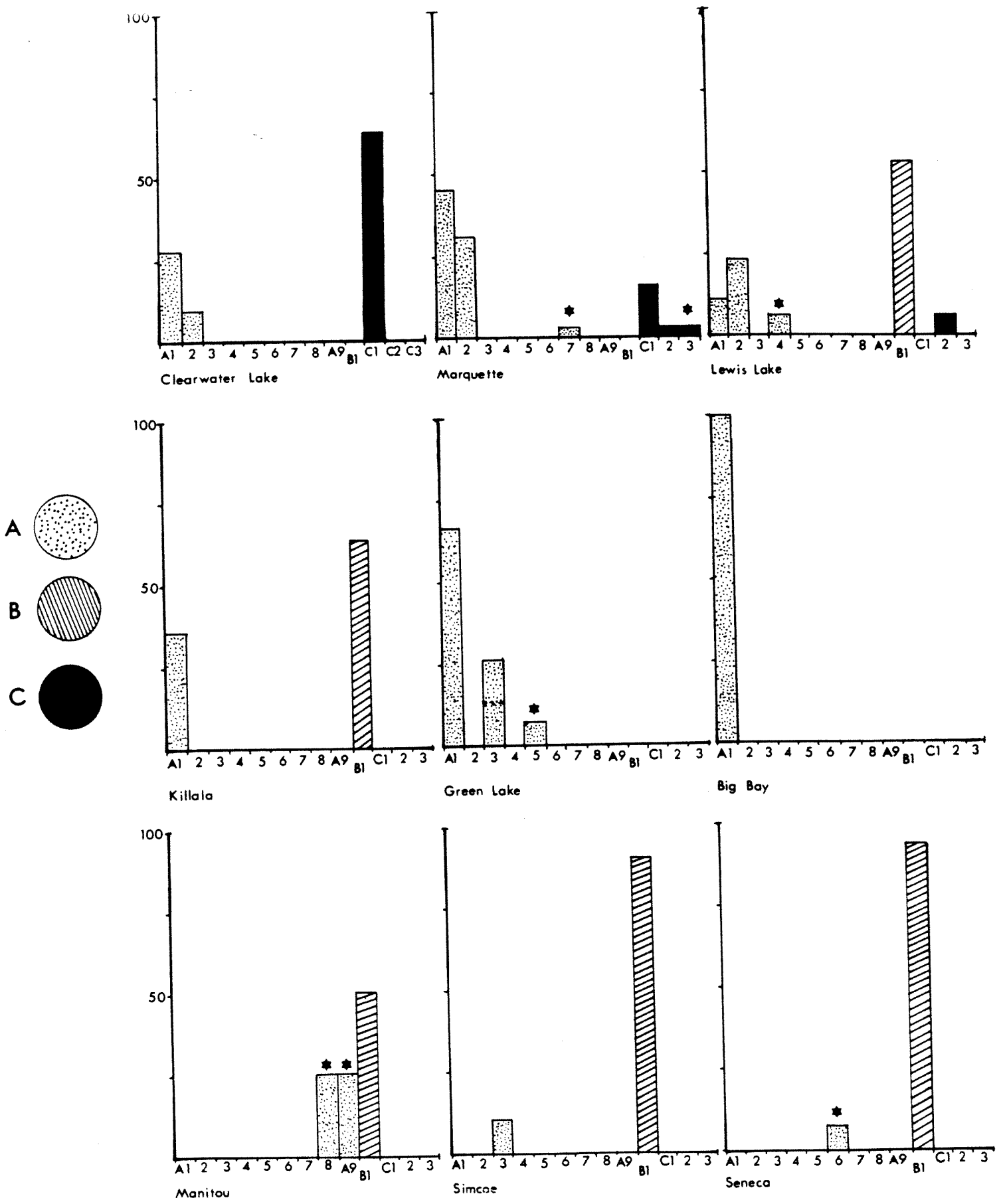


FIGURE 7. Proportions of the 13 mitochondrial clones found among the 9 brood stocks surveyed. The multiple samples for the Green Lake, Lewis Lake, and Marquette stocks were pooled.

* - indicates clones unique to a particular strain.

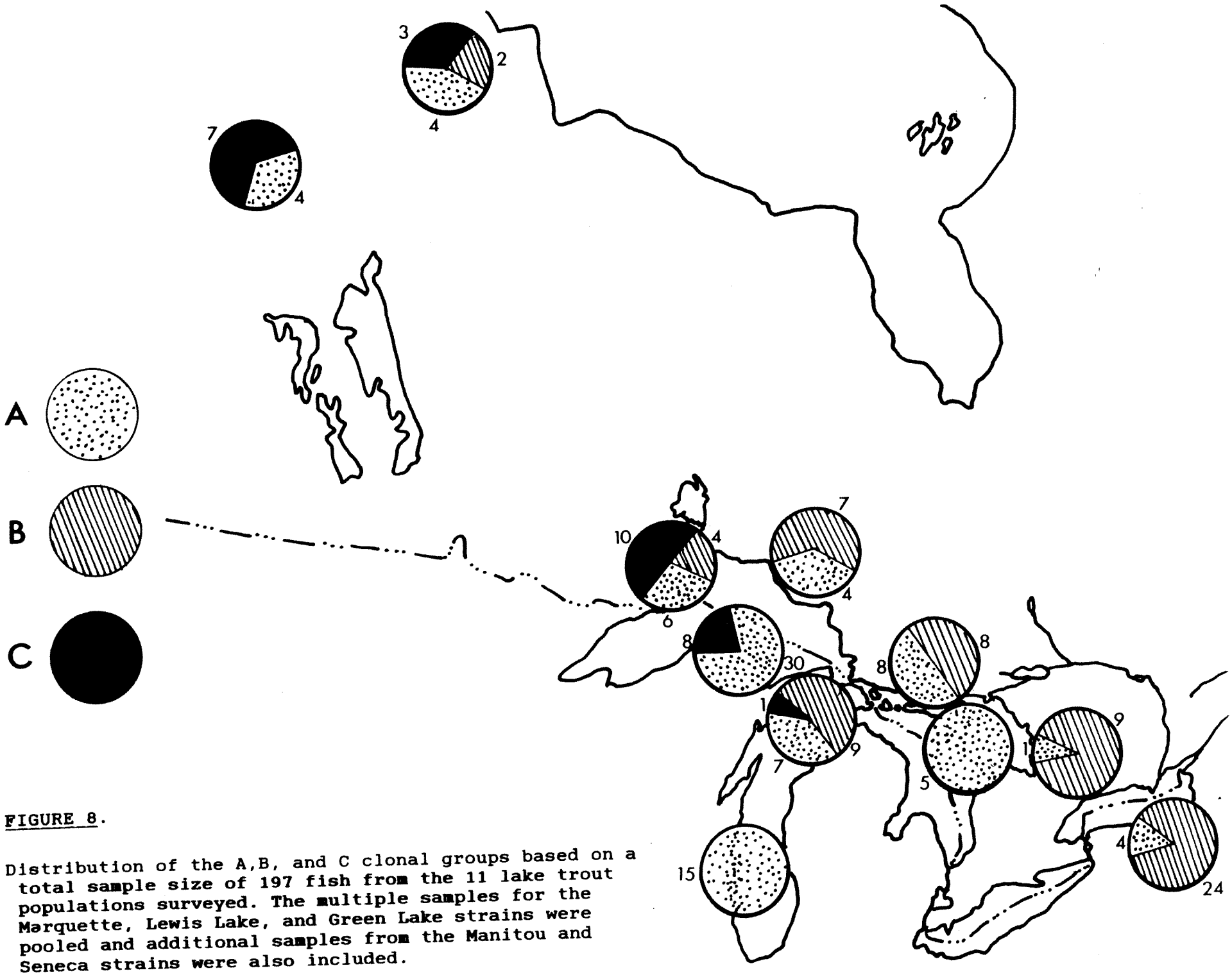


FIGURE 8.

Distribution of the A,B, and C clonal groups based on a total sample size of 197 fish from the 11 lake trout populations surveyed. The multiple samples for the Marquette, Lewis Lake, and Green Lake strains were pooled and additional samples from the Manitou and Seneca strains were also included.

APPENDIX 1: MITOCHONDRIAL DNA TECHNIQUES

Isolation of Mitochondrial DNA

Between 2-5 grams of chopped liver were homogenized in 30 mLs of sucrose grinding buffer (.25M sucrose, 10mM Tris pH 7.5, 1mM EDTA pH 8.0, 10mM NaCl) using a teflon homogenizer attached to an electric drill. The homogenate was transferred to 50 mL sorvall centrifuge tubes and centrifuged 3 times for 5 min. at 750 x G at 4°C, discarding the pellet each time. These 3 spins effectively removed cellular debris and most nuclei. The supernatant was finally spun for 20 min. at 18,700 x G at 4°C to pellet mitochondria. Mitochondria were then resuspended (app. 4 mL/gram mitochondria) in TE (10mM Tris pH 7.6, 1mM EDTA). SDS was then added (final conc. 1%) to lyse the mitochondria. When the lysate had cleared, a saturated CsCl solution was added (1/6 of the total volume). This solution was placed for a minimum of one hour at 4°C to precipitate proteins, SDS, some nuclear DNA, and RNA. At this point samples could be held for periods of up to two months, but, they were usually kept for only 1-2 weeks.

Further purification of the mitochondrial DNA was accomplished by isopycnic density centrifugation. For this step, the precipitated lysate solution was centrifuged for 20 min. at 20,500 x G at 4°C. One and a half mLs of the supernatant were removed and 0.5 mLs of propidium iodide (2 mg/mL) was added to it. (Propidium iodide differentially intercalates into the structures of mitochondrial and nuclear DNA creating slight differences in their relative buoyancies. This dye also causes nucleic acids to fluoresce under U-V illumination allowing for

the labelled DNA. The ethanol was then removed. This first wash effectively removed most of the unincorporated nucleotides. One millilitre of 70% ethanol (-20^{o}C) was added to the sample, mixed gently, and re-centrifuged for 10 minutes. The supernatant was again decanted, removing more unincorporated nucleotides and any remaining salt. The pellets were then lyophilized and resuspended in 15 uL of loading dye (8% sucrose, 0.05% bromophenol blue, in 1X TBE). The samples were then ready to be electrophoresed.

Samples were split into 7.5 uL aliquots and simultaneously electrophoresed utilizing agarose and polyacrylamide gels in a TBE (89mM Tris, 89mM Boric Acid, 2mM EDTA pH 8.0) buffer system. Agarose gels (1.2%) allowed for the resolution of DNA fragments from 20,000 to app. 500 base pairs, while acrylamide gels (4%) allowed for the resolution from 1000 to app. 26 base pairs.

Upon completion of electrophoresis, gels were dried to a 3MM filter paper backing and then exposed to X-ray (Fuji-RX) film overnight. The restriction fragments appeared as sharp black bands (see Fig. 2) on these autoradiographs. Fragment sizes were estimated from the autoradiographs utilizing a program developed by Keiser (1982, modified by P. Grewe) and run on an Apple II plus computer in conjunction with a HIPAD (model DT-11A, Houston Instruments) digitizing pad. Three classes of restriction endonucleases (4,5, and 6 base) were employed in this study. Six base enzymes have 6 base pairs in their palindromic recognition sequence and thus recognize fewer sites than do 4 and 5 base endonucleases. The latter two classes of endonucleases, by recognizing many more sites, make it possible to sample a larger

percentage of the mitochondrial genome with fewer digests than is possible with the 6 base endonucleases. Most 6 base enzymes produced patterns which could be resolved by agarose gels alone. Agarose did not allow enough resolution of small fragments to distinguish all phenotypes produced by 4- and 5-base enzymes as well as some 6-base enzymes and in these cases acrylamide gels were invaluable. The use of acrylamide gels also saved time completing digests of 6-base enzymes. With acrylamide gels affording the resolution of all restriction fragments, double digests could be preformed on each sample, effectively cutting in half the time to do each 6-base endonuclease.

Determining restriction site homology of 4 and 5 base enzymes was nearly impossible. This was quite easily done, however, for the 6 base endonucleases through simple double digests. Once homology was confirmed, the data could be used in calculating Nei's d for phylogenetic analysis.

Visualization of Restriction Fragments

Three different methods are commonly used to visualize restriction fragments upon completion of electrophoresis : 1) staining of the gel with an intercalating dye such as ethidium bromide or propidium iodide which causes DNA to fluoresce upon U.V. illumination ; 2) endlabelling restriction fragments prior to electrophoresis, followed by autoradiography and 3) probing of southern blots with radiolabelled mtDNA followed by autoradiography.

The use of an intercalating dye or end-labelling of fragments both require mtDNA samples which are free of contaminating nuclear DNA. This usually entails purification via

many hours of ultra-centrifugation. The number of samples which can be processed per centrifuge run is limited to six and ultracentrifugation ordinarily serves as the rate limiting step. Use of an intercalating dye requires much larger quantities of DNA, than does autoradiography to visualize restriction fragments and small fragments are not easily observed. However, using the technique of probing, large amounts of nuclear DNA contamination can be tolerated and ultracentrifugation is not required. Detection of minute quantities of mtDNA is possible with probing, but it is difficult to detect restriction fragments which are less than 500 base pairs long with this technique. Some of the fragments produced by 6 base enzymes and most fragments from 4 and 5 base enzymes fall below this 500 base pair limit. End-labelling allows the detection of minute quantities of DNA as well as resolution (when acrylamide gels are used) of fragments down to 26 base pairs. Although pure samples of mt DNA are required, the extra resolution afforded by this technique made it the method of choice for this initial study of the lake trout.

APPENDIX 2: STANDARD FRAGMENT LENGTHS

Lambda cut by Hind III	Lambda cut by Hind III / Eco RI	Lambda cut by Hind III / Eco RI + Hind III	pBR 322 cut by Hpa II
23.130	21.226	23.130	.622
9.416	5.148	21.226	.527
6.561	4.977	9.416	.404
4.357	4.268	6.561	.309
2.322	3.529	5.148	.242
2.027	2.027	4.977	.238
.564	1.904	4.357	.217
.125	1.584	4.268	.201
	1.375	3.529	.190
	.947	2.322	.180
	.827	2.027	.160
	.564	1.904	.147
	.125	1.584	.122
		1.375	.110
		.947	.090
		.827	.076
		.564	.067
		.125	.034
			.026
			.015
			.009

Various standards used to determine restriction fragment sizes. Lambda phage and pBR322 were purchased from Bethesda Research Laboratories. Fragment sizes (Kilobase pairs) are as reported by the BRL Catalogue and Reference Guide (1985).

APPENDIX 3

Dichotomous Key For Lake Trout Brood Stock Identification Based on Restriction Phenotypes of Mitochondrial DNA

1.	A) presence of A8 and/or A9 clones	- MANITOU
	B) both A8 and A9 absent	- 2.
2.	A) proportion of B1 clone > 80%	- 3.
	B) B1 clone < 80%, A1 or A2 present	- 4.
3.	A) A3 clone present, A6 clone absent	- SIMCOE
	B) A3 clone absent, A6 clone present	- SENECA
4.	A) A1 clone present, A2 absent	- 5.
	B) A1, A2, and "C" clones present	- 7.
5.	A) B1 clone present	- KILLALA
	B) B1 clone absent	- 6.
6.	A) A5 clone present, A3 common	- GREEN LAKE
	B) A1 clone present and > 90% of sample	- BIG BAY*
7.	A) B1 clone present	- LEWIS LAKE
	B) B1 clone absent	- 8.
8.	A) C2 and C3 absent	- CLEARWATER LAKE
	B) C2 and C3 present	- MARQUETTE

Assumptions: 1) current frequencies reflect actual brood stock frequencies.
2) frequencies of sampled populations reflect input variation from stocked strains.

*- At present only the A1 clone has been found in the Big Bay strain. Hopefully other clones will be discovered which can further identify and confirm the presence of this strain in a sample of fish.