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Divergence of mitochondrial DNA among the sub-family Salmoninae with special reference to the genus Salvelinus.

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DIVERGENCE OF MITOCHONDRIAL DNA AMONG THE SUB-FAMILY SALMONINAE WITH SPECIAL REFERENCE TO THE GENUS SALVELINUS

by

Peter Martin Grewe

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ABSTRACT

DIVERGENCE OF MITOCHONDRIAL DNA

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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 9 brood-stocks along with an additional 47 fish from 4 natural populations. Seventeen restriction endonucleases were employed to analyse all brood stock fish as well as 17 fish from the Yukon Territory population. Two of these enzymes (HindIII and BamHI) were used to examine 21 fish from the Hare Island (Lake Superior) and 9 fish from the North Knife Lake populations.

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 one strain (Manitou). It contained two genomes, one of which was less frequent and shorter by 20 base pairs than the other ones examined.

The 17 restriction enzymes resolved 13 mitochondrial clones which fell into 3 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 of the 13 mitochondrial clones were unique to a particular strain. In addition there were dramatic shifts in the relative proportions of the 6 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.

Mitochondrial DNA was also isolated from two additional members of the genus Salvelinus (alpinus and fontinalis) and from the closely related taxon, Hucho hucho. Restriction sites recognized by fourteen endonucleases were mapped for each of the species and percent sequence divergence was estimated between species pairs. A phylogeny of Salvelinus based on restriction analysis of mitochondrial DNA, using Hucho as an outgroup, is presented. In all respects this phylogeny supports the classical taxonomy of the genus Salvelinus.

Finally, sequence divergence data of the current study was merged with three other published salmonid mtDNA phylogenies to produce a composite distance phenogram representing the phylogeny of the subfamily Salmoninae. Divergence time estimates, for the break points between the major taxa of this group, indicate that the Salmoninae genera are of recent origin, late Miocene to early Pliocene, and that the present species originated during the late Pliocene to early Pleistocene.

DEDICATION

This thesis is dedicated to my parents for guiding me during my reckless youth and providing an atmosphere that prepared me for the hardships of the real world. I would also like to add a special note of thanks to my dear friend Jeanne Wachna who saw me through some hard times during the completion of this work.

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I am indebted to my supervisor, Dr. Paul Hebert, who was a constant source of support and has introduced me to the exciting field of population genetics. I am also sincerely grateful to my other comittee members, Dr. Bob M'Closkey and Dr. John Jacobs for their guidence and patience throughout the course of this project.

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PREFACE

The following is designed to introduce the reader to the organization of this thesis, which consists of two interrelated, yet, autonomous chapters. The first chapter utilizes the restriction analysis of mitochondrial DNA (mtDNA) to examine variation among populations of the lake trout and then expands on the application of this technique as a fisheries management tool to assist lake trout rehabilitation effort in the Great Lakes. The second chapter again uses restriction analysis of mtDNA, however, as a systematic tool to infer relationships at a higher taxonomic level. Both chapters have been written as separate entities containing their own abstract, introduction, methods and materials, results, and discussion sections, with a separate set of tables and figures following each chapter. The first chapter also has an additional section entitled "Management Implications" which deals with current and future uses of the restriction site data set. Finally there is a common bibliography and set of appendices referenced by both chapters.

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CHAPTER I

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

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 (12 fish) and Seneca (13 fish) 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 assessment 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 focused 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 of 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 structure, 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. First, 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. Second, the resolution capability of mitochondrial DNA studies is enhanced by the fact that mitochondrial DNA evolves at ten times the rate of the nuclear genome (Brown et al., 1979).

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 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).

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 (Figure 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. Analysis of variance, 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.

The survey of mitochondrial DNA diversity revealed 13 mitochondrial clones among the brood stock fish examined and one

additional clone in the Atlin Lake sample from British Columbia. The analysis of mtDNA 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, only four fish were examined, and more analyses are required before drawing further conclusions. The Green Lake strain contained approximately two mitochondrial clones despite its confinement to hatcheries for at least three generations (Krueger et al., 1983) and subjection to several transfers with associated 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.). The 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 ones sampling intensity of the mitochondrial genome and 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. First, how many individuals must be analyzed to detect all the clones present in a specific stock? Second, what proportion of the mitochondrial genome needs to be surveyed in order to resolve all mitochondrial variants? Currently, we are developing a method to conduct 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 et al., 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 1.67 million years ago.

My 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 et al., 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.

TABLE 1. Lake trout brood stock surveyed by the study of Grewe and Hebert (1987).

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	(Sup)	10	75'	- Jordan River N.F.H., Nov. 84.
(domestic)		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*	Bst EII	G'GTGAC,C
	C'CCGG,G**		G'GTAAC,C
	C'TCGG,G		G'GTTAC,C
	C'CCGA,G		G'GTCAC,C
Bam HI	G'GATC,C	Pvu II	CAG'CTG
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 fo 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)		
81' (Sup)	8	(3) (3) (1) (1)		C3 (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
Sebastes atrovirens	17,300 ± 400	(1985)
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
Stizostedion vitreum	18,475 ± 300	(1986)
Stizostedion canadense	16,702 ± 259	
Stizostedion lucioperca	16,736 ± 277	
Salvelinus namaycush	16,800 ± 200	Present study

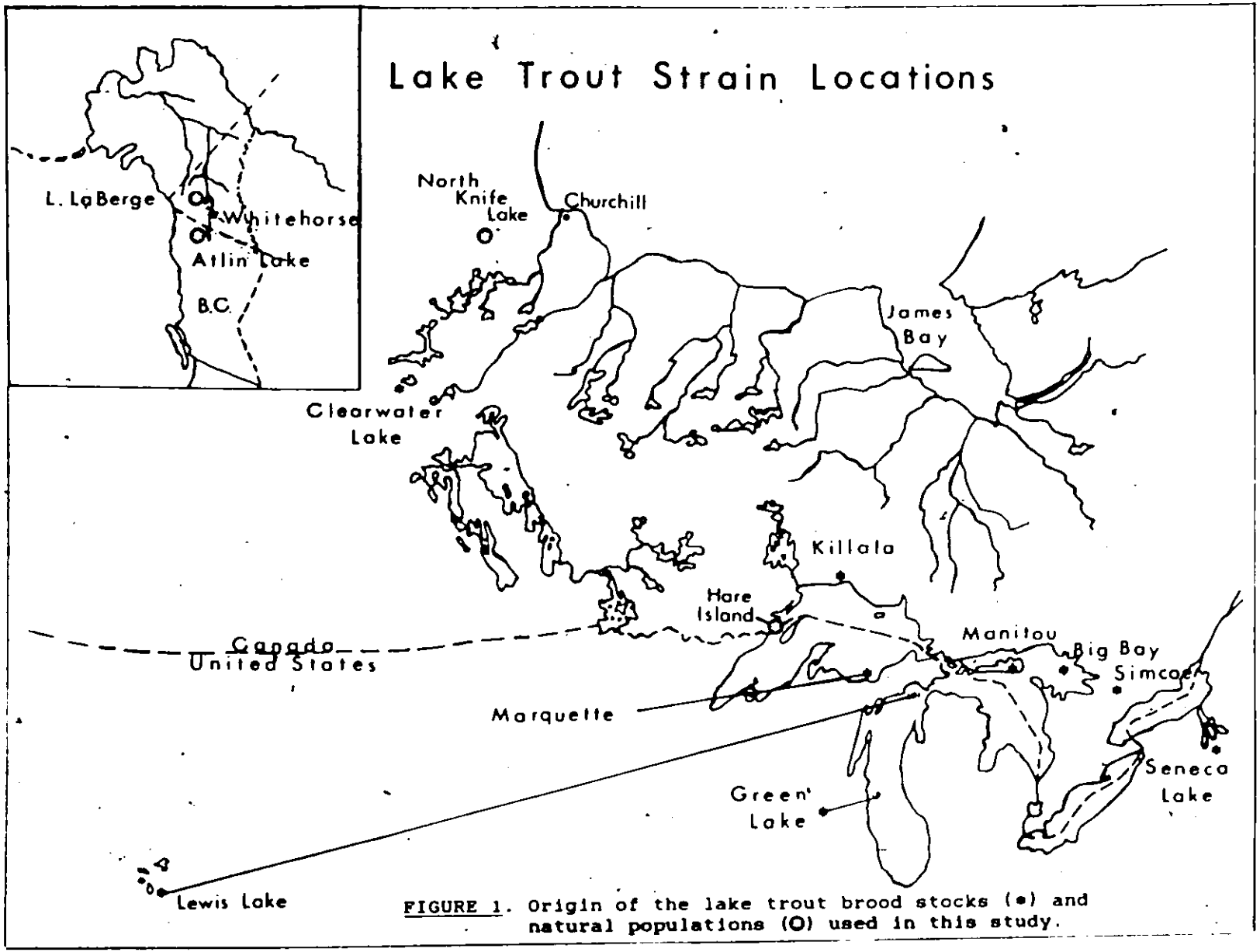


FIGURE 1. Origin of the lake trout brood stocks (•) and natural populations (O) used in this study.

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.

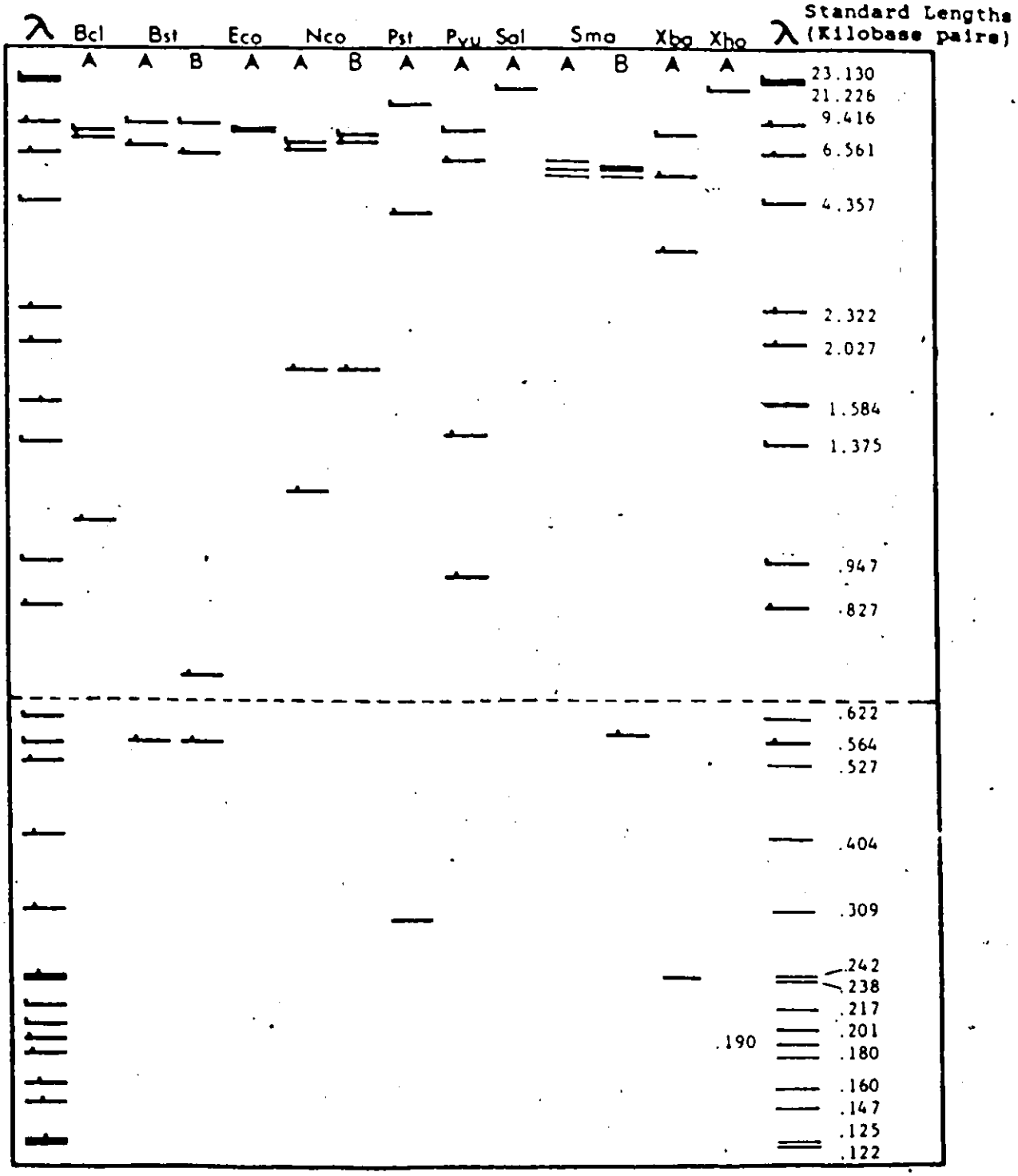

λ
Bcl
Bst
Eco
Nco
Pst
Pvu
Sal
Sma
Xba
Xho
λ
Standard Lengths (Kilobase pairs)
A A B A A B A A A A B A A
23.130
21.226
9.416
6.561
4.357
2.322
2.027
1.584
1.375
.947
.827
.622
.564
.527
.404
.309
.242
.238
.217
.201
.190
.180
.160
.147
.125
.122

FIGURE 2. (a)

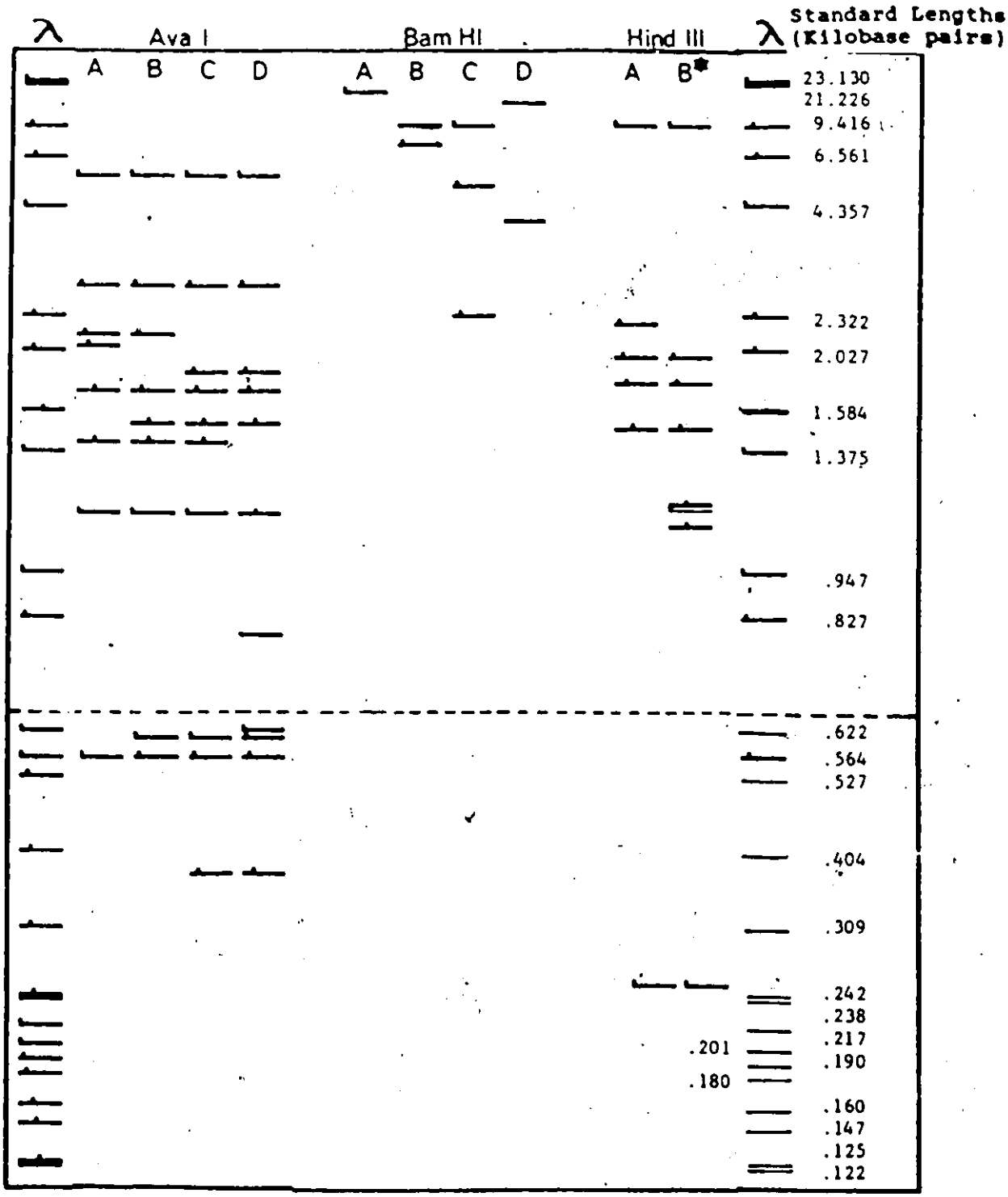

λ
Ava I
A B C D
Bam HI
A B C D
Hind III
A B*
λ
Standard Lengths
(Kilobase pairs)
23.130
21.226
9.416
6.561
4.357
2.322
2.027
1.584
1.375
.947
.827
.622
.564
.527
.404
.309
.242
.238
.217
.201
.190
.180
.160
.147
.125
.122

FIGURE 2. (b)

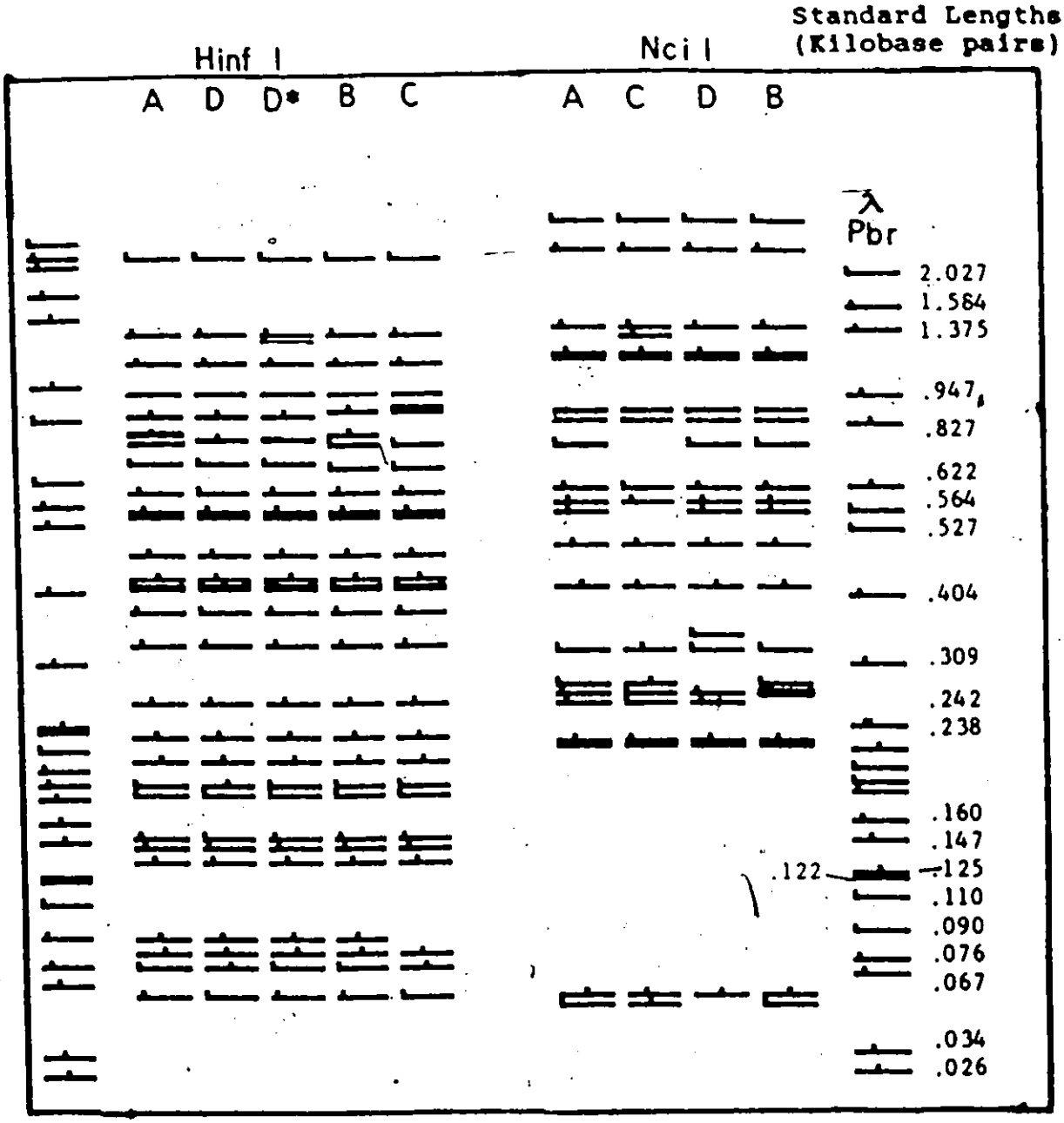

Standard Lengths
(Kilobase pairs)
Hinf I
A D D* B C
Nci I
A C D B
λ
Pbr
2.027
1.584
1.375
.947
.827
.622
.564
.527
.404
.309
.242
.238
.160
.147
.122
.125
.110
.090
.076
.067
.034
.026

FIGURE 2. c)

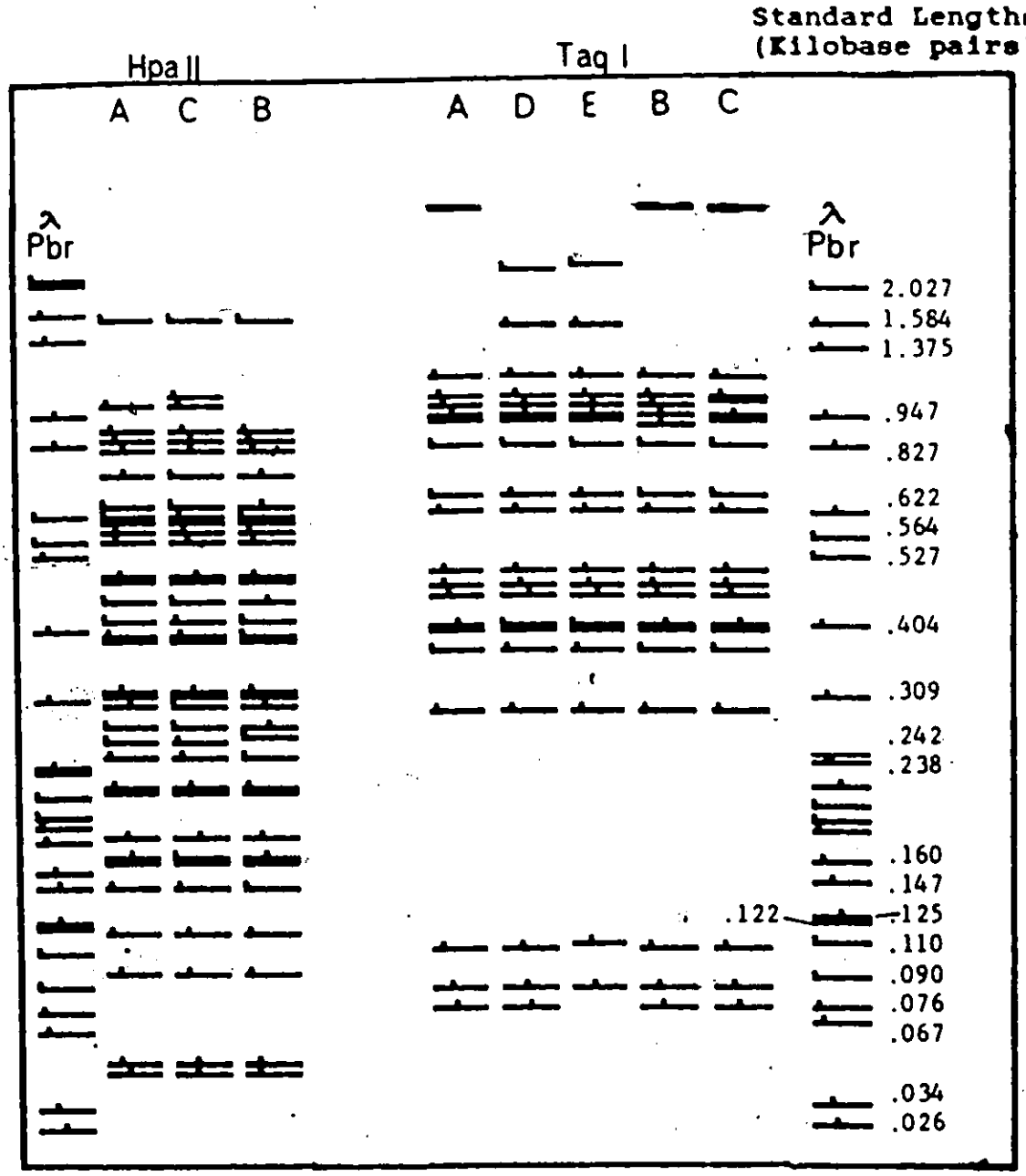

Standard Lengths
(Kilobase pairs)
Hpa II
Taq I
A C B
A D E B C
λ Pbr
λ Pbr
2.027
1.584
1.375
.947
.827
.622
.564
.527
.404
.309
.242
.238
.160
.147
.122
.125
.110
.090
.076
.067
.034
.026

FIGURE 2. d)

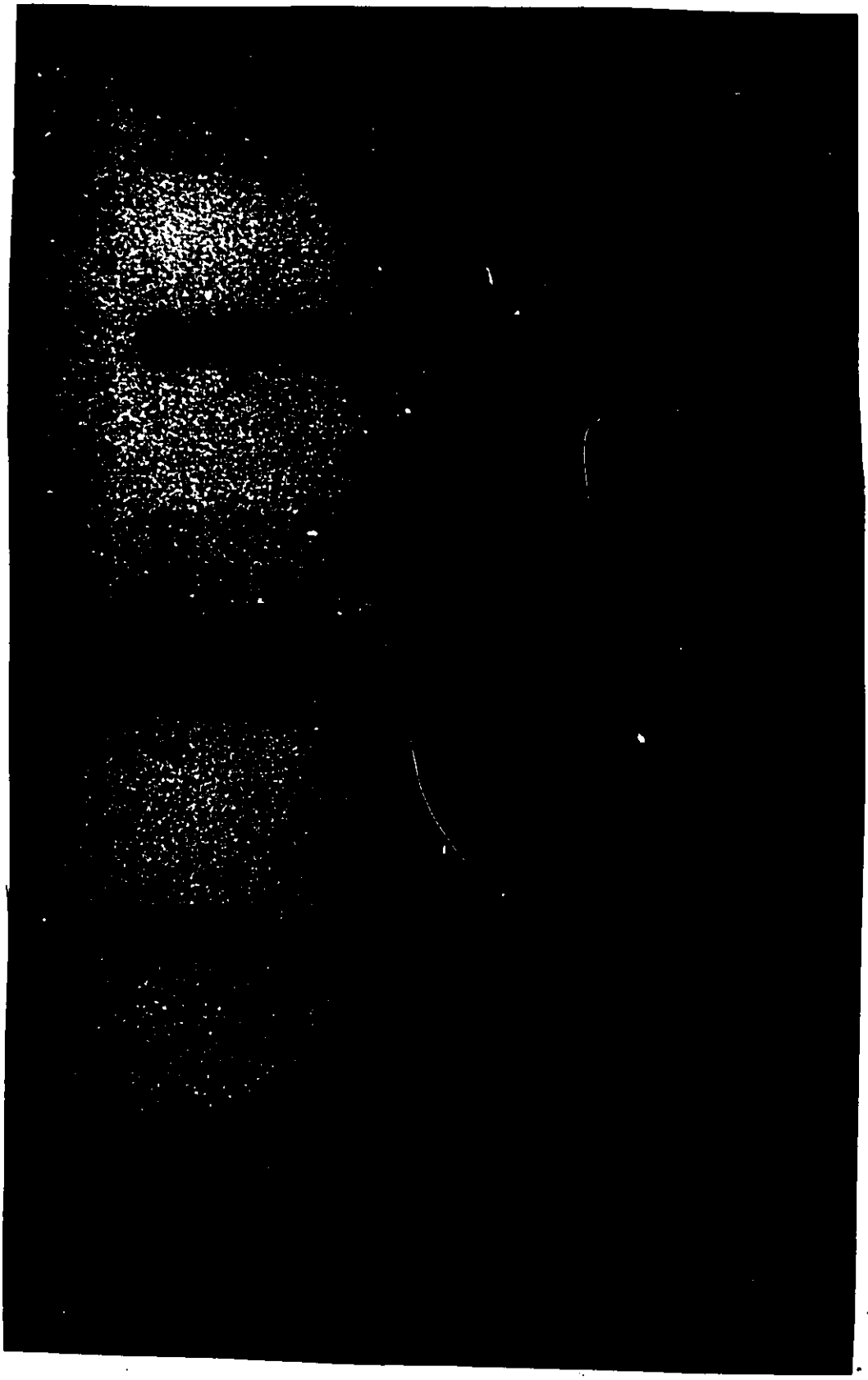


FIGURE 2. (e)

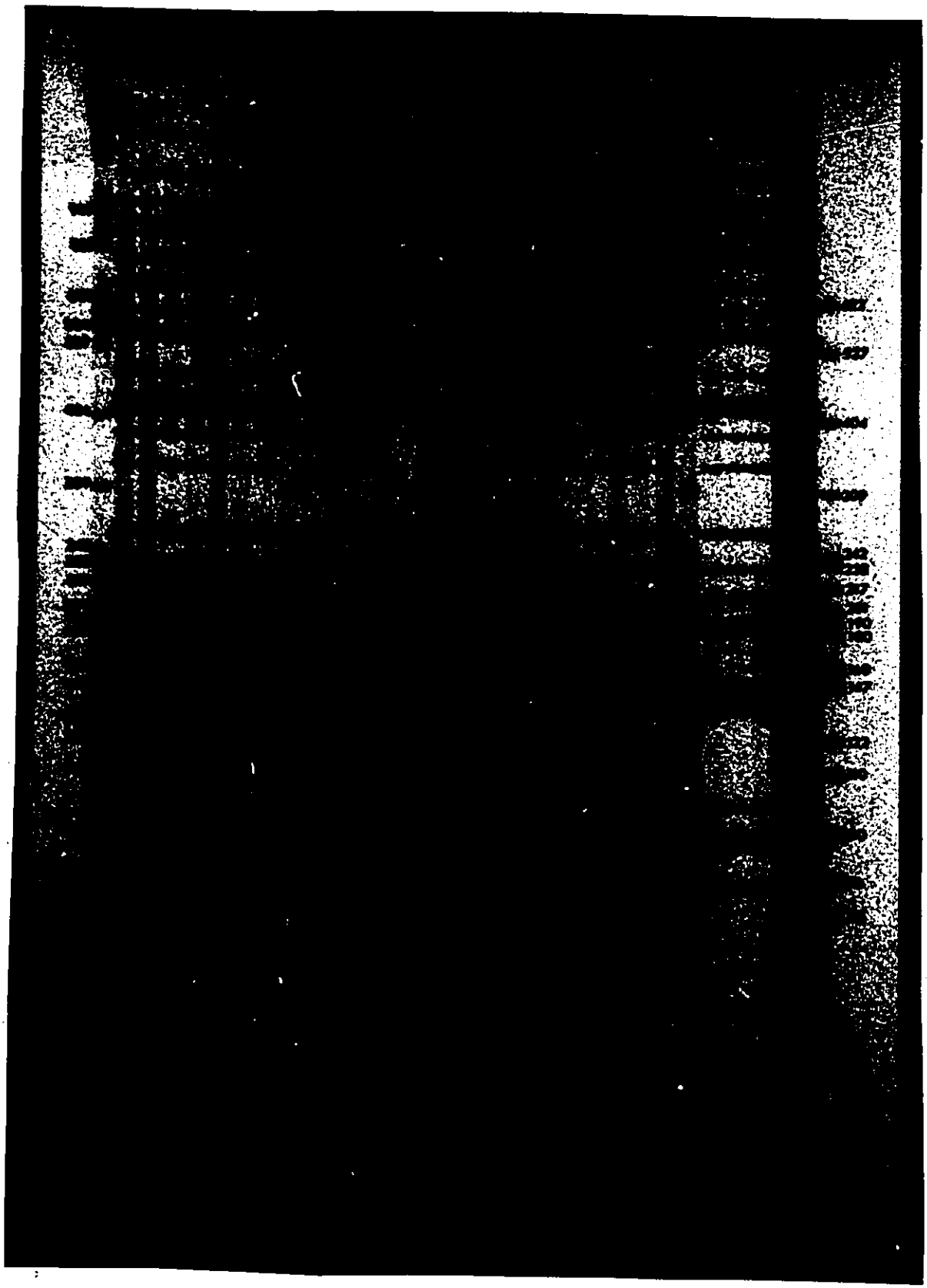


FIGURE 2. (f)

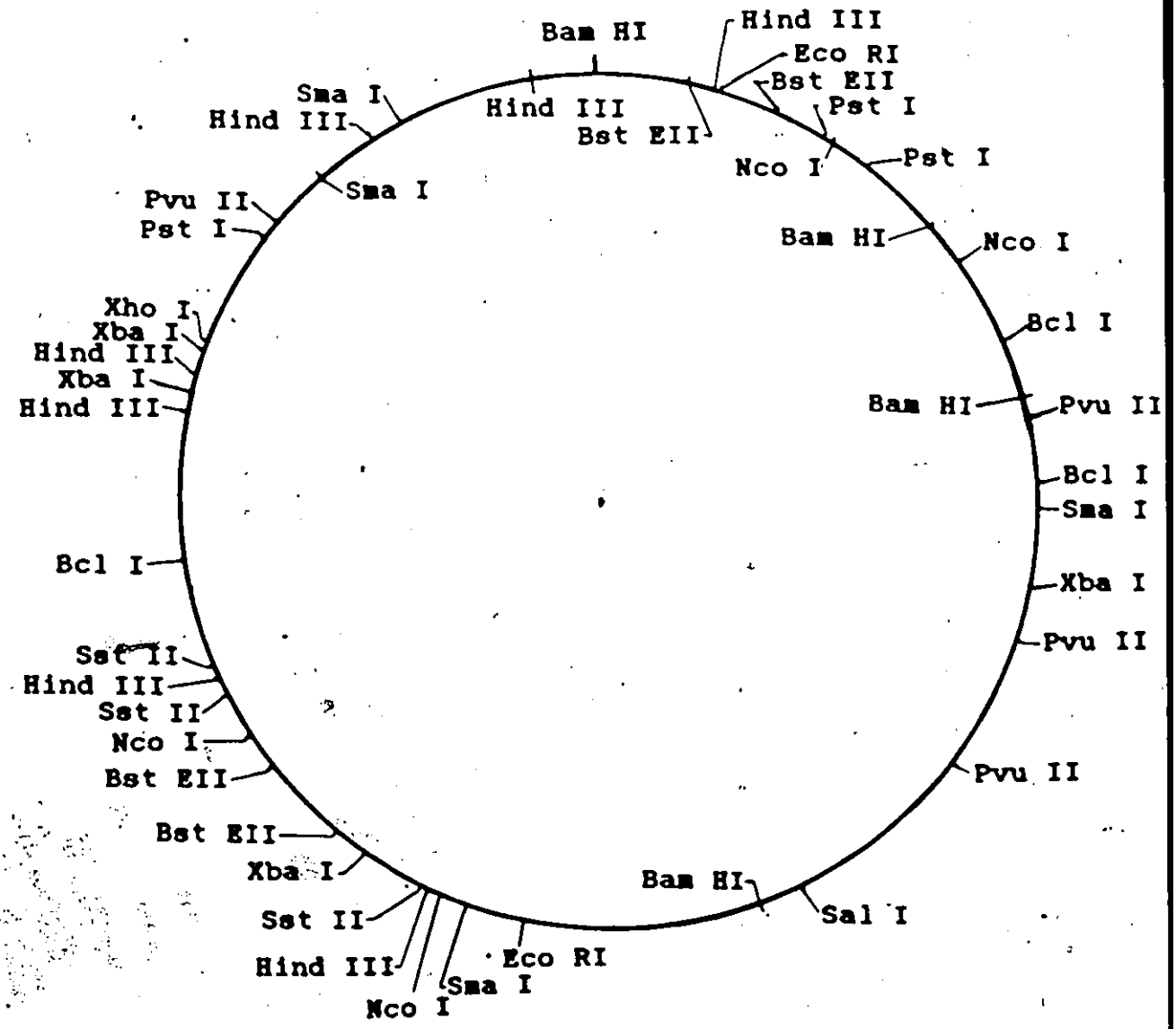


FIGURE 3. Restriction site map of the lake trout mitochondrial genome. Variable sites have been labeled inside the circle.

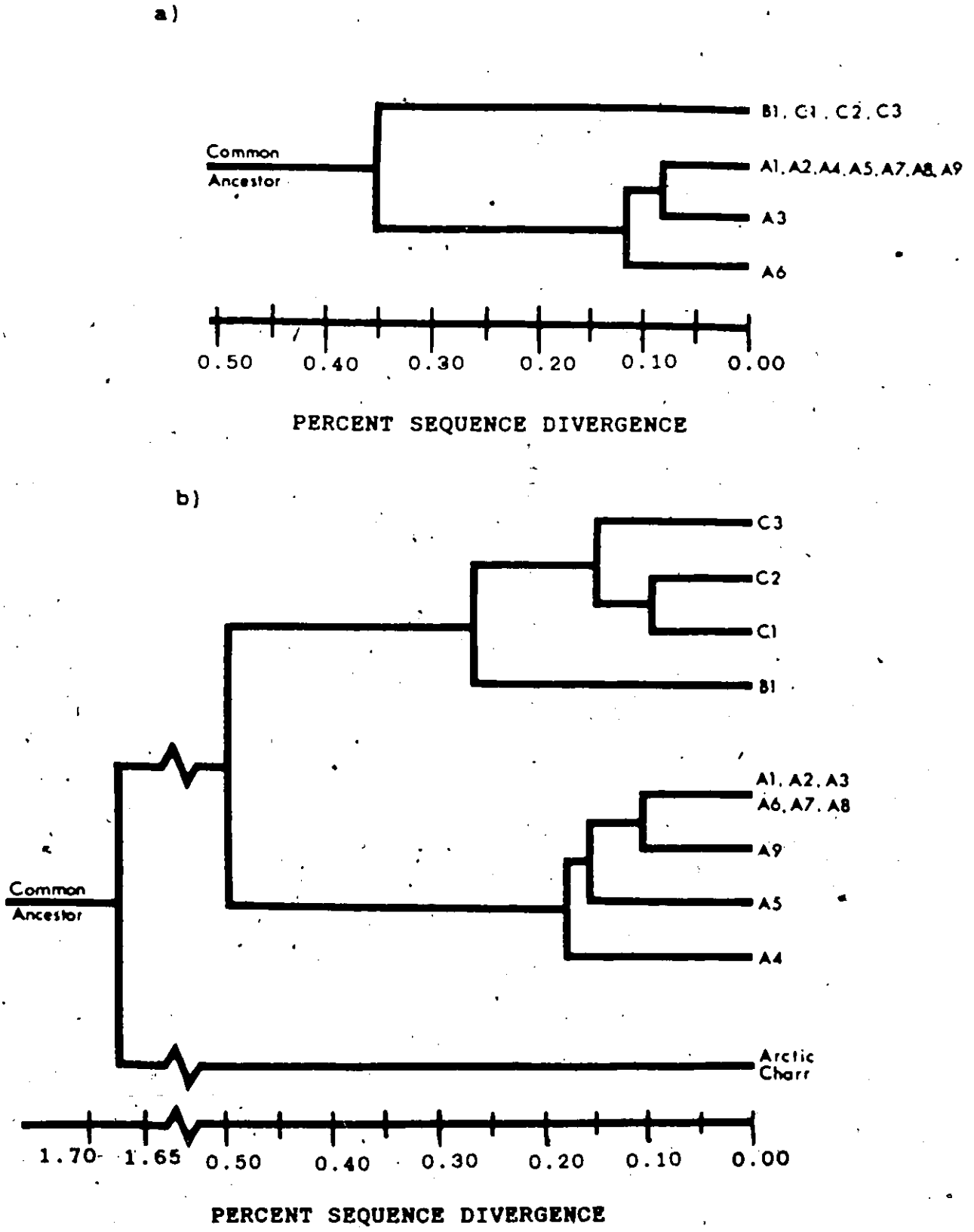


FIGURE 4. UPGMA generated ohenograms based on the maximum likelihood estimate of the number of nucleotide differences/site (d) constructed for (a) Nci I and (b) the 6 base enzyme data.

NOTE: (distance values plotted as 1/2 d)

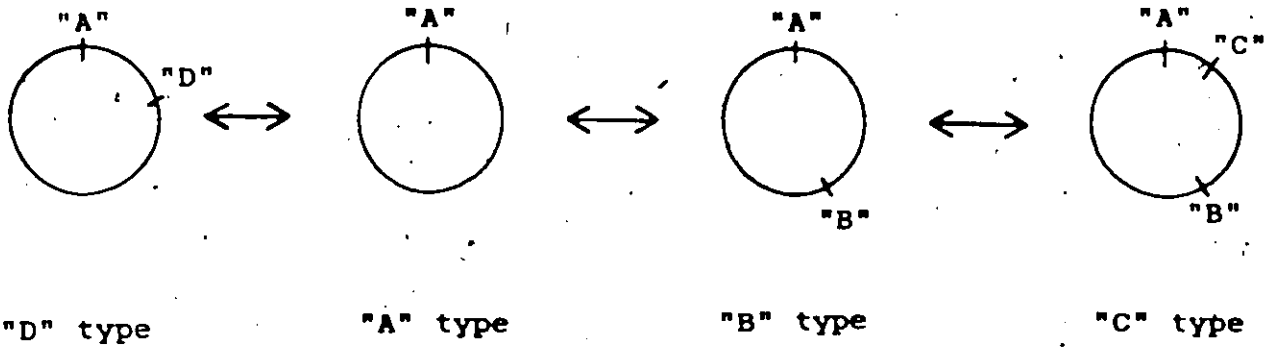


FIGURE 5. Changes occuring in the lake trout mtDNA molecules in order to move from the BamHI "D" type to the "C" type.

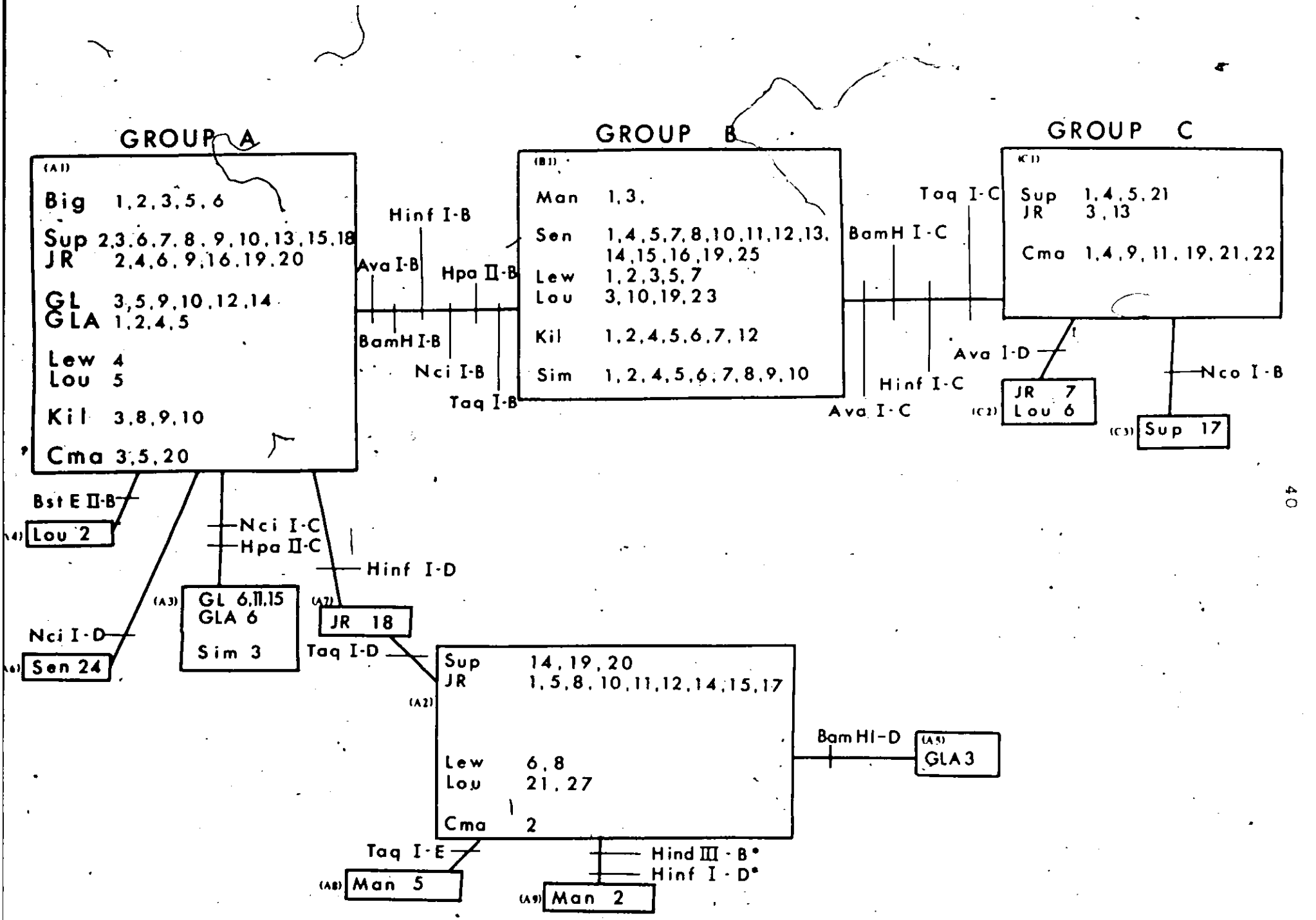


FIGURE 6. Phenogram produced by simple parsimony analysis outlined in the methods section.

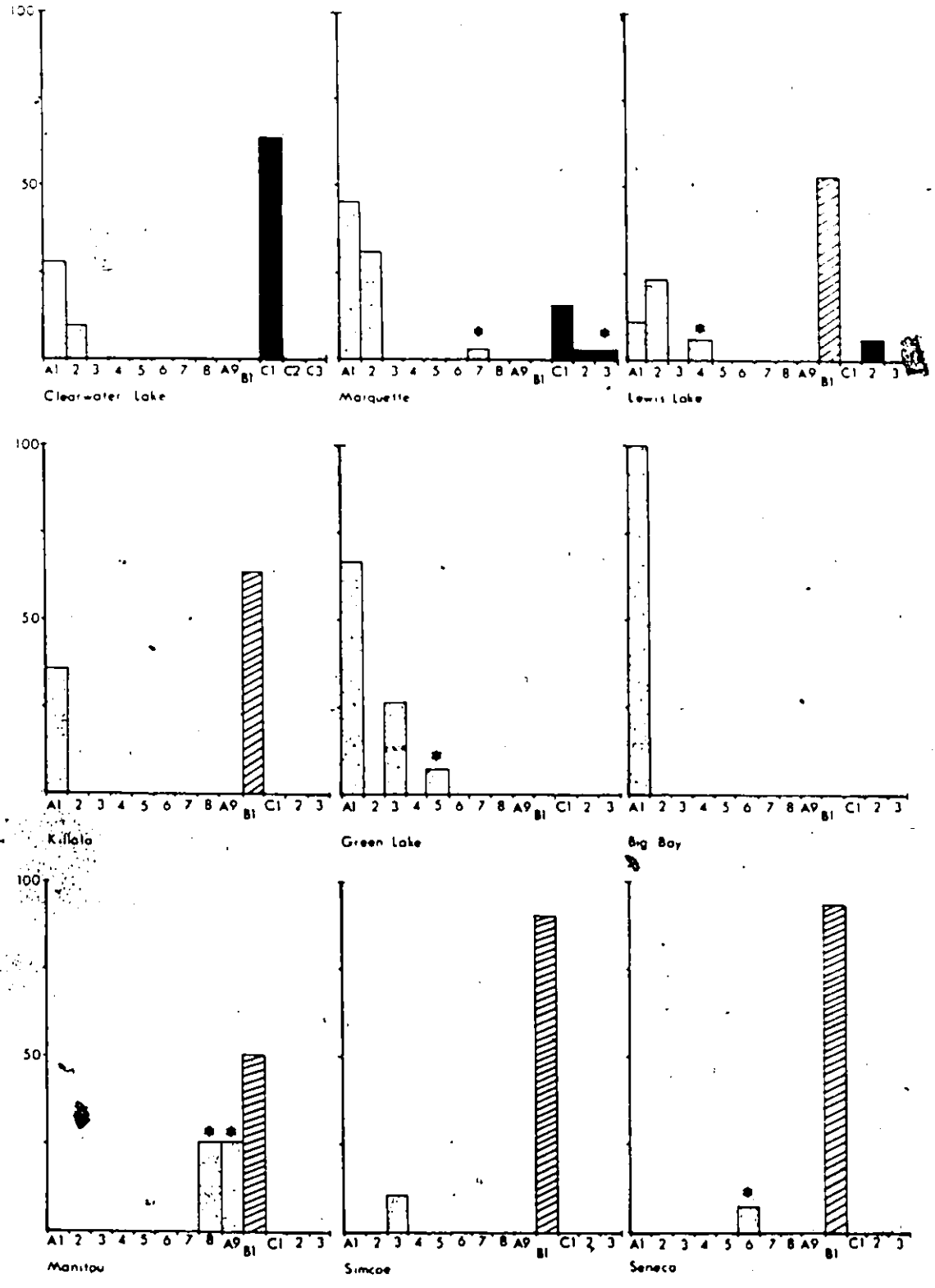


FIGURE 7. Proportions of the 13 mitochondrial clones found among the 9 brood stocks surveyed. The multiple samples for the Green Lake, Lewis Lake, and Marquette stocks were pooled.

* - indicates clones unique to a particular strain.

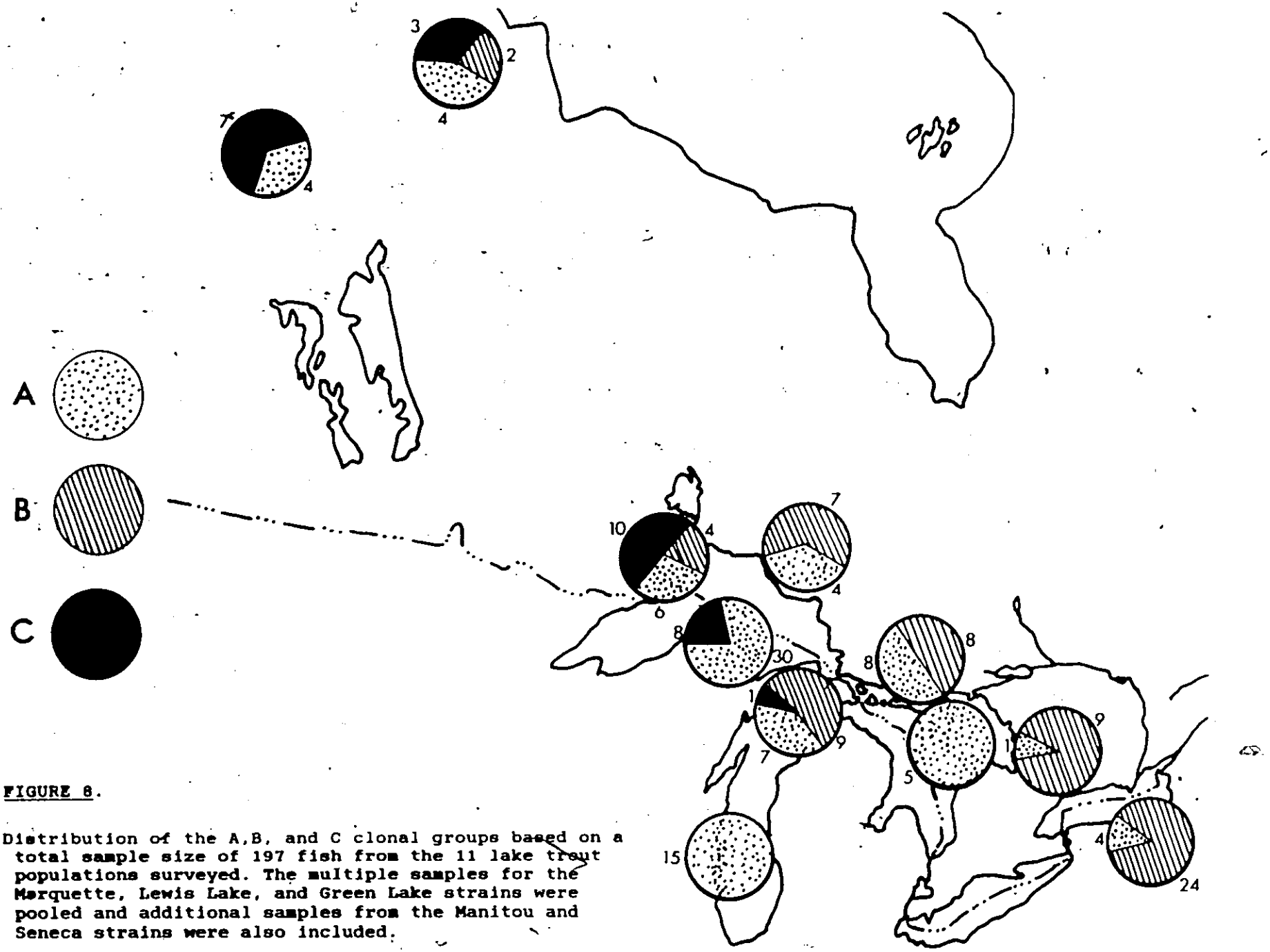


FIGURE 8.

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.

CHAPTER TWO

PHYLOGENETIC RELATIONSHIPS AMONG THE SALMONINAE AS INFERRED FROM MITOCHONDRIAL DNA DIVERGENCE

ABSTRACT

Mitochondrial DNA was isolated from three members of the genus Salvelinus (namaycush, alpinus, fontinalis) and from the closely related taxon, Hucho hucho. Restriction sites recognized by fourteen endonucleases were mapped for each of the species and percent sequence divergence was estimated between species pairs. A phylogeny of Salvelinus based on restriction analysis of mitochondrial DNA, using Hucho as an outgroup, is presented. In all respects this phylogeny supports the classical taxonomy of the genus Salvelinus.

Finally, sequence divergence data of the current study was merged with three other published salmonid mtDNA phylogenies to produce a composite distance phenogram representing the phylogeny of the subfamily Salmoninae. Divergence time estimates, for the break points between the major taxa of this group, indicate that the salmonine genera are of recent origin, late Miocene to early Pliocene, and that the present species originated during the late Pliocene to early Pleistocene.

INTRODUCTION

The family Salmonidae consists of the three subfamilies, Coregoninae, Thymallinae, and Salmoninae. Six genera are present in the latter (Brachymystax, Hucho, Onchorynchus, Salmo, Salmothymus, and Salvelinus). The current taxonomy of the salmonids is based on studies of morphology (Norden, 1961; Vladykov, 1963; Behnke, 1965; Medvedeva and Savvaitova, 1980), including analysis of meristics (Forester and Pritchard, 1935; McPhail, 1961; Behnke, 1972; Cavender, 1980) and colour patterns (Rounsefell, 1962; Behnke, 1979), supplemented by karyological (Simon, 1963, 1964; Gold et al., 1972), and ontogenetic studies (Balon, 1980; Pavlov, 1980). More recently allozyme electrophoresis (Tsuyuki and Roberts, 1966; Utter et al., 1973; Loudenslager and Gall, 1980; Milner et al., 1981), DNA-DNA hybridization (Mednikov and Akhundov, 1975; Gharrett et al.,1977; Hanham and Smith, 1980; Mednikov et al., 1980; Schmidtke and Kandt, 1981), and chromosome banding (Phillips and Ihssen, 1987) studies have been used to further resolve the phylogeny of species belonging to this group.

The taxonomy of the Salmoninae through examination of traits encoded by the nuclear genome have proven difficult, especially when differentiating among Salvelinus congeners (Behnke, 1980; Savvaitova, 1980). These difficulties have been attributed to the recent origin and resulting limited degree of genetic divergence among the Salmoninae; most modern genera appear to be of Pliocene or Pleistocene origin (Neave, 1958; Norden, 1961; Behnke, 1965). Evidence of their recent speciation is demonstrated by the viability of intergeneric and fertility of intrageneric hybrids

(Buss and Wright, 1958). Discrimination of taxa at the specific level is also complicated by intraspecific phenotypic plasticity. For example, eggs from a single arctic charr can give rise to three distinct morphotypes (Nordeng, 1983). More recent efforts, which have attempted to use allozymes to resolve taxonomic difficulties encountered by morphological studies, have been complicated by the polyploid nature of the salmonid genome (Allendorf et. al., 1979; Utter, 1981).

Restriction analysis of mitochondrial DNA is a recognized alternative, to studies involving the nuclear genome, for the assessment of phylogenetic relationships between closely related taxa. Advantages of using this technique include maternal inheritance (Avise et al., 1979), and elevated rate of mutation (Brown, 1979). It has already proven useful for examining conspecific relationships among a variety of taxa (Avise et al., 1979; Kessler and Avise, 1984; Avise et al., 1984; Crease, 1986). Most mtDNA studies have dealt with aspects of population dynamics, but others have utilized mtDNA data sets to infer phylogenetic relationships at higher taxonomic levels in birds (Kessler and Avise, 1984), mammals (Ferris et al., 1981), and in fish (Berg and Ferris, 1984; Gyllenston and Wilson, 1987; Thomas et. al., 1986; Billington and Hebert, 1986).

Two techniques are employed to infer phylogenies from restriction site data. One uses parsimony analysis programs such as PAUP (Swofford, 1985) or PHYLIP (Felsentein, 1985) to represent phylogenies as a Wagner trees and separates the taxa by the numbers of character state changes required (ie. restriction site changes to move from one taxon to the next). The other uses

estimates of percentage sequence divergence (Nei et. al., 1979) followed by UPGMA analysis (Sneath and Sokal, 1973; Nei et. al., 1985) to represent the phylogenies as bifurcating trees on which the nodes represent the percent sequence divergence between taxa. The approximate times of divergence can also be estimated from sequence divergence data by using the values calculated for the rate of primate mtDNA sequence divergence (Brown et al., 1979). However, these estimates should be interpreted with caution (Avise et al., 1987). While phylogenetic trees can be interpreted by parsimony programs, such as PAUP (Swofford, 1985) or PHYLIP (Felsenstein, 1985), divergence time estimates cannot be made from these types of output.

This paper attempts to draw a phylogeny, utilizing both sequence divergence estimates and parsimony analysis, for the three Salvelinus subgenera (Cristivomer, Baione, and Salvelinus) using Hucho hucho as the outgroup. Results of the two methods are discussed relative to the accepted framework of classical taxonomy for the genus Salvelinus. A composite phylogeny for the major genera of the subfamily Salmoninae, based on the present as well as other published salmonid mitochondrial DNA phylogenies, is also presented.

MATERIALS AND METHODS

The lake trout specimens analyzed in this study were obtained from hatchery brood stock, with the exception of the wild caught Lake Simcoe (Ontario), Lewis Lake (Wyoming), Atlin Lake (British Columbia), and Lake LaBerge (Yukon Territory) fish (Table 1). The geographic origin of each strain is shown in Figure 1. Arctic charr specimens were obtained from Tarsuk Arm (Baffin Island) and Lake Windemere (Britain). The brook trout specimens were obtained from Lake Nipigon (Ontario) and Dickson Lake (Ontario). The Hucho hucho specimen was obtained from the Turiek River (Czechoslovakia).

Mitochondrial DNA was prepared from fresh liver tissue (for extraction details see Appendix 1). The tissue was homogenized in a sucrose buffer and mitochondria were isolated from this suspension by differential centrifugation. The mitochondrial pellet was then resuspended in TE (10mM Tris pH 7.6, 1mM EDTA) and the mitochondria lysed by the addition of sodium dodecyl sulfate. Following lysis mitochondrial DNA was further purified from contaminating nuclear DNA, RNA, and proteins by CsCl isopycnic density ultracentrifugation (Lansman et al., 1981). The mitochondrial DNA band was removed from the gradient tube by bottom puncture and dialysed in a 1/10 TE solution. Samples were then frozen or used immediately for restriction analysis.

Fourteen restriction endonucleases (Table 2) were employed to analyze all 143 lake trout, 2 arctic charr, 2 brook trout, and a single Hucho hucho. Digestions were conducted using the buffer system and incubation temperatures specified by the supplier (Bethesda Research Laboratories). Samples were then end-

labelled with 32P 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). 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 (Whatman 3MM), and exposed overnight to X-ray (Fuji-RX) film. Sizes of the mtDNA fragments appearing on these autoradiographs were estimated 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.

Appropriate double digest were used to confirm homologies of restriction sites and to complete restriction site maps for the mitochondrial genomes of the fish involved. From these data a restriction site presence/absence matrix was created (Appendix 3).

The lengths of restriction fragments greater than 11 kb were

difficult to estimate accurately on 1.2% agarose gels. 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 sizes of the various mitochondrial genomes were then estimated by comparing the sums of restriction fragments produced by the fourteen 6-base enzyme digests.

The maximum likelihood estimates of the number of nucleotide differences per site (d) were calculated following Nei and Tajima (1983). Divergence estimates between taxa were reported as percent sequence divergence ("d") while the distance phenogram, constructed by UPGMA (Sneath and Sokal, 1973), was plotted with each lineage being exactly half this value (1/2 "d") between neighbouring taxa (see Nei and Tajima, 1985). Standard error values for the branching points of the distance phenogram were calculated by the method presented by Nei et al. (1985). Divergence times were estimated using the mitochondrial DNA divergence clock for primates (2% sequence divergence per million years) calibrated by Brown et al. (1979). In other words each lineage, on the distance phenogram, is evolving away from its neighbour at 1% per million years, with a combined sequence divergence rate of 2% per million years between neighbouring taxa.

A parsimony network or "Wagner Tree" was estimated using the branch and bound search routine of Hendy and Penny (1982), as modified by Swofford (1985) for the PAUP program. All OTU's (operational taxonomic units) were treated as unordered and the

huchen (Hucho hucho hucho) was used as an outgroup to root the parsimony network. Initially the A-BAM, A-HIND, C-NCO, and C-SMA clones of S. namaycush were exempted from the analysis. These clones differed from the major clones A-LAKE and C-LAKE by only single site substitutions. These substitutions allowed placement of the clones at several positions on the "lake trout" branch of the trees when using the branch and bound method. Swapping of these branches did not affect overall tree length or consistency. Therefore only the major lake trout clones were used in the initial analysis to take advantage of the "branch and bound" method which is guaranteed to find the "most parsimonious" tree(s). Additional information, obtained from four and five base restriction enzymes (Grewe and Hebert, 1987), was utilized to place these clones on the final parsimony network. A final tree length and consistency index was then recalculated by inputing this tree topology back into PAUP using the "topology" option. For details of the input file see Appendix 4.

RESULTS

Restriction Patterns

Twelve mitochondrial clones were found among the four species of Salmoninae examined. The two brook trout examined had identical restriction patterns for each endonuclease used and will henceforth be referred to as a single brook trout mitochondrial clone. Sixty-eight restriction sites were recognized among these clones by the suite of 14 endonucleases used and each site was mapped (Figure 1 and Appendix III).

Thirty-seven of fourty four restriction sites were conserved among the eight lake trout mitochondrial clones, while thirty-three of fourty sites were conserved between the charr from the Canadian Arctic and the United Kingdom. By contrast, only twenty eight of fifty one sites were conserved among the three Salvelinus congeners, and only twenty three of these sites were conserved between Salvelinus and Hucho hucho. Single site gains/losses were required to move between patterns of the Salvelinus mtDNA clones, while multiple site gains/losses were required to move from any of the Salvelinus patterns to those observed in the Hucho specimen.

Mitochondrial Genome Size

Double digests employing combinations of the enzymes Eco RI, Sal I, and Sst II produced fragments of equal mobility in all Salvelinus examined. Thus, the mitochondrial genomes of these species, by comparison of their digestion profiles relative to those of the lake trout, were estimated to be approximately 16,800±200 base pairs in length. The Hucho hucho mitochondrial genome was, however, approximately 700 base pairs longer than

those of Salvelinus. This increase in length appears to be the result of a single insertion which mapped into the area between the conserved (for all species) Sma I and Eco RI restriction sites (Figure 1 and Appendix III).

Localization of the D-loop Region

It is possible, by observing the location of Sst II restriction sites, to locate the mtDNA D-loop region or origin of heavy strand replication of the salmonid species examined in this study. Sst II produced three fragments in each of the Salvelinus and Hucho mitochondrial genomes examined (250, 1750, and 14,800 bp). Two of these restriction sites (S1 and S2, Figure 1) define a 1750 bp fragment and occur in the 16S rRNA and 12S rRNA molecule respectively. These sites appear to be conserved in all sequenced vertebrate mtDNA genomes (Berg and Ferris, 1984; H. Hotz, C. Spolsky, and T. Uzzell, pers. comm.; Beckman Micro Genie data files). The third Sst II site (S3, Figure 1), located 250 bp downstream of S2, was conserved in all salmonids, but is not present in other vertebrates, which possess only two sites. However, inspection of sequence data for mice (Bibb et al., 1981) and humans (Anderson et al., 1981) reveals a sequence (CCTCGG), located 253 and 252 bp (Figure 2), downstream from the S2 Sst II site, which differs by one nucleotide from the Sst II regognition sequence (CCGCGG). This result provides additional evidence of the homology of all three sites and permits location of the D-loop region. Specifically, the D-loop regions in mice and humans begin approximately 500-700 bp upstream of the S1 site and span a region of approximately 1000 bp. In the case of salmonids, this would place the origin of the D-loop between the second conserved

would place the origin of the D-loop between the second conserved Eco RI site (E2, Figure 1) and the sole conserved Sma I site (M, Figure 1). It is significant that there is a 5000 bp region upstream of the E2 site which lacks any conserved sites. Significantly, the proposed origin of the D-loop region is the same area to which the insertion was mapped for the Hucho mitochondrial genome (see Appendix III).

Nei's Estimate of Genetic Distance (UPGMA)

Table 3 lists the total number of restriction sites for each distinct mitochondrial clone, and the number of sites it shared with other mitochondrial clones. These data were used to calculate the percent substitution (percent sequence divergence) between the respective species pairs (Table 4). Estimates of intraspecific sequence divergence ranged from 0.22 ± 0.22 %, between S. namaycush clones, to 1.68 ± 0.70 %, between the two clones of S. alpinus. Differences between taxa were considerably larger than those within a taxon. Estimates between brook trout and the arctic charr lineage, and between lake trout and the lineage leading to brook trout/arctic charr, were 3.47 ± 0.70 and 3.94 ± 0.70 %, respectively. The sequence divergence between Salvelinus and Hucho was estimated to be 8.00 ± 1.60 %.

The values from Table 4 are represented as a distance phenogram in Figure 3. There are no significant branching points among the eight lake trout mtDNA clones. The lake trout mitochondrial clones and two arctic charr specimens form two cohesive groups, significantly different from each other, and the brook trout lineage. At present, arctic charr and brook trout appear most closely allied on the distance phenogram. The break

points defining the relationships between these three species, however, are not significant to ± one standard error and they represent an unresolved trichotomy. Conversely, the break point between Salvelinus and Hucho was significant at ± one standard error.

Parsimony Analysis

The "branch and bound" ("BANDB") algorithm of PAUP revealed two equally parsimonious trees, with similar consistency indexes In contrast to the distance phenogram, both parsimony networks placed the BLAKE clone on the lineage leading to the CLAKE clone rather than on the lineage leading to the ALAKE clone. The first parsimony network (Figure 5) was most similar to the distance phenogram, placing brook trout intermediate to the arctic charr clones. The second parsimony network (Figure 6) placed the brook trout clone branching off the lineage that lead to British charr, twice as far from the lake trout lineage as the Baffin charr clone. This is in stark contrast to the distance phenogram obtained by UPGMA.

After the initial analysis, the extra lake trout clones were added onto the parsimony network utilizing additional 4- and 5-base enzyme data obtained by Grewe and Hebert (1987). The number of steps required and the consistency indexes, for each of the parsimony networks (Figure 5 and 6), were 57 and 0.846 respectively.

DISCUSSION

The length of the mitochondrial genome in both Salvelinus namaycush and the three other Salvelinus taxa was approximately 16,800 ± 200 base pairs (Grewe and Hebert, 1987). The value concurs well with other length estimates for salmonid mitochondrial genomes listed in Table 5 (Berg and Ferris, 1984; Birt et all., 1986; and Thomas et al., 1986) and is similar to those for other fish species. However, the mitochondrial genome of Hucho hucho was approximately 700 bp. longer. Such size increases are not unusual even within a single taxon. For example, in a sample of Great Lake's walleye (Stizostedion vitreum), a 1700 base pair insertion was found within the mitochondrial genome of two individuals (Billington and Hebert, 1986). Bermingham et al. (1986) report that the mtDNA of bowfin (Amia calva) varied by as much as 900 base pairs overall and by 700 base pairs within a single river system. The size inserts ordinarily map to the boundry of the D-loop region. Similarily, in the present case, the length insert in Hucho mapped to the vicinity of the D-loop region.

Before considering the phylogeny, which was inferred from the mtDNA analysis, it is necessary to discuss the taxonomic relationships recognized on the basis of previous studies. Classical taxonomy now recognizes the lake trout, brook trout, and arctic charr as representatives of the three subgenera of Salvelinus : S. (Cristivomer) namaycush, S. (Baione) fontinalis, and S. (Salvelinus) alpinus, respectively (Behnke, 1965 and references cited therein).

S. namaycush, the sole member of the subgenus Cristivomer,

is endemic to North America with a distribution closely matching the maximum extent of Wisconsin ice sheets (Figure 7). Within this range many diverse populations can be found. The lack of speciation has been attributed to its deepwater existence, resulting in less exposure to cosmic radiation and a lower mutation rate (Rounsefell, 1962; Kahn and Qadri, 1971). Alternatively, Behnke (1972) suggested, that the specialization of the lake trout as a deepwater lacustrine predator with narrow environmental tolerances, has prevented it from adapting to new niches. Grewe and Hebert (1987) suggest, based on mitochondrial data, that lake trout are derived from small refugial populations (briefly isolated during the Pleistocene glacial advances), which have expanded their range to cover portions of the North American continent with suitable habitats. In areas of secondary contact, such as in the Great Lakes, the once unique populations have introgressed to varying degrees thereby creating the various forms and mtDNA clonal combinations found today. The mtDNA data (Grewe and Hebert, 1987) provide no evidence that mutation rate of the lake trout mitochondrial genome is lower than that of other vertebrates, in contradiction to Rounsefell's hypothesis (Rounsefell, 1962). Therefore, Behnke's (1972) hypothesis seems more reasonable in explaining the lack of speciation in this subgenus. Despite the lack of apparent speciation (perhaps due to introgression and/or narrow niche requirements), two subspecies of lake trout are recognized. These are the lean or typical lake trout S. namaycush namaycush and the siscowet or the "fat" lake trout S. namaycush siscowet. The two forms are apparently reproductively isolated and occur sympatrically in Lake Superior,

with the siscowet exploiting the deeper portions of the lake (>100m). Perhaps Lake Superior is the only deep body of water with enough environmental heterogeneity to allow for speciation of the lake trout to occur.

Salvelinus fontinalis, the brook trout, is the single representative of the subgenus Baione. The brook trout is endemic to northeastern North America, but, has now been widely introduced outside of its native range (Figure 8). The aurora trout, S. timagamiensis (Henn and Rikenbach, 1925), was initially described as a separate species in this subgenus, but, it has since been relegated to subspecific status, S. fontinalis timagamiensis (Sale, 1967; Qadri, 1968; Behnke, 1980). Behnke (1972) suggests S. agassizi, the "silver charr", was a second member of the subgenus Baione, but, is now likely extinct.

According to Behnke (1980), "virtually all of the problems regarding determination of relationships and recognition of taxa within the genus Salvelinus concern the widely distributed (Figure 9) Salvelinus alpinus complex, which includes all of the subgenus Salvelinus except for S. leucomaenis". Two major species are recognized in the "alpinus" complex, S. alpinus (arctic charr) and S. malma (dolly varden trout). Two other minor species are also recognized, S. confluentus (bull trout), a species broadly distributed in western North American, and S. profundus, a species restricted to one or a few lakes in Europe. Three additional, albeit "tentative" species, also belong to the subgenus Salvelinus. These are S. drjagini (the deepwater charr of Lake Taimyr), S. taimyricus, and the stone charr of Kamchatka

(Behnke, 1980; Savvaitova, 1980).

In North America and Asia (particularly in Kamchatka) confusion may arise when assigning some charr populations to either S. malma or S. alpinus. This has led Savvaitova (ref. cited in Behnke, 1972) to conclude that "S. malma is only a polymorphic form of S. alpinus and that S. malma should be considered a synonym of S. alpinus" (for arguments see Behnke, 1972; Behnke, 1980; Savvaitova, 1980). It is clear, however, that S. malma and S. alpinus occuring sympatrically in Alaskan lakes, represent two valid species (DeLacy and Morton, 1943; McPhail, 1961; Caveder, 1980).

Several subspecies (the number of which fluctuates) of S. alpinus have been described, but, in this paper discussion will be confined to S. alpinus inhabiting North America and Europe. Moreover, due to the taxonomic confusion surrounding the validity of the specific/subspecific status of various forms of arctic charr found throughout the range of the "alpinus" complex (Figure 9), the outline of taxa and nomenclature provided by Behnke (1980) will be used to describe the relationships between the forms of arctic charr relevent to this study.

Arctic charr were first described from Swedish Lapland by Linnaeus. Charrs of northern Sweden, characterized by approximately 21 - 27 gill rakers, are hence recognized as S. alpinus alpinus. These include the sympatric freshwater resident, dwarf, and anadromous forms, which have been shown to be members of a single gene pool (Nordeng ,1983). Charrs of this type are also found in the waters of the Kola Peninsula, Karelia, and Norway.

Behnke (1980) suggests that most arctic charr inhabiting Great Britain and Ireland belong to the nominate subspecies. In some localities, such as Lake Windemere, two populations occur which are meristically identical, yet, differ with respect to spawning season and their allele frequency at two allozyme loci (Child, 1984). These sympatric populations, typical of British charr, show only slight meristical differences from the Swedish charr (Behnke 1972) and have been classified as S. alpinus alpinus. The charr from Lake Coomarsaharn in Ireland are, however, characterized by high gill raker number (26 - 32), typical of charr from the Alpine Lakes of Europe and charr from the Taimyr Peninsula. Moreover, the presence of ciscoe, similar to Coregonus autumnalis (arctic ciscoe), in some Irish lakes (including Lake Coomarsaharn) is evidence of a westward movement of fishes from the Arctic Ocean to the British Isles via the sea during postglacial times (Behnke, 1980). Thus, the charr of Lake Coomarsaharn are more likely to represent S. alpinus derived from the area of the Taimyr Peninsula which are recognized by Behnke (1980) as S. alpinus erythrinus.

Charr found in the alpine lakes of Europe exhibit a similar diadromous life history as S. alpinus alpinus of Sweden (Behnke, 1980). Sympatric stocks of "alpine" charr are characterized by a normal predatory form and a dwarf form. These forms differ from typical S. alpinus alpinus by their higher meristic counts and are recognized by Behnke (1980) as a unique subspecies, S. alpinus salvelinus. These "alpine" charr coexist in the Bodensee (Lake Constance) with the highly divergent charr, S. profundus,

which diverged from the S. alpinus lineage "no later than early to mid-Pleistocene" (Behnke, 1980).

In North America, the eastern Arctic S. alpinus, which occurs from the MacKenzie River to Hudson Bay, bears a close resemblance to S. alpinus erythrinus of the Taimyr Peninsula. Behnke (1980), however, has suggested the temporary use of S. alpinus stagnalis for these fish, reserving this name until the taxonomic status of Greenland arctic charr has been determined.

Relict populations of S. alpinus in eastern North America, occuring at the southern-most range of S. alpinus, include the Quebec Red Trout, and the Blueback and Sunapee of Maine. These populations, which have been examined morphologically and allozymically (Kircheis, 1980), appear to be more closely related to each other than to other subspecies of S. alpinus and are recognized as a distinct subspecies, S. alpinus oquassa. Behnke (1980) also refers to another form of Arctic charr from Labrador, which appears to be the result of hybridization between the eastern Arctic and eastern North American forms (S. alpinus oquassa and S. alpinus stagnalis respectively). However, a subspecific name has not been designated for these charr.

Members of two arctic charr subspecies (stagnalis and alpinus) were included in this study. In future, studies should aim to compare mtDNA of the other putative subspecies of the S. alpinus complex.

The genus Hucho was also examined by this study. The huchens are broadly distributed across Eurasia (Figure 10) and are restricted to fresh water, with the exception of Hucho perryi. Formerly, they were placed in either the genus Salmo or

Salvelinus, but, most authors now agree with its independent generic status. The genus Hucho is the most closely allied to Salvelinus of all the Salmoninae genera (Behnke, 1965; Holcik, 1982). It can be broken into two subgenera, Hucho and Parahucho, respectively. The latter contains only one species H. (Parahucho) perryi, while the former contains H. (Hucho) bleekerii, (Chinese huchen) H. (Hucho) ishikawai (Korean huchen), H. (Hucho) hucho hucho (Danubian huchen), and H. (Hucho) hucho taimen (the taimen). The specimen examined by this study was the Danubian huchen.

The distance phenogram clearly distinguishes the three subgenera (Salvelinus, Baione, and Cristivomer) of the genus Salvelinus (Figure 3). Based on 2% sequence divergence per million years (Brown et al., 1979), S. namaycush mtDNA clones have been diverging from each other since the mid Pleistocene (450,000 ybp) to the Holocene (last 10,000 years). British (S. alpinus alpinus) and Baffin (S. alpinus stagnalis) charr last shared a common ancestor approximately 850,000 ybp (early Pleistocene). The three subgenera of Salvelinus last shared a common ancestor approximately 1.5 - 2.5 million ybp. when they simultaneously diverged from each other. This value agrees with the divergence time, "late Pliocene to early Pleistocene", hypothesized for the separation of these groups from a common ancestral Salvelinus (Behnke, 1980). The genus Hucho is clearly differentiated from Salvelinus, with these two genera separating from the proto- hucho/salvelinus ancestor approximately 4 million ybp (middle to late Pliocene). A fuller account of the

systematics of Hucho's affinities to Salmo/Salvelinus are provided by Holcik (1982).

Of the two equally parsimonious networks found by PAUP, network 1 represents a phylogeny agreeing with classical taxonomy and the mtDNA distance data, which propose that the phylogenies of the three Salvelinus subgenera are equidistant from a common ancestor. The alternative phylogeny of network 2 proposed that brook trout, almost twice as distant from lake trout (25 steps) as Baffin charr (only 14 steps), are at the same distance from lake trout as the genus Hucho (28 steps). It also indicates that Baffin charr are an intermediate form between lake trout and brook trout with the latter species sharing a closer relationship with British charr than Baffin charr. If such distances truly existed between brook trout and its congeners, as are represented by the parsimony network 2 (Figure 2), they surely would have manifested themselves morphologically allowing for easy taxonomic distinction between these species. Chromosome data (R. Phillips pers. comm.) and production of the fertile hybrid "splake" indicate a closer relationship between brook and lake trout, further supporting the parsimony network 1 (Figure 1), mtDNA distance data, and classical taxonomy of Salvelinus. Perhaps further information on interspecific hybrids between these three species will clarify the situation. Meanwhile the phylogenetic network presented in Figure 1 must be assumed to be the most plausible one. It is interesting to note that this phylogeny places Hucho closer to S. alpinus than to S. namaycush (27 vs. 30 steps respectively), although, these values may not be significantly different from each other.

In a broader taxonomic sense, it is possible to create a phylogeny for the genera of the subfamily Salmoninae, excluding Brachymystax and Salmothymus, by combining the results of this study together with prior mtDNA studies (Berg and Ferris, 1984; Thomas et al., 1986; Gyllensten and Wilson, 1987). The studies of Berg & Ferris (1984) and that of Gyllensten and Wilson (1987) both examined Salmo (Salmo) trutta (brown trout), Salmo (Parasalmo) gairdneri (rainbow trout), and Salvelinus fontinalis (brook trout), finding approximately 10 and 12 percent sequence divergence (respectively) between the Salvelinus line and the lineage leading to Salmo and Parasalmo. It should also be noted that Onchorychus split off from the subgenus Parasalmo after the latter had split off from the lineage leading to the subgenus Salmo (Berg and Ferris, 1984; Thomas et al., 1986). From these results, Salvelinus appears to be an old lineage, diverging from the rest of the Salmoninae approximately five to six million ybp. Hucho diverged from the Salvelinus lineage just after this break point, approximately three to four million ybp (8.0 $\pm$ 1.6% sequence divergence). By superimposing these values, and utilizing percent sequence divergence data of salmonid species obtained in the present study along with those examined by Berg and Ferris (1984), Thomas et. al. (1986), and Gyllensten and Wilson (1987), a composite distance phenogram can be created to approximate the phylogeny of the subfamily Salmoninae, based on restriction analysis of mitochondrial DNA (Figure 11). However, caution should be exercised when interpreting these results, because comparing these four studies to create a composite

phenogram is akin to superimposing photographs of the same object all taken from different angles.

Using the mtDNA clock calibrated by Brown et al. (1979), one can obtain a relative measure of the age of the Salmoninae. Estimates of "true" divergence times for the phylogenetic break points between the major taxa, however, are difficult due to the paucity of fossil remains. This lack of physical evidence is possibly due to either a lack of interest among paleontologists with fishes of the late Tertiary or to the preferred habitat of salmonid fishes, not being conducive to fossilization (Norden, 1960). Thus, it was not possible to calibrate a salmonid mtDNA clock.

Assuming, that salmonid mtDNA is evolving at the same rate as the primate mtDNA, a minimum age of the Salmoninae can be estimated by observing the value of percent sequence divergence between the most divergent taxa (ie. the breakpoint when all the Salmoninae genera last shared a common ancestor). This value (about 12.0% sequence divergence) converts to approximately 6 million ybp, which is more recent than those estimated using the few fragmentary fossil remains available for dating the origin of these fish. Behnke (1965) suggested that the Salmoninae had diverged from the ancestral Salmonidae lineage by the Miocene (12-25 million ybp), while Cavender (1980) states that evidence from Nevada represents the oldest (at least 10 million years) known fossil Salvelinus. It should be noted that the sequence divergence values, reported between the respective Salmoninae lineages, are well within the linear portion (linear for values <15%) of the sequence divergence (vs. time of divergence) curve

estimated by Brown et al. (1979) and are hence not underestimates of the true amount of divergence present among these taxa. Relatively speaking, the genera of the Salmoninae are of recent origin diverging from a common ancestor within the last 5 - 6 million years (late Miocene to early Pliocene), with most of the respective species appearing only recently, during the last 1.5 million years (late Pliocene to early Pleistocene). This latter statement is in agreement with the classical notions regarding the time frame during which speciation of the major Salmoninae genera occured (Norden, 1961; Behnke, 1965; Behnke, 1980 and references cited therein).

In conclusion, restriction analysis of mitochondrial DNA appears to be a valuable systematic tool for examining the difficult and often times confusing issue of Salmonid taxonomy, particularly with regards to the S. alpinus complex. Moreover, due to the asexual nature of the mitochondrial genome, phylogenetic histories of closely related taxa will not be marred by introgression. Some of the taxonomic problems examined by this study, however, are still unresolved such as the relationship between the three subgenera of Salvelinus. Examination of more brook trout and arctic charr populations should enable a better estimate of intraspecific variation by indicating how well each specimen represents each of the species lineages. Finally, although intuitively obvious, the use of additional restriction endonucleases, should lend more resolution to this issue.

Regardless, the use of mtDNA analysis has brought an exciting semblance of order to a once confusing issue. An

attempt should now be made to complete restriction analysis of the remaining Salmoninae genera, Brachymystax and Salmothymus. Next, the valid species of Salvelinus such S. malma, S. confluentus, S. profundus and S. leucomaenis should be examined along with their recognized subspecies such as S. alpinus oquassa, S. namaycush siscowet, and S. fontinalis timagamiensis.

I agree with Behnke (1980) that a cooperative effort is required in order to analyze the various forms of arctic charr of the S. alpinus complex. Along these same lines, agreement must be reached as to what degree of sequence divergence is necessary to constitute separation and identification of a valid subspecies. Perhaps sequence divergence estimates between Baffin and British charr may be used as a guideline. It will be interesting to compare these values to those obtained between British/Baffin charr and S. profundus (characterized by Behnke as a definite species and estimated to have diverged from alpinus during Pleistocene times). Specific nomenclature should also be developed to identify stocks being dealt with by various researchers. With regards to mtDNA analysis, the use of certain key restriction enzymes and mapping reference points should be standardized in order to provide direct and unambiguous comparison between various research groups. The choice of restriction enzymes may be arbitrary, but, restriction maps could be aligned using the conserved Sst II restriction sites.

As a final note, it should be possible to extract mtDNA from small tissue samples of museum specimens using the techniques of Barker et al. (1986). With this technology specimens, used by the various authors (Behnke, 1980. Saavaitova, 1980, Cavender, 1980)

to make their taxonomic decisions, could be examined for comparison of their mtDNA patterns. Taxonomic status of extinct species such as the "silver charr", S. (Baione) agassizi, could also be ascertained. Hopefully future research utilizing restriction analysis of mitochondrial DNA for phylogenetic reconstruction, will lead to a better understanding of the taxonomic relationships among the members of the Salmoninae.

TABLE 1. Species used in the study and their origin.

SPECIES	no.	year class	origin of fish and date obtained
Hucho hucho	1	unknown	- Turiek Hatchery, Bratislava, Czechoslovakia, by Jurek Holcik, May 87.
S. alpinus	1	unknown	- collected from the Tarsuk Arm, Baffin Island, N.W.T., by Brent Glynn, Aug. 85.
S. alpinus	1	unknown	- collected from Lake Windemere, Lake District, Britain, by Neil Billington, Dec. 85.
S. fontinalis	2	unknown	- Maple Research Station, Ontario, July 86.
S. namaycush (by strain)			
Manitou	4	80'	- Maple Research Station, Ontario, May 85.
Big Bay	5	80'	- Maple Research Station, Ontario, 85.
Simcoe	10	unknown	- collected from ice-fishermen on Lake Simcoe and identified as Simcoe stock by personnel at Sibbald Point assessment unit, Feb. 86.
Killala	11	83'	- Hatchery, Ontario, Aug. 85.
Seneca	15	78'	- Allegheny N.F.H., Nov. 84.
Green Lake	9	75'	- Jordan River N.F.H., Oct. 84.
	6	75'	- Jordan River N.F.H., Oct. 85.
Lewis Lake	8	82'	- Jackson N.F.H., Aug. 85.
	9	unknown	- wild caught fish from Lewis Lake Sept. 85.
Clearwater Lake Manitoba	11	82'	- Great Lakes Fish. Comm., June 85.
Marquette	10	75'	- Jordan River N.F.H., Nov. 84.
(domestic)	8	81'	- Jordan River N.F.H., Nov. 84.
	20	77'	- Jordan River N.F.H., Nov. 85.
Atlin Lake	10	unknown	- collected from Atlin Lake, British Columbia by P. Etherton, April 86.
Lake LaBerge	4	unknown	- collected from Lake LaBerge, Yukon Territory by P. Etherton, April 86.

6-BASE ENZYMES

Bam HI	G'GATC,C
Bcl I	T'GATC,A
Bgl II	A'GATC,T
Bst EII	G'GTGAC,C G'GTAAC,C G'GTTAC,C G'GTCAC,C
Eco RI	G'AATT,C
Hind III	A'AGCT,T
Nco I	C'CATG,G
Pst I	C,TGCA'G
Pvu II	CAG'CTG
Sal I	G'TCGA,C
Sma I	CCC'GGG
Sst II	CC,GC'GG
Xba I	T'CTAG,A
Xho I	C'TCGA,G

TABLE 2. Restriction enzymes used and their recognition sites.

	ALAKE	A-BAM	A-BST	AHIND	BLAKE	CLAKE	C-NCO	C-SMA	BAFFN	BRITT	BROOK	HUCHO
ALAKE	38	38	38	38	38	38	38	38	29	31	31	24
A-BAM		39	38	38	38	38	38	38	29	31	31	24
A-BST			39	38	38	38	38	38	29	31	31	24
AHIND				39	38	38	38	38	29	31	31	24
BLAKE					39	39	38	39	29	31	31	24
CLAKE						40	39	40	29	32	32	24
C-NCO							39	39	29	31	31	23
C-SMA								41	29	32	32	24
BAFFN									35	33	30	23
BRITT										38	33	25
BROOK											41	23
HUCHO												38

Table 3. Restriction site data used to compute Nei's d. Values on the diagonal are the number of restriction sites per clone, with the other values refering to number of restriction sites in common between the various clones.

	ALAKE	A-BAM	A-BST	AHIND	BLAKE	CLAKE	C-NCO	C-SMA	BAFFN	BRITT	BROOK	HUCHO
ALAKE	-----	.0038	.0036	.0033	.0022	.0066	.0066	.0066	.0394	.0394	.0394	.0799
A-BAM	.0022	-----	.0038	.0038	.0038	.0066	.0066	.0066	.0394	.0394	.0394	.0799
A-BST	.0022	.0043	-----	.0036	.0036	.0066	.0066	.0066	.0394	.0394	.0394	.0799
AHIND	.0022	.0043	.0043	-----	.0036	.0066	.0066	.0066	.0394	.0394	.0394	.0799
BLAKE	.0022	.0043	.0043	.0043	-----	.0066	.0066	.0066	.0394	.0394	.0394	.0799
CLAKE	.0043	.0065	.0065	.0065	.0021	-----	.0032	.0021	.0394	.0394	.0394	.0799
C-NCO	.0066	.0088	.0088	.0088	.0043	.0021	-----	.0032	.0394	.0394	.0394	.0799
C-SMA	.0065	.0085	.0085	.0085	.0042	.0021	.0042	-----	.0394	.0394	.0394	.0799
BAFFN	.0383	.0406	.0406	.0406	.0406	.0406	.0406	.0450	-----	.0168	.0347	.0799
BRITT	.0339	.0361	.0361	.0361	.0361	.0330	.0361	.0351	.0168	-----	.0347	.0799
BROOK	.0404	.0425	.0425	.0425	.0425	.0393	.0425	.0413	.0394	.0300	-----	.0799
HUCHO	.0766	.0788	.0788	.0788	.0788	.0809	.0859	.0830	.0770	.0698	.0901	-----
	ALAKE	A-BAM	A-BST	AHIND	BLAKE	CLAKE	C-NCO	C-SMA	BAFFN	BRITT	BROOK	HUCHO

Table 4. Maximum likelihood estimate of mtDNA sequence divergence among Salvelinus and Hucho species.

Note: The estimated sequence divergence between species is given above the diagonal, with actual values given below.

TABLE 5. 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
Sebastes atrovirens	17,300 ± 400	(1985)
Sebastes caurinus	17,400 ± 400	
Sebastes melanostomus	17,200 ± 400	
Sebastes mystinus	16,900 ± 400	
Scomber japonicus	17,200 ± 400	
Amia calva	16,000 to 16,900	Bermingham & Avise (1986)
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
Stizostedion vitreum	18,475 ± 300	(1986)
Stizostedion canadense	16,702 ± 259	
Stizostedion lucioperca	16,736 ± 277	
Salvelinus namaycush	16,800 ± 200	Grewe & Hebert (1987)
S. fontinalis	16,800 ± 200	Present Study
S. alpinus alpinus	16,800 ± 200	"
S. alpinus stagnalis	16,800 ± 200	"
Hucho hucho hucho	17,500 ± 200	"

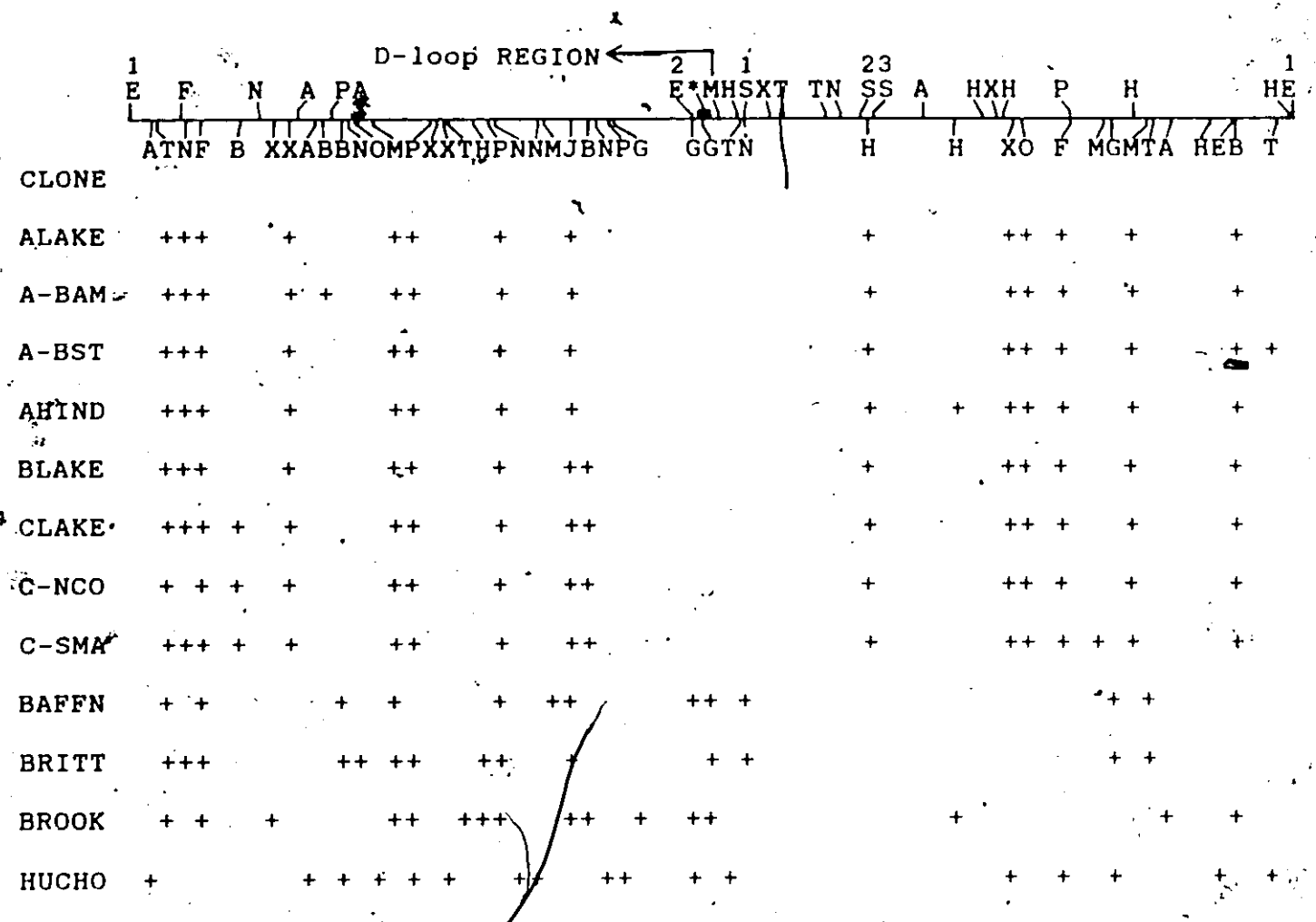


FIGURE 1. Mitochondrial DNA cleavage map of the clones examined by this study. The molecule has been depicted linearly, opened at an arbitrary Eco RI site.
Enzymes: A, Bcl I; B, Bam HI; E, Eco RI; F, Pst I; G, Bgl II; H, Hind III; J, Sal I; M, Sma I; N, Nco I; O, Xho I; P, Pvu II; S, Sst II; T, Bst EII; X, Xba I.
* indicates the area to which the Hucho hucho insert was mapped. Polymorphic sites are identified below the map and are indicated by a plus sign when present.

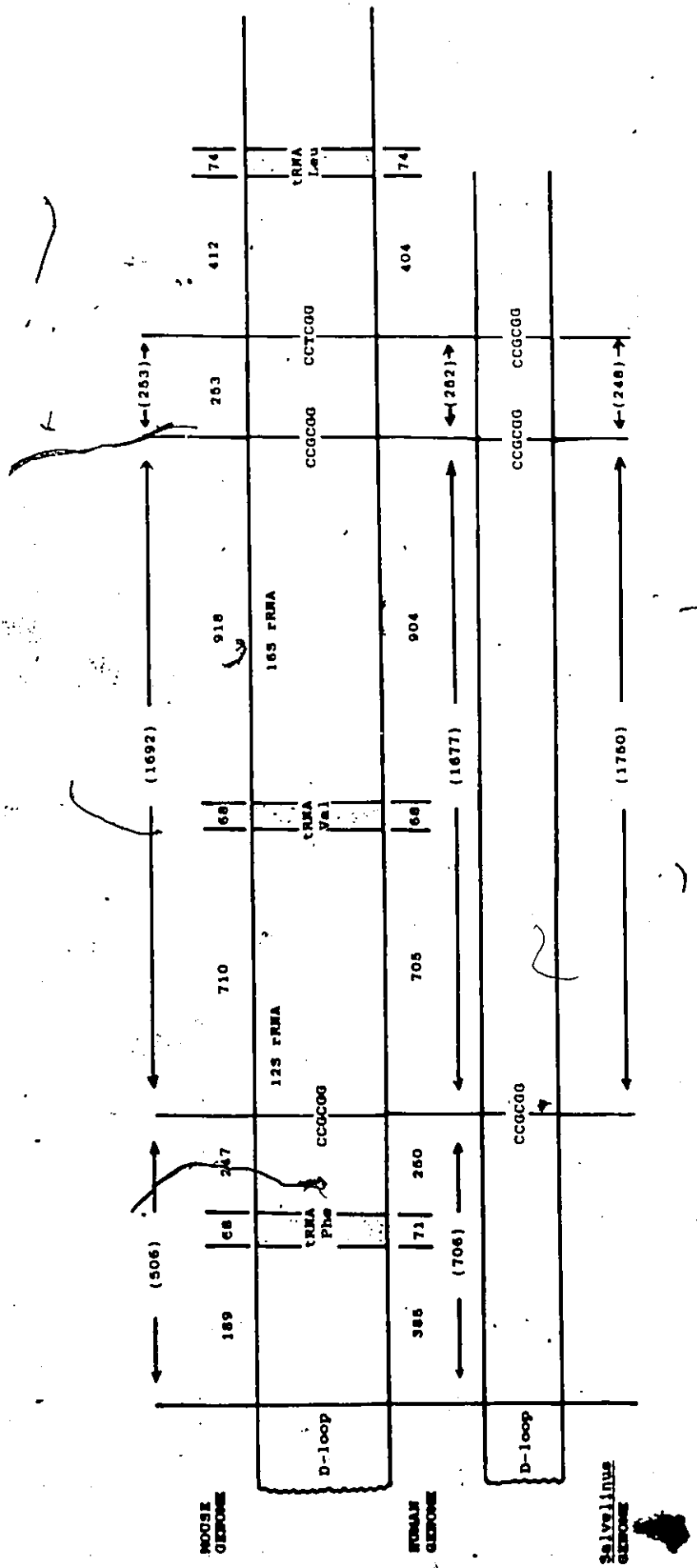


FIGURE 2. Location of Set II sites, in relation to the D-loop region, based on the published sequences of mouse (Bibb et al., 1981) and human (Anderson et al., 1981). Lengths between sites are in base pairs.

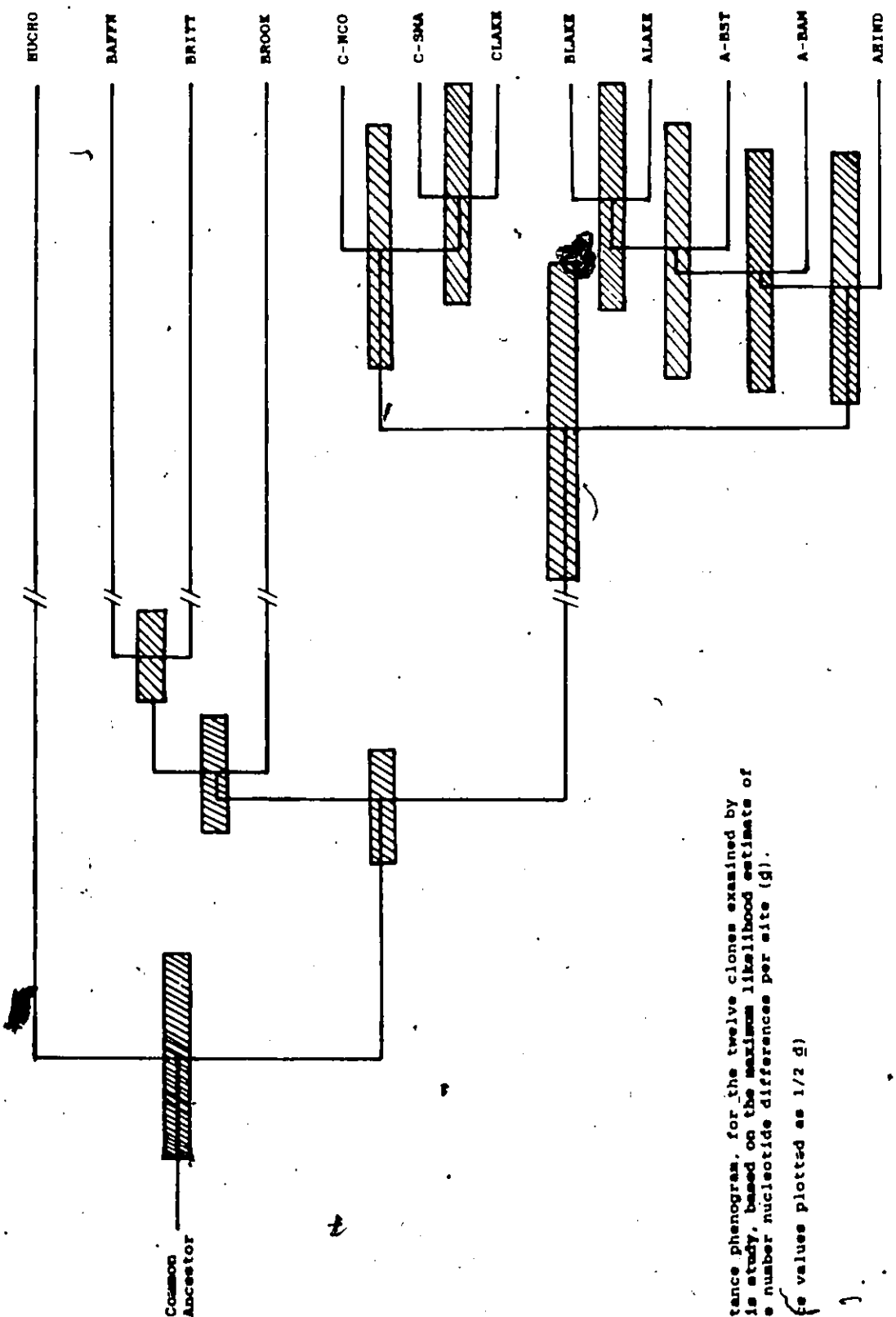


FIGURE 3. Distance phenogram, for the twelve clones examined by this study, based on the maximum likelihood estimate of the number nucleotide differences per site (d).

NOTE: (distance values plotted as 1/2 d)

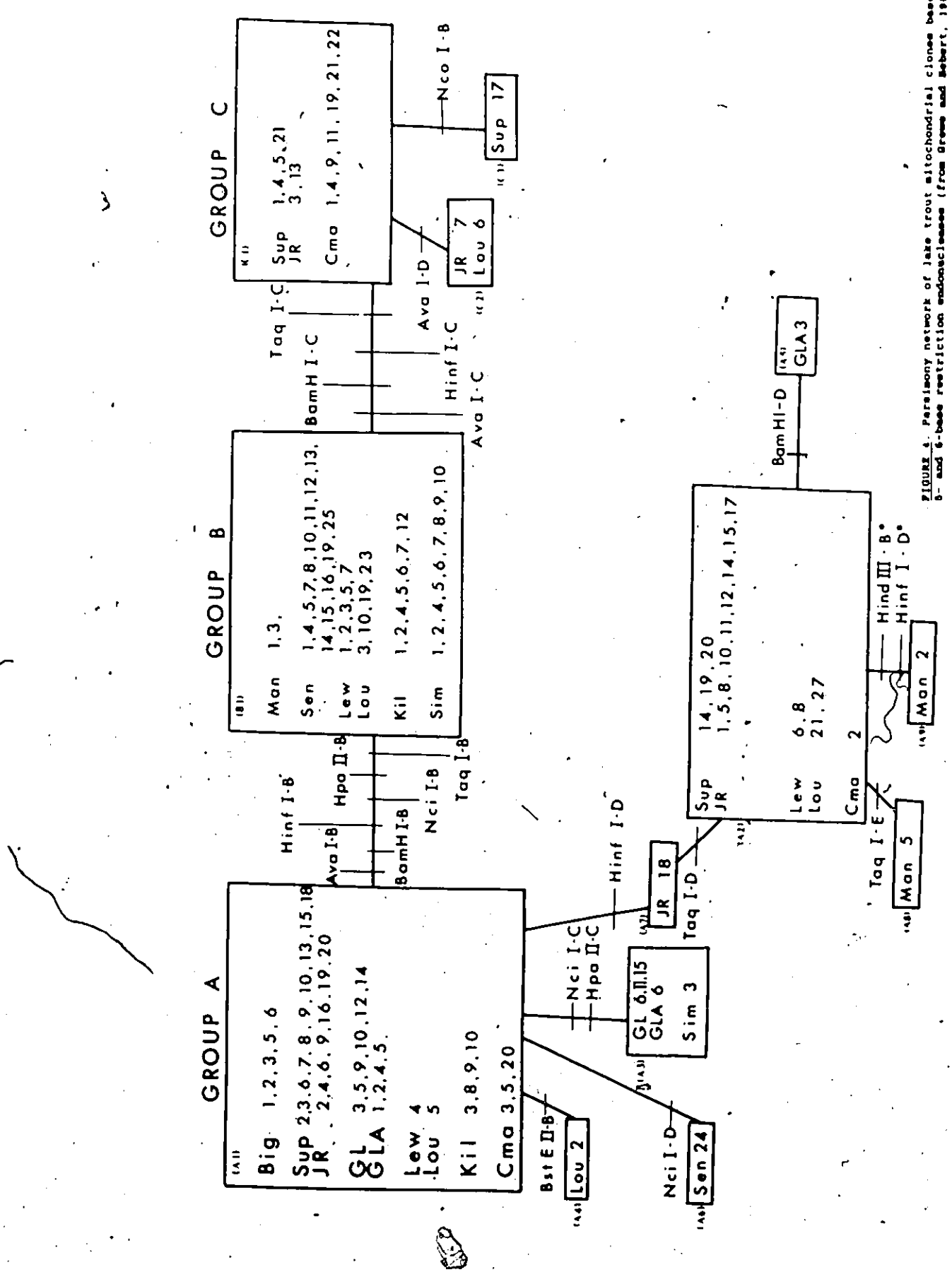
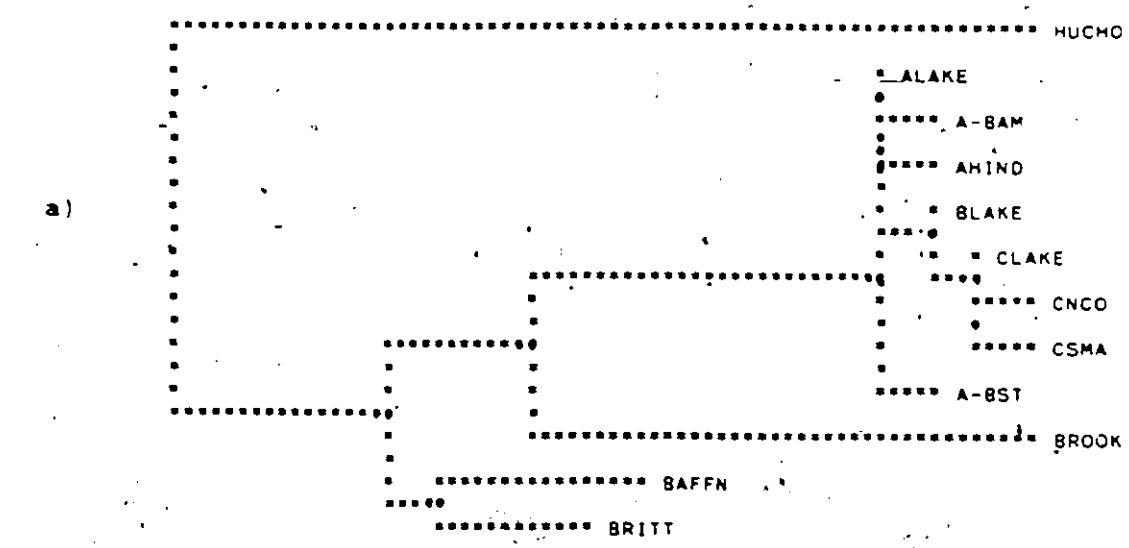


FIGURE 4. Parsimony network of lake trout mitochondrial clones based on 4- and 6-base restriction endonucleases (from Grewe and Hebert, 1987).

Statistics for tree no. 1

Length = 57.000
Consistency index = 0.842

Tree no. 1 rooted using designated outgroup Hucho



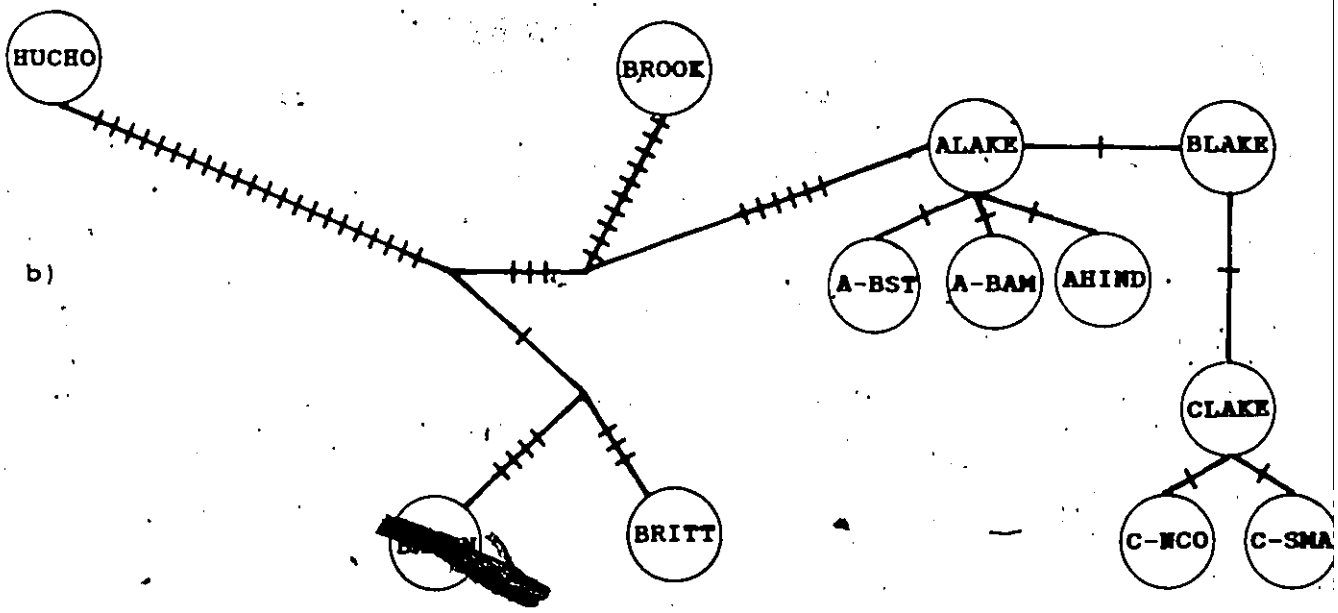


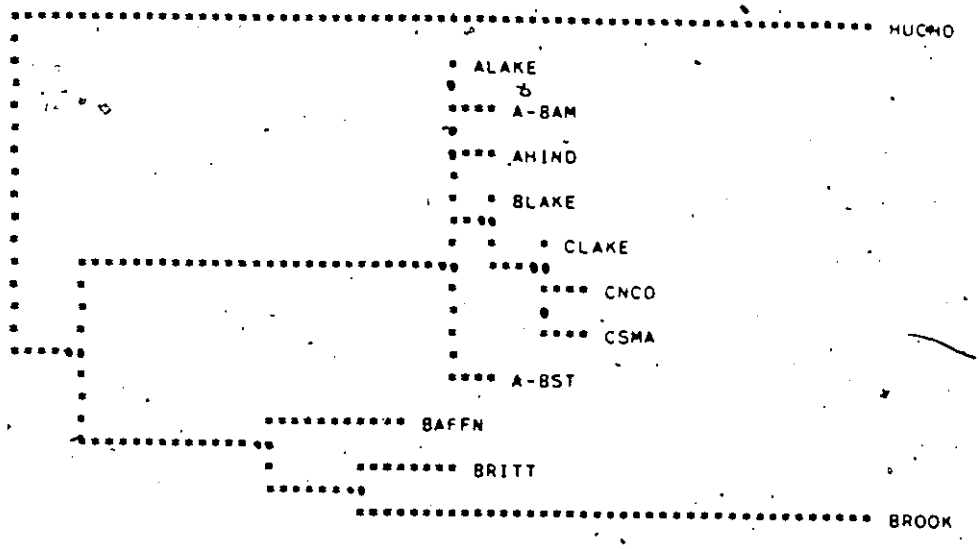
FIGURE 5. Parsimony network #1 depicting the relationships between the species examined by this study: a) drawn by PAUP as a dendrogram (branch lengths to scale; and b) drawn as a Wagner tree with slashes indicating a character state change.

Statistics for tree no. 2 .

Length = 57.000
Consistency index = 0.842

Tree no. 2 rooted using designated outgroup Hucho

b)



a)

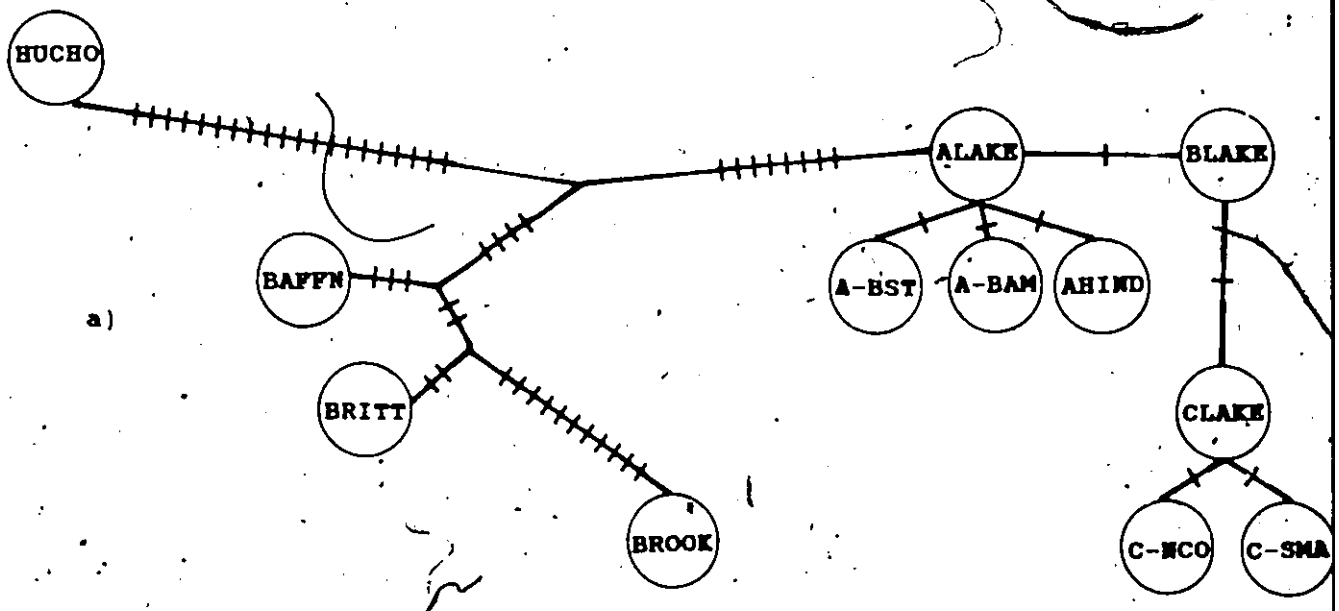


FIGURE 6, Parsimony network #2 depicting the relationships between the species examined by this study: a) drawn by PAUP as a dendrogram (branch lengths to scale; and b) drawn as a Wagner tree with slashes indicating a character state change.

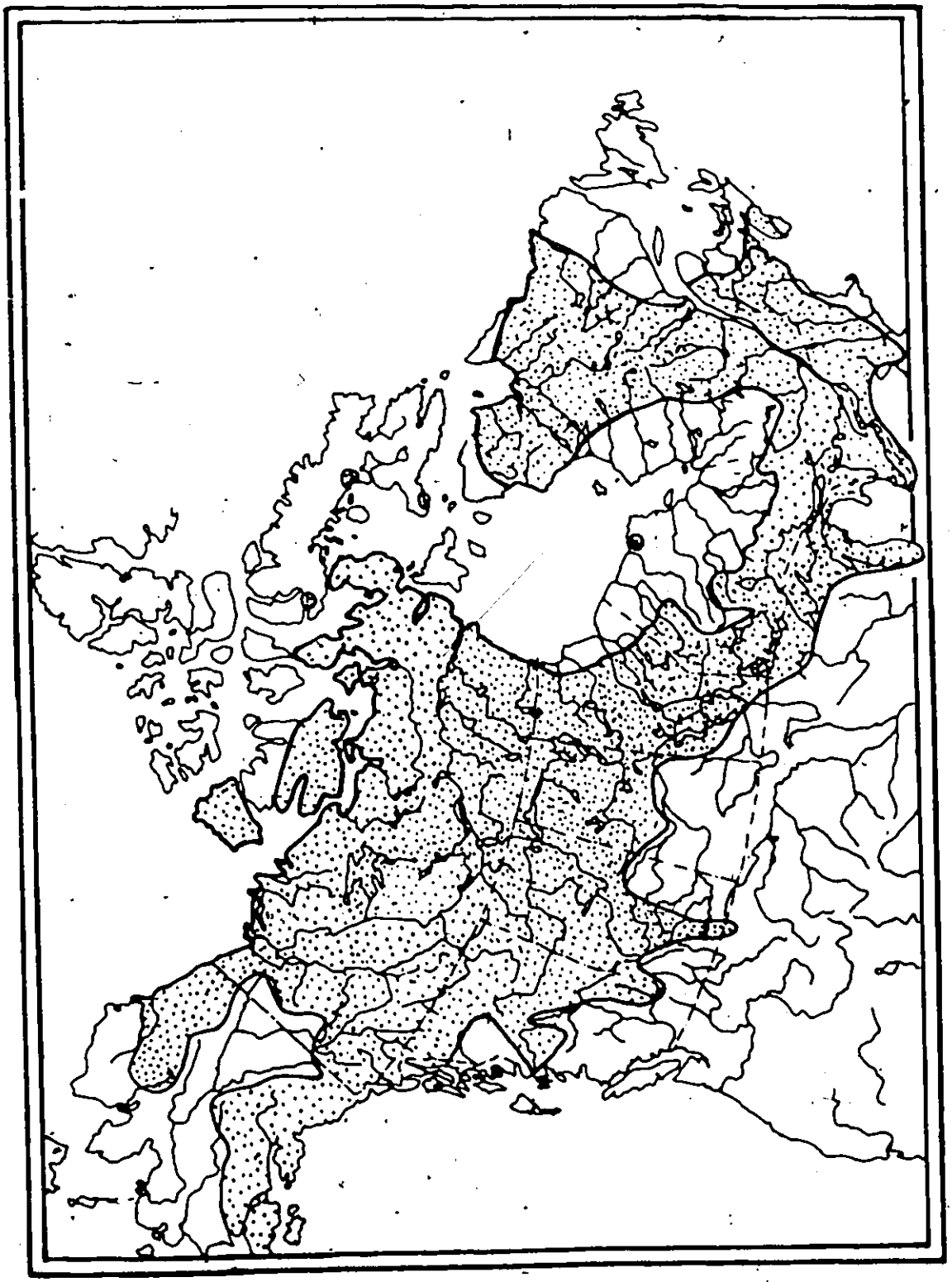


FIGURE 7. Native distribution of the lake trout in North America.
(from Scott and Crossman, 1973).

FIGURE 8. Native distribution of the brook trout in North America. (from Scott and Crossman, 1973)

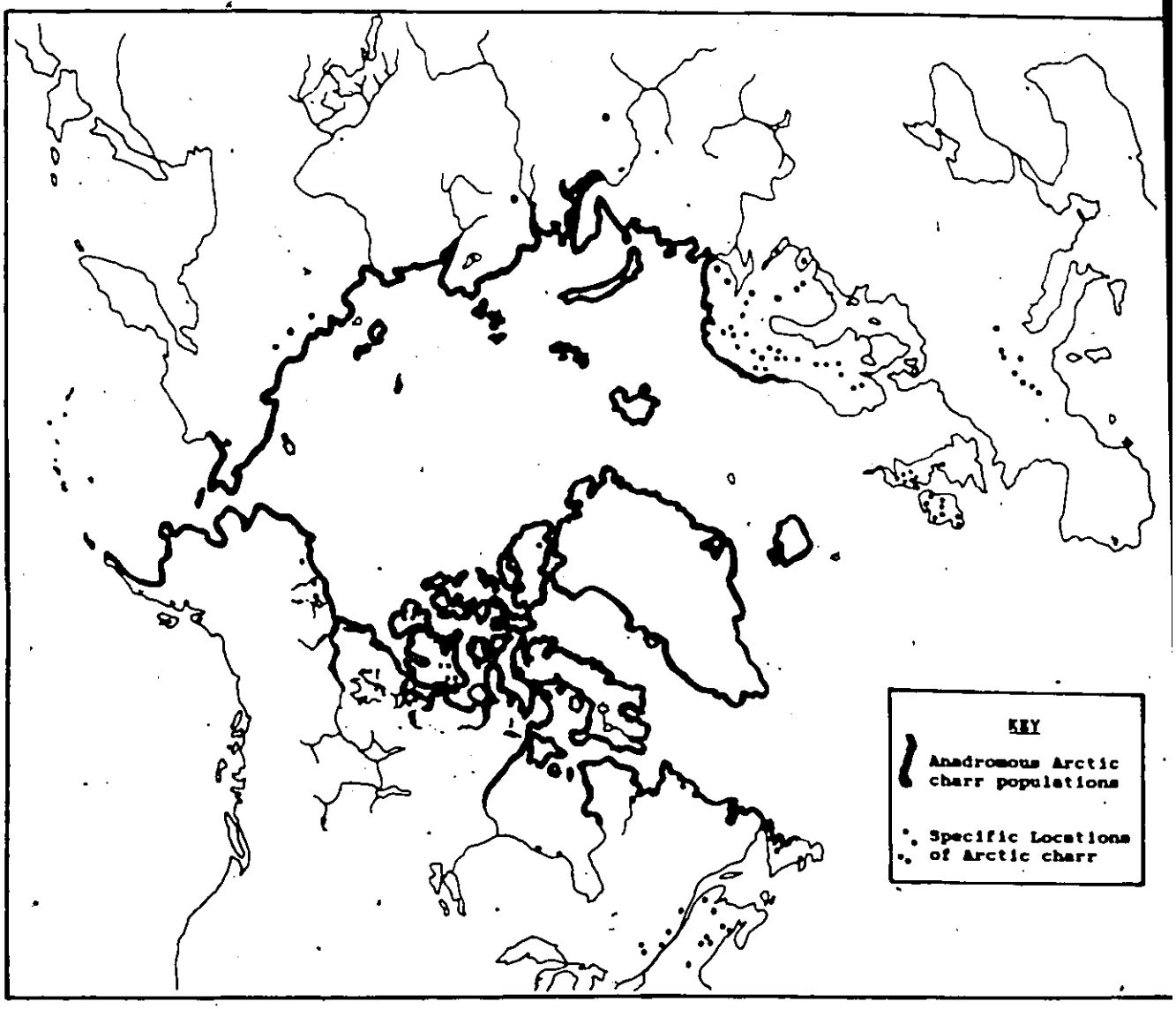


FIGURE 9. Distribution of Arctic charr. The absence of marking in the Pacific Basin does not necessarily indicate that S. alpinus does not occur there, but its exact distribution must be determined by the appropriate investigations. (from Johnson, 1980)

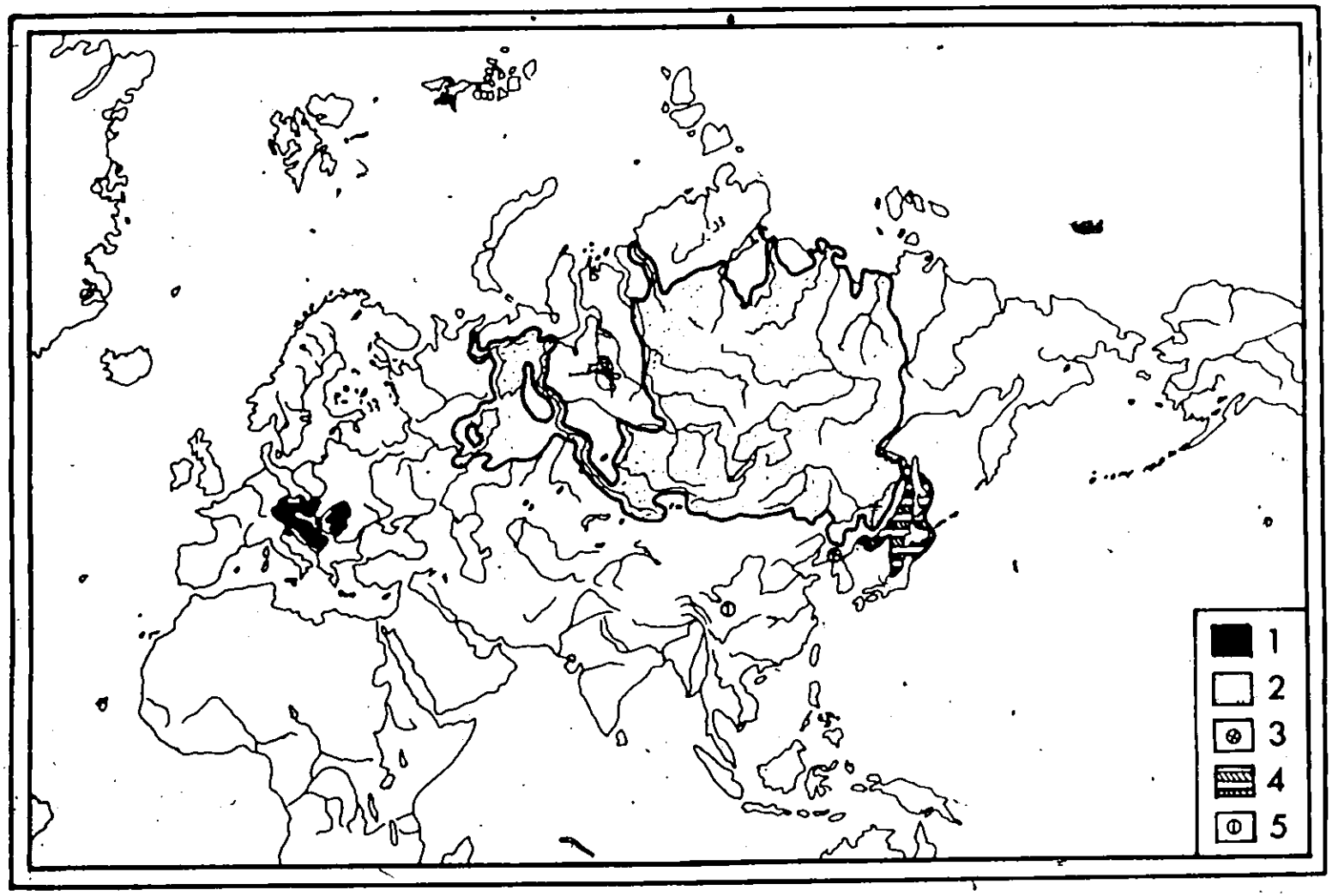


FIGURE 10. Distribution of particular species of Hucho. 1. Hucho hucho hucho; 2.Hucho hucho taimen; 3. Hucho ishikawai; 4. Hucho perryi; 5. Hucho bleekeri (from Holcik, 1982).

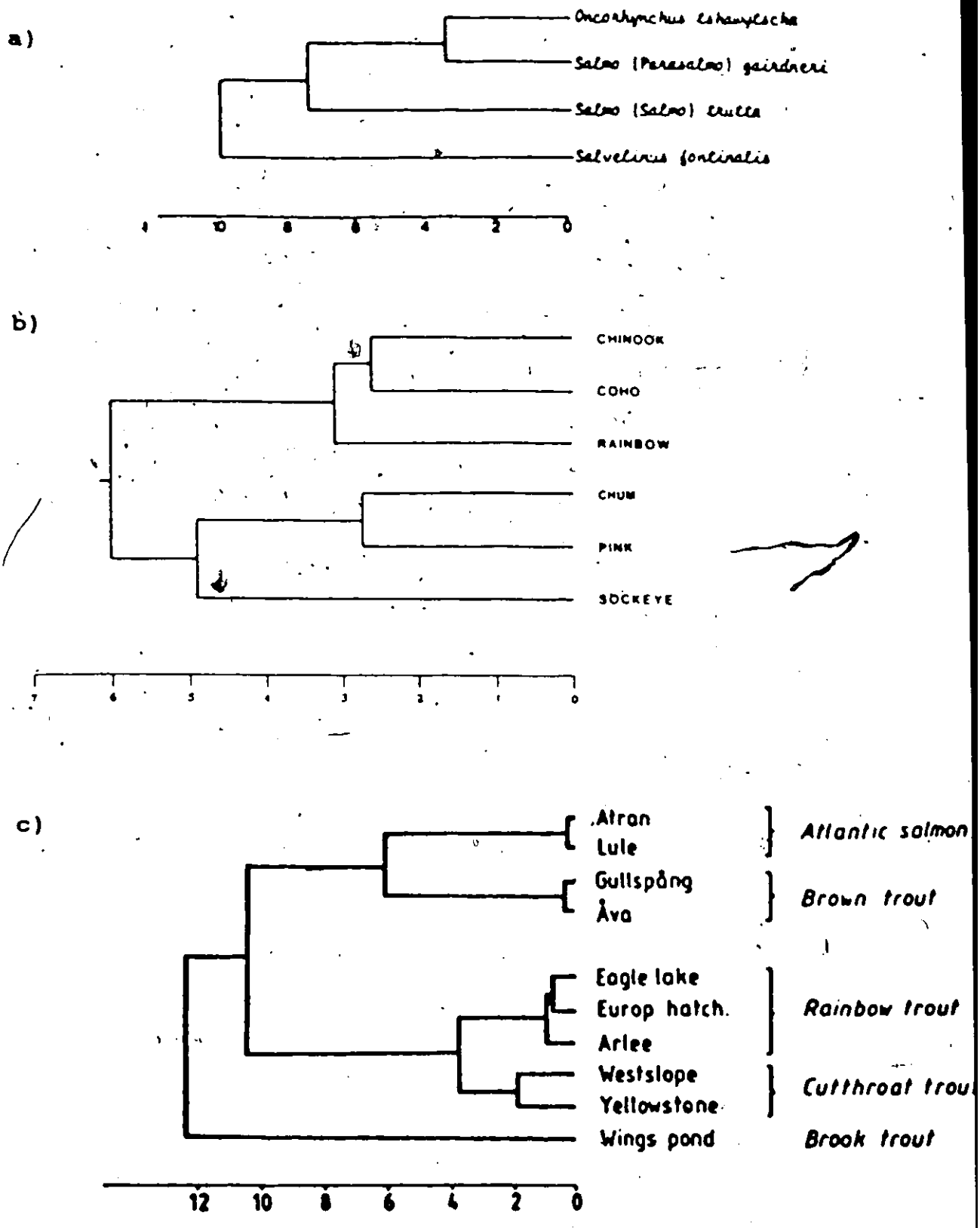


FIGURE 11. Distance phenograms from the three published salmonid phylogenies based on percentage sequence divergence of mitochondrial DNA restriction analysis:
(a) from Berg and Ferris, 1984;
(b) from Thomas et al., 1986;
(c) from Gyllensten and Wilson, 1987.
Scales are in percent sequence divergence.

NOTE: Distance values plotted as d.

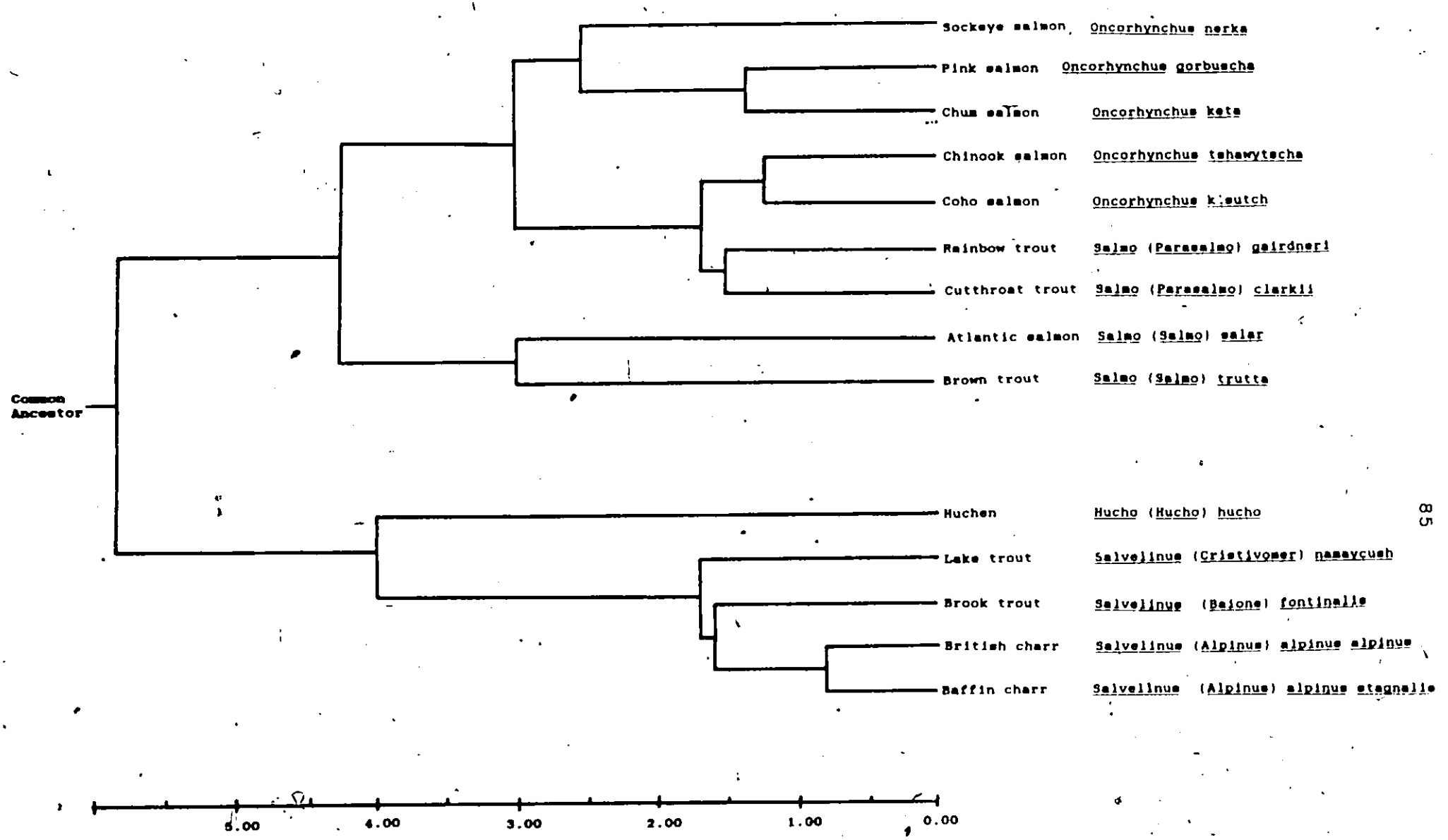


FIGURE 12. Composite distance phenogram, of the Salmoninae, based on percent sequence divergence of mitochondrial DNA.

NOTE: (distance values plotted as 1/2 d)

APPENDIX I: 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

direct visualization of the DNA). After addition of the dye, the density was adjusted to 1.57 g/mL with the addition of solid CsCl. A density gradient was established after 48-60 hours of centrifugation at 36,000 r.p.m. at 20°C in a Beckman L5-65 centrifuge using an SW60-Ti rotor. After centrifugation samples were visualized under U-V illumination. The mitochondrial DNA band appeared 5 mm. under the nuclear/nicked-circular DNA band (Lansman et al. 1981) and was removed by bottom puncture. Approximately 400-500 uL of solution were withdrawn from each tube and the propidium iodide was then removed using n-butanol (saturated with NaCl and water). Samples were susequently dialysed for 24 hours in dialysis solution I (1M NaCl, 10mM Tris-Hcl pH 8.0, 1mM EDTA pH 8.0) followed by two twelve hour washes in dialysis II (one-tenth TE). When purified in this manner, mt DNA can be kept indefinitely at -20°C until required for restriction analysis.

Restriction Analysis

Two microlitres of each sample were digested (total vol. 15 uL) with one of the restriction endonucleases (Table 2) in the appropriate buffer system according to the supplier (Bethesda Research Laboratories). Samples were digested for one and a half hours and then end-labelled with ^{32}P radio-labelled nucleotides (usually only dCTP and dATP were used) using the fill in reaction of the large (Klenow) fragment of DNA Polymerase I (Maniatis, 1982). Samples were then precipitated at -70°C for one hour by adding 300 uL of a yeast tRNA carrier mix (1 ug yeast tRNA, .3M Na-Acetate) and 1 mL of 100% ethanol. Samples were then centrifuged, in an Eppendorf centrifuge, for 20 minutes to pellet

the labelled DNA. The ethanol was then removed. This first wash effectively removed most of the unincorporated nucleotides. One millilitre of 70% ethanol (-20°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 II: STANDARD FRAGMENT LENGTHS

Lambda cut by Hind III	Lambda cut by Hind III / Ecb 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 III: RESTRICTION SITE MAPS FOR THE SALMONID MITOCHONDRIAL GENOMES EXAMINED BY THIS STUDY

a) lake trout Salvelinus namaycush

b) Baffin charr Salvelinus alpinus stagnalis

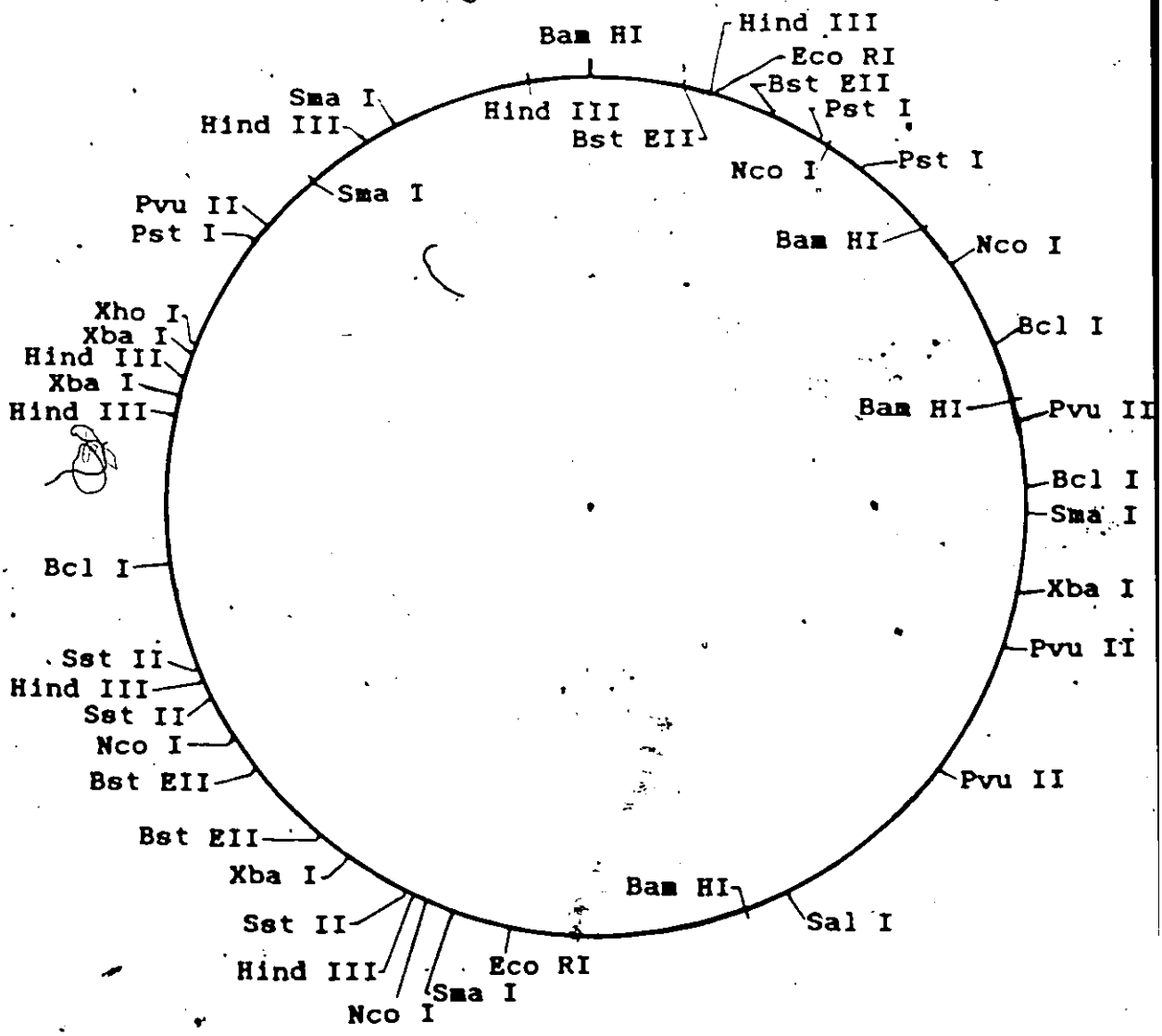
c) British charr Salvelinus alpinus alpinus

d) brook trout Salvelinus fontinalis

e) sites conserved among species of Salvelinus examined

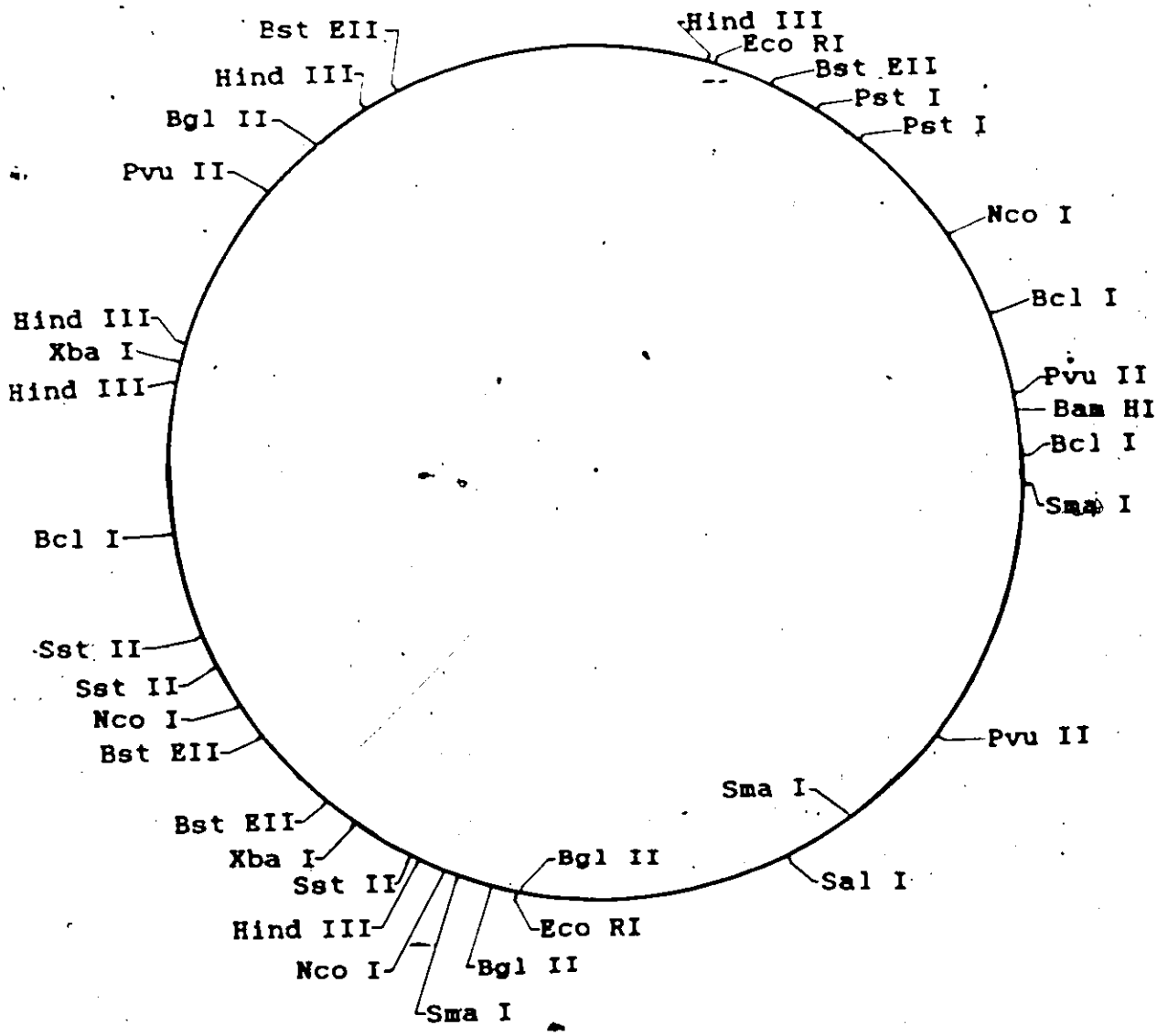
f) huchen Hucho hucho

a)

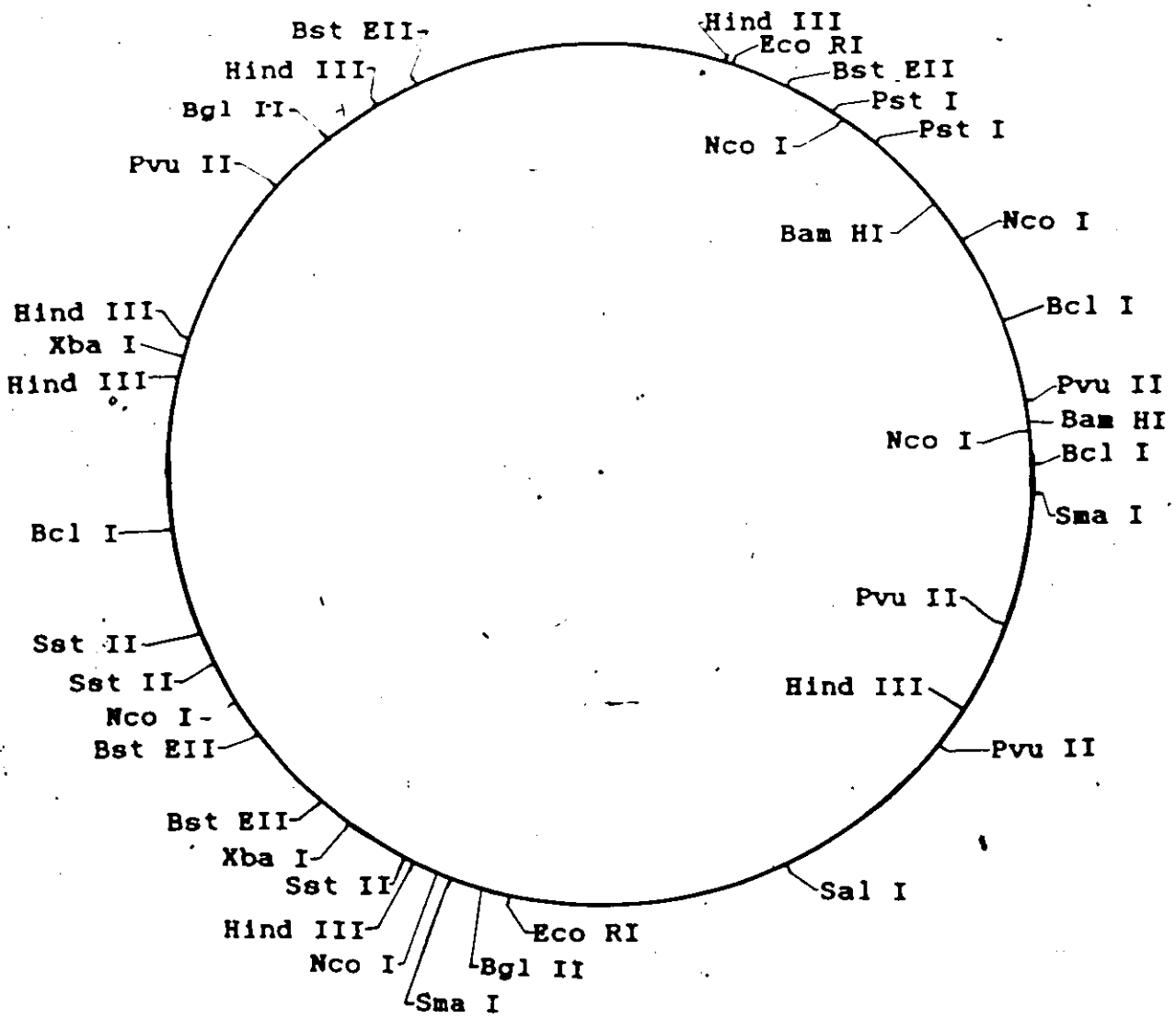

Bam HI
Hind III
Eco RI
Bst EII
Pst I
Pst I
Nco I
Bam HI
Nco I
Bcl I
Bam HI
Pvu II
Bcl I
Sma I
Xba I
Pvu II
Pvu II
Sal I
Bam HI
Eco RI
Sma I
Nco I
Hind III
Sst II
Xba I
Bst EII
Bst EII
Nco I
Sst II
Hind III
Sst II
Bcl I
Hind III
Xba I
Hind III
Xho I
Xba I
Pst I
Pvu II
Sma I
Hind III
Sma I
Bst EII

lake trout

b)

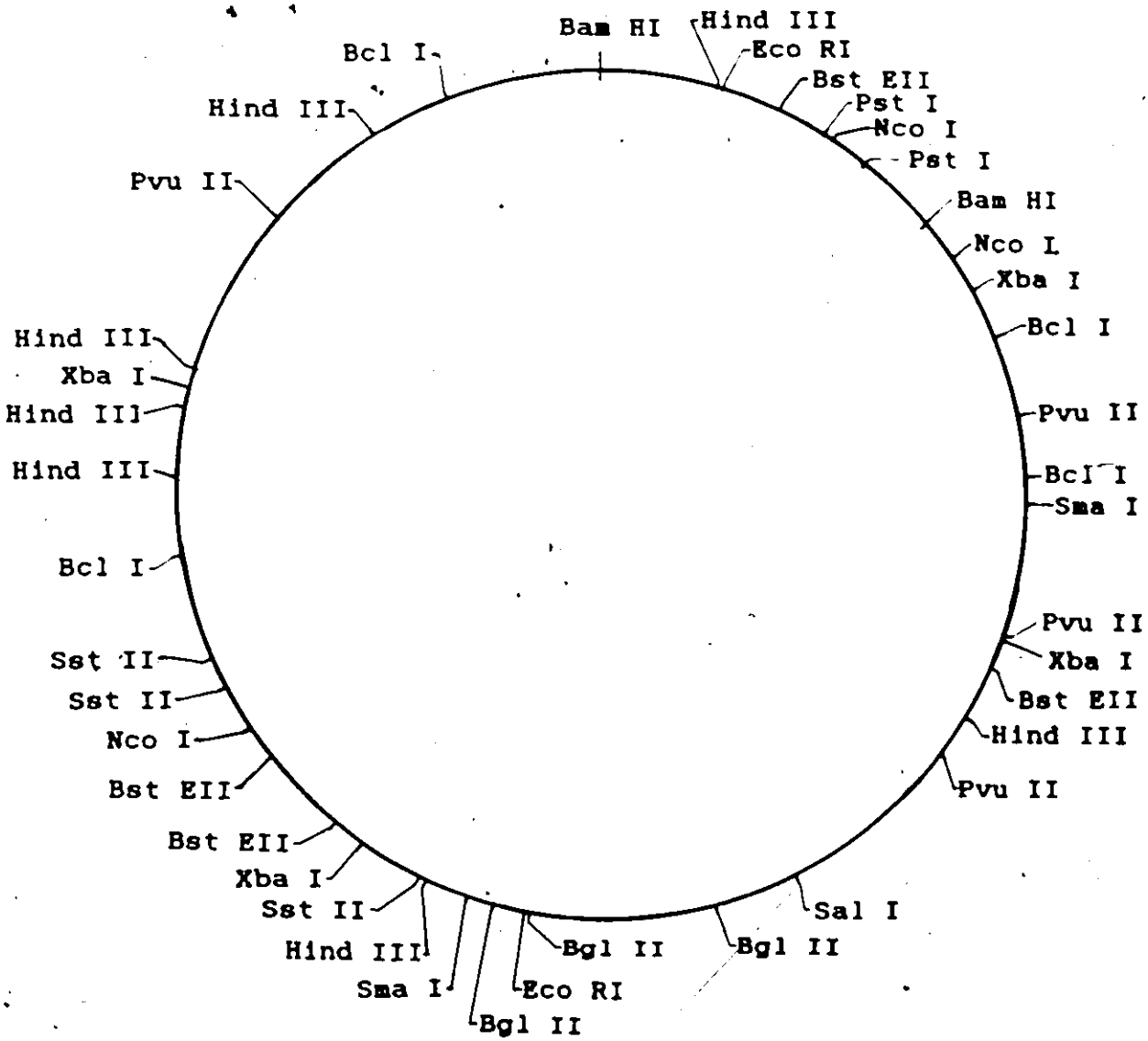


Baffin charr.



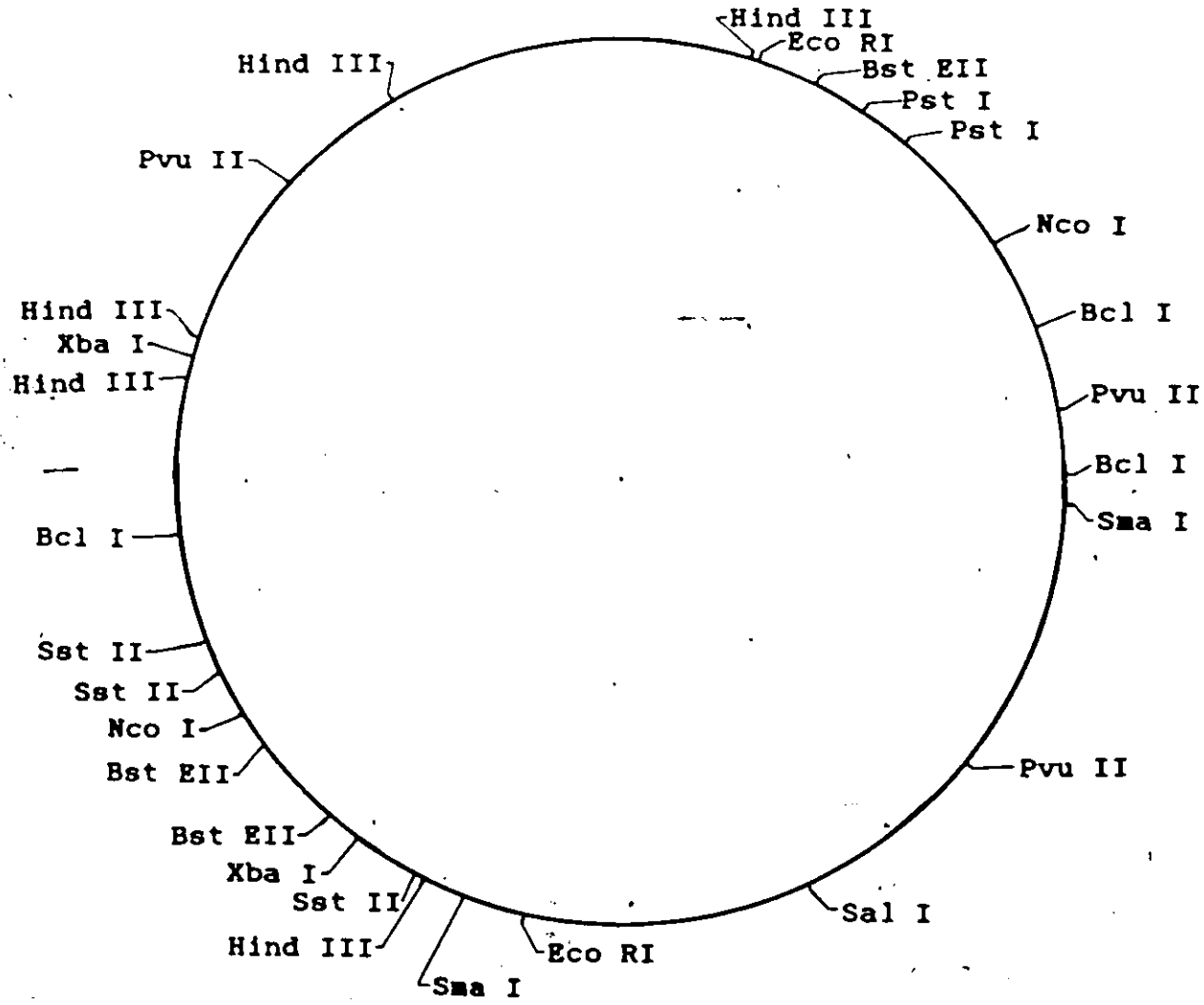
British charr

d)



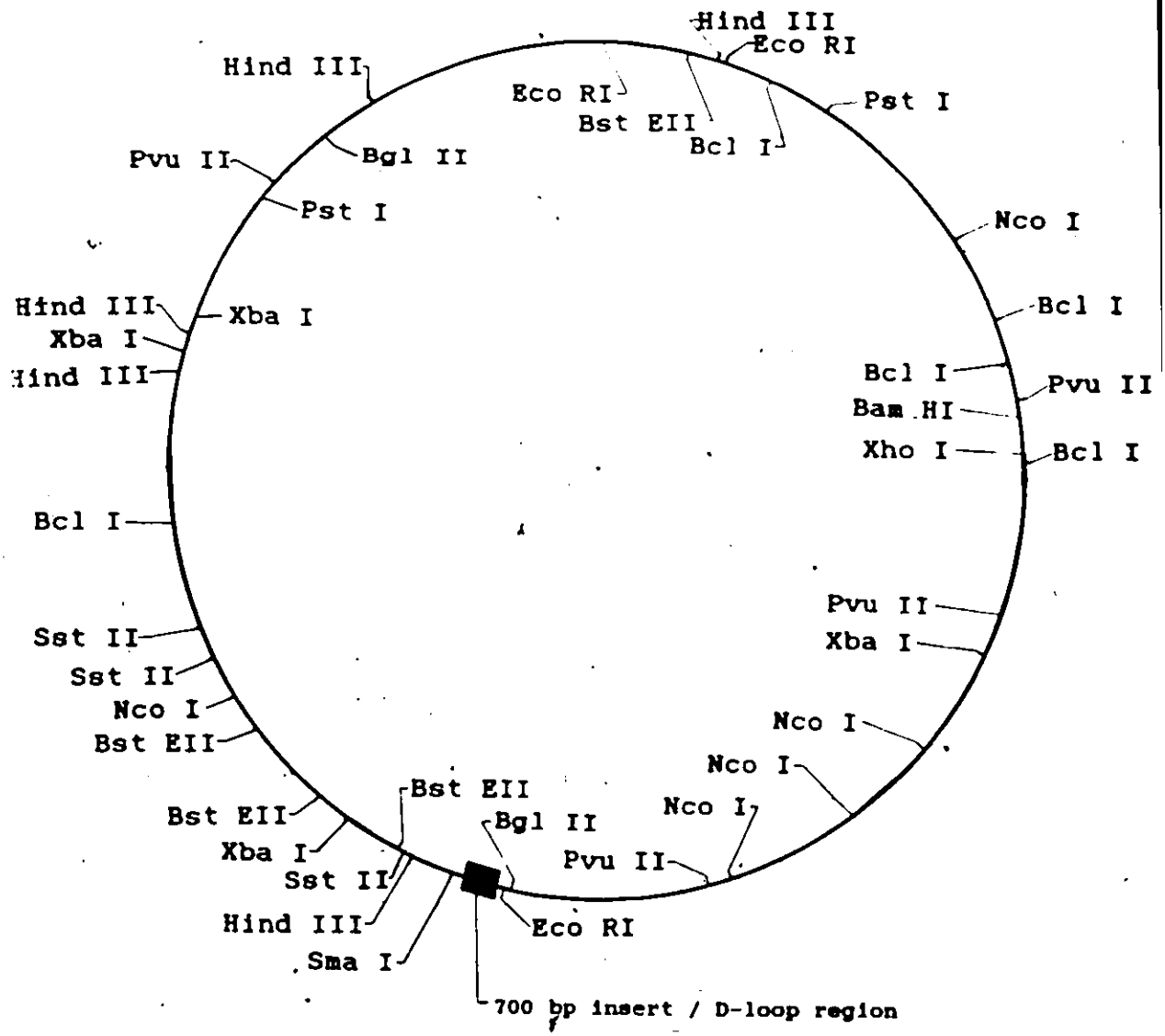
brook trout

e)



sites conserved between Salvelinus species

f.)


Hind III
Eco RI
Pst I
Nco I
Bcl I
Pvu II
Bam HI
Xho I
Bcl I
Pvu II
Xba I
Nco I
Nco I
Nco I
Bst EII
Bgl II
Pvu II
Eco RI
700 bp insert / D-loop region
Sma I
Hind III
Sst II
Xba I
Bst EII
Bst EII
Nco I
Nco I
Sst II
Sst II
Bcl I
Hind III
Xba I
Hind III
Pst I
Bgl II
Pvu II
Hind III
Eco RI
Bst EII
Bcl I

huchen

APPENDIX IV

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.

APPENDIX V. Restriction site presence/absence matrix

ENZYME		ALAKE	ARAM	ARST	AHIND	BLAKE	CLAKE	CNCO	CSMA	BAFEN	BRITT	BROOK	HYDRO
	A	0	0	0	0	0	0	0	0	0	0	0	1
	B	0	0	0	0	0	0	0	0	0	0	0	1
Bcl	C	1	1	1	1	1	1	1	1	1	1	1	1
I	D	1	1	1	1	1	1	1	1	1	1	1	1
	E	1	1	1	1	1	1	1	1	1	1	1	0
	F	0	0	0	0	0	0	0	0	0	0	1	1
	A	1	1	1	1	1	1	1	1	1	1	1	1
	B	1	1	1	1	1	1	1	1	0	1	1	1
Pvu	C	1	1	1	1	1	1	1	1	1	1	1	1
II	D	1	1	1	1	1	1	1	1	1	1	1	0
	E	0	0	0	0	0	0	0	0	0	0	0	1
Eco	A	0	0	0	0	0	0	0	0	0	0	0	1
RI	B	1	1	1	1	1	1	1	1	1	1	1	1
	C	1	1	1	1	1	1	1	1	1	1	1	1
SalI	A	1	1	1	1	1	1	1	1	1	1	1	0
	A	0	0	1	0	0	0	0	0	0	0	0	1
	B	1	1	1	1	1	1	1	1	1	1	1	0
	C	0	0	0	0	0	0	0	0	0	0	1	0
Bst	D	1	1	1	1	1	1	1	1	1	1	1	1
EII	E	1	1	1	1	1	1	1	1	1	1	1	1
	F	0	0	0	0	0	0	0	0	1	1	0	0
	G	0	0	0	0	0	0	0	0	0	0	0	1
Xho	A	1	1	1	1	1	1	1	1	1	1	0	0
I	B	0	0	0	0	0	0	0	0	0	0	0	1
Pst	A	1	1	1	1	1	1	1	1	1	1	1	1
I	B	1	1	1	1	1	1	1	1	1	1	1	0
	C	1	1	1	1	1	1	1	1	0	0	0	1
	A	1	1	1	1	1	1	1	1	1	1	1	1
	B	0	0	0	0	0	0	0	0	0	1	1	0
Hind	C	1	1	1	1	1	1	1	1	1	1	1	1
III	D	1	1	1	1	1	1	1	1	0	0	0	0
	E	0	0	0	0	0	0	0	0	0	0	1	0
	F	1	1	1	1	1	1	1	1	1	1	1	1
	G	1	1	1	1	1	1	1	1	1	1	1	1
	H	1	1	1	1	1	1	1	1	1	1	1	1
	I	0	0	0	1	0	0	0	0	0	0	0	0
	A	1	1	1	1	1	1	1	1	0	0	1	0
Bam	C	0	0	0	0	0	1	1	1	0	1	1	0
HI	D	0	1	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	1	1	1	1	0	0	0	0
	E	0	0	0	0	0	0	0	0	1	1	0	1
	A	0	0	0	0	0	0	0	0	0	0	1	0
Bgl	B	0	0	0	0	0	0	0	0	1	0	1	0
II	C	0	0	0	0	0	0	0	0	1	1	1	1
	D	0	0	0	0	0	0	0	0	1	1	0	1
	A	1	1	1	1	1	1	1	1	1	1	1	0
Sma	B	0	0	0	0	0	0	0	0	1	0	0	0
I	C	1	1	1	1	1	1	1	1	1	1	1	1
	D	1	1	1	1	1	1	1	1	0	0	0	0
	E	0	0	0	0	0	0	0	1	0	0	0	0
	A	0	0	0	0	0	0	0	0	0	0	0	1
	B	0	0	0	0	0	0	0	0	0	0	1	0
	C	1	1	1	1	1	1	1	1	0	0	0	0
Xba	D	0	0	0	0	0	0	0	0	0	0	1	0
I	E	1	1	1	1	1	1	1	1	1	1	1	1
	F	1	1	1	1	1	1	1	1	1	1	1	1
	G	1	1	1	1	1	1	1	1	0	0	0	1
	A	1	1	1	1	1	1	1	1	1	1	1	1
Sst	B	1	1	1	1	1	1	1	1	1	1	1	1
II	C	1	1	1	1	1	1	1	1	1	1	1	1
	A	1	1	1	1	1	1	0	1	0	1	1	0
	B	1	1	1	1	1	1	1	1	1	1	1	1
	C	0	0	0	0	0	0	0	0	0	1	0	0
Nco	D	0	0	0	0	0	0	0	0	0	0	0	1
I	E	0	0	0	0	0	0	0	0	0	0	0	1
	F	0	0	0	0	0	0	0	0	0	0	0	1
	G	1	1	1	1	1	1	1	1	1	1	0	0
	H	1	1	1	1	1	1	1	1	1	1	1	1

TOTAL- 69 SITES

APPENDIX VI. PAUP input file.

```
*                          FISHY.PAU

*          ALL TROUT CLONES AND SPECIES DATA FOR PAUP

* trout clones baffin brit brook HUCHO
*      ! DATA PAGE 1              !#     DATA PAGE 2     #*   DATA PAGE 3  *
* BLC PVU ECO SAL BST XHO PST HIND BAM BGL SMA XBA SST NCO

*

*        !!  !     !!    !!    !  ! !  !!!            !      !! !!!      !

PARAM NOTU=12 NCHAR=68 ROOT=OUTGROUP ;
DATA (A6,A1,68I1);
HUCHO *111100110011110100110101101101001110000010011001001000111111110011101
ALAKE  001110111100111010110011010111101101110100000000101100010111111111000011
BLAKE  001110111100111010110011010111101101110100100000101100010111111111000011
CLAKE  001110111100111010110011010111101101110101000001011000101111111111000011
BAFFN  001110101100111010111000110101001110Q000101111110000001101110100001l
BRITT  001110111100111010111000110111001110010010011101000000110111111100011
BROOK  001111111100111011110000110111011110110001110101000101110111111000001
A-BST  001110111100111110110010111110110111010000000010110001011111111000011
A-BAM  001110111100111010110010111110110111010100000010110001011111111000011
AHIND  001110111100111010110010111110110111110000000010110001011111111000011
CNCO   001110111100111010110010111110110111010100000010110001011111101000011
CSMA   001110111100111010110010111101101110110100000101110010111111111000011

UNORDERED 1-8 ;
DELETE  3,4,7,13,14,19,20,25,28,30,33,34,35,48,55,56,58,59,60,68;
INTERACT;

* CHECK TOPOLOGY #1
TOPOLOGY (1(((((2(9,10))(3(4(11,12))))8)7)(5,6))) ;
GO/ ROOT=OUTGROUP BEEP;
INTERACT;

* CHECK  TOPOLOGY #2
TOPOLOGY (1((8((2(9,10))(3(4(11,12)))))(5(6,7)))) ;
GO/ ROOT=OUTGROUP BEEP;
INTERACT;

GO/BANDB ROOT=OUTGROUP BEEP ;
END;
```

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