MITOCHONDRIAL DNA POLYMORPHISM IN AMERICAN SHAD (*Alosa sapidissima*) AND ITS IMPLICATIONS FOR POPULATION STRUCTURE

by

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ABSTRACT

Analysis of mitochondrial DNA (mtDNA) sequence variation among 244 American shad (Alosa sapidissima) from 14 rivers spanning the (Florida-Quebec) range of the species revealed several unusual features of shad mtDNA polymorphism. Two types of heteroplasmy, one involving a length polymorphism and the other a restriction site are common in shad. The length polymorphism involves a novel tandem triplication of a 1.5-kb sequence in the D-loop region. Both forms of heteroplasmy stem from multiple mutational events. The mtDNA data indicate that shad populations are reproductively discrete, and suggest that differences in the reproductive traits of northern and southern shad populations have evolved since the Pleistocene. Low mtDNA sequence variation in shad may stem at least in part from Pleistocene population reductions. A fossil calibration supports an mtDNA divergence rate in shad at least one order of magnitude slower than the prevailing estimate for vertebrates.

RESUME

La variabilité des séquences de nucléotides de l'ADN mitochondriale (ADNmt) a été analysé pour 244 spécimens de l'alose savoureuse (Alosa sapidissima) provenent de 14 rivières qui parcour le domaine geographique de l'espèce (de la Floride au Québec). Plusiers caractéristiques remarquable de l'ADNmt chez l'alose ont été revelé au niveau du polymorphisme. Deux types de hétéroplasmie se trouvent frequemment chez l'alose: l'un caractèrisé par un polymorphisme de longeur et l'autre par un polymorphisme de site de restriction. Le polymorphisme de longeur est une triplication en tandem notée pour la première fois pour une sequence de 1.5 millier de paires de bases dans la région de la "D-loop". Les deux types d'hétéroplasmie proviennent de plusiers mutations. Les données indiquent ques les populations reproductives d'alose sont isolées et suggèrent que les traits reproductifs qui distinguent les populations du nord de celles du sud ont évolué depuis l'ère de la pléistocène. Le peu de variabilité des séquences nucléotidiques de l'ADNmt de l'alose peut être attribué, en partie, à la réduction des populations durant la pléistocène. L'évidence paléontologique est en accord avec un taux de divergence de l'ADNmt d'au moins dix fois plus lent que celui qui est présentement accepté pour les animaux vertébrés.

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PREFACE

Remarks on Style and Authorship

As required by the Guidelines Concerning Thesis Preparation, the following article is quoted:

"The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text (see below), of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interests of cohesion.

It is acceptable for theses to include as chapters authentic copies of papers already published, provided these are duplicated clearly on regulation thesis stationery and bound as an integral part of the thesis. Photographs or other materials which do not duplicate well must be included in their original form. In such instances, connecting texts are mandatory and supplementary material is almost always necessary.

The inclusion of manuscripts co-authored by the candidate and others is acceptable but the candidate is required to make an explicit statement on who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims, e.g. before the Oral Committee. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear. Candidates following this option must inform the Department before it submits the thesis for review."

A general introduction and conclusion have been added in accord with the guidelines, and figures and tables have been inserted into the text to improve readability. Chapter 1 has been published (Bentzen, P., W.C. Leggett and G.G. Brown. 1988. Length and restriction site heteroplasmy in the mitochondrial DNA of American shad (Alosa

sapidissima). Genetics 118:509-518). Chapter 2 has been submitted to the Canadian Journal of Fisheries and Aquatic Sciences (Bentzen, P., G.G. Brown and W.C. Leggett. Mitochondrial DNA polymorphism and its implication for the interpretation of population structure and life history variation). Chapters 3 and 4 will be submitted to scientific journals with myself as senior author and W.C. Leggett and G.G. Brown as co-authors.

My two co-supervisors, who are the co-authors of the above manuscripts, acted in an advisory and editorial context and provided the logistic support necessary for me to conduct the work. The data, analyses and ideas presented in these manuscripts are my own.

In addition to several seminars presented to the Department of Biology, McGill University, many of the results contained in this thesis have been presented at the scientific meetings and professional seminars listed below:

Bentzen, P. 1988. Mitochondrial DNA polymorphism in American shad (Alosa sapidissima). Dept. of Biology, Dalhousie University, Halifax, N.S., Aug. 1988. (invited seminar).

Bentzen, P. 1988. Mitochondrial DNA polymorphism in American shad and its implications for population structure. West Vancouver Lab., Dept. of Fisheries and Oceans, June 1988. (seminar)

Bentzen, P. 1988. Geographic variation in the frequencies of mitochondrial genotypes of American shad. Society for the Study of Evolution, Ann. Meeting, June 1988, Asilomar CA. (contributed paper)

Bentzen, P., and W.C. Leggett. 1988. The application of mitochondrial DNA analysis to stock discrimination. Canadian Conference For Fisheries Research, Jan. 1988. (invited presentation; publ. abstr.)

Bentzen, P., and G.G. Brown. 1987. Biogeographic and molecular aspects of heteroplasmy in shad mitochondrial DNA. Genetics Society of America, Ann. Meeting, June 1987, San Francisco, CA. (contributed paper)

Bentzen, P. 1987. Biogeographic and molecular aspects of heteroplasmy in shad mitochondrial DNA. Canadian Society of Zoologists, Ann. Meeting, May 1987, Montreal, Que. (contributed paper)

Bentzen, P. 1987. The application of mitochondrial DNA analysis to biogeographic and stock discrimination studies. Bedford Institute of Oceanography, Dartmouth, N.S., March 1987. (invited seminar)

In addition to the above, the results of a collaboration between D.A. Roff and myself (Roff, D.A. and P. Bentzen. The statistical analysis of mitochondrial DNA data: chi-square and the problem of small

samples.) has been submitted to the scholarly journal Molecular Biology and Evolution, but has not been included in this thesis.

Contributions to Original Knowledge

This thesis makes the following original contributions to knowledge:

1) The molecular basis of a major mtDNA length polymorphism is characterized. This is the second major length polymorphism to be extensively characterized in vertebrates, and the first in fishes. The tandem triplication of a substantial (1.5 kb) DNA sequence that is described is the only such polymorphism reported for vertebrate mtDNA. (chapter 1)

2) The existence of widespread length and restriction site heteroplasmy in American shad mtDNA is documented. This is the first evidence of widespread restriction site heteroplasmy in populations. It is the first characterization of frequencies of heteroplasmy over the entire range of a species. (chapter 1)

3) Evidence that both forms of heteroplasmy in shad mtDNA stem from multiple origins is presented. This is first evidence of multiple mutational origins for individual types of heteroplasmy within a species. This point, taken in combination with the non-random geographic distribution of the major length polymorphism in shad mtDNA provides the first evidence of selection acting on a mitochondrial polymorphism. (chapter 1)

4) The distributions of shad mtDNA genotypes are characterized over the entire geographic spawning range of the species. These data provide the first evidence that American shad are genetically differentiated on at least a regional basis, and thus provide the first corroborating evidence for the hypothesis that latitudinal variation in the reproductive traits of shad is genetically mediated. These data also provide the first indication that temporal effects (timing of spawning migration) can be an important determinant of gene flow in shad. (chapter 2)

5) The low magnitude of intraspecific mtDNA sequence variation in shad is documented. It is suggested that this low level of genetic variation in shad, and similarly low levels of mtDNA variation in other temperate diadromous fishes, may stem from population reductions during the Pleistocene. (chapter 2)

6) Evidence that two European species of shad, A. fallax and A. alosa, have exchanged mtDNAs is presented. This is the first evidence of reciprocal transfer of mtDNA between two species. (chapter 3)

7) Evidence that the mean rate of divergence of the mitochondrial genomes of two subgenera of shad has been 0.15-0.2% per million years is presented. This is the first evidence that the rate of mtDNA divergence in vertebrates can be substantially lower than a rate of 2% per million years estimated in other vertebrate groups. (chapter 4)

8) A phylogeny of five species of shad based on their mitochondrial genetic relationships is presented. This is the first biochemical analysis of the evolutionary relationships of this group of fishes. (chapter 4)

ACKNOWLEDGEMENTS

I am grateful, first of all, for help from the following people and organizations in obtaining shad: M. J. Dadswell, P. Dumont, G. Judy, J. G. Loesch, A. J. Lupine, Y. Mailhot, J. W. McCord, G. G. Melvin, J. Provost, the South Carolina Wildlife and Marine Resources Department, and the Department of Fisheries and Oceans, Canada (Moncton). I am especially grateful for the kind assistance of two people whom I have never met, M. W. Aprahamian and A. Belaud, in obtaining samples of European shad. I also thank E. DeBlois for translating the abstract and D. Denti and P. Bentzen Sr. for technical assistance at various stages of this project.

Financial support for this study came from an NSERC operating grant to W. C. Leggett and an FCAR grant to G. G. Brown. During my Ph.D. research I received financial support from an FCAR (Quebec government) fellowship, Rigler and McConnell fellowships (McGill), GIROQ, and an NSERC operating grant to W. C. Leggett.

I am grateful to my co-supervisors, Dr. W. C. Leggett and Dr. G. G. Brown, for the years of stimulating interactions, and especially, for giving me the opportunity to do this sort of research in the first place.

Finally, I want to thank my parents for all sorts of support, and for failing to voice impatience during what has been a rather prolonged tour through student life, and also Daniela, whose frequently voiced impatience probably encouraged me to actually finish this thesis.

GENERAL INTRODUCTION

Two principal themes form the basis of this thesis. One encompasses the characterization of mitochondrial DNA (mtDNA) polymorphism in a clupeid fish, the American shad (Alosa sapidissima) and some of its congeneric relatives. This theme is present in every chapter of the thesis, and progresses in hierarchical fashion through molecular, organismal, population and interspecific attributes of mtDNA variation. The second theme concerns the implications of mtDNA polymorphism for the population biology and evolutionary genetics of American shad, and is dealt with primarily in chapter 2. This second theme formed the original motivation of this project, but assumed a secondary role when my early analyses of shad mtDNA began to suggest that the then current paradigm for mtDNA polymorphism and evolution was flawed and in need of further evaluation. Below I describe some of the historical and conceptual antecedants of this study.

Why mtDNA analysis?

The study of mtDNA polymorphism as an approach to evolutionary genetics began only a decade ago when studies began to reveal high levels of intraspecific mtDNA sequence variation in several species (Upholt and Dawid 1977, Avise et al. 1979a,b, Brown and Simpson 1981). Since then research in this field has grown at an exponential rate, fueled both by methodological advances and a widening appreciation of the unique attributes of mtDNA.

The primary methodological development that has facilitated the use of mtDNA analysis in evolutionary genetics has been the discovery and

subsequent commercial distribution of a wide range of type II restriction endonucleases. These enzymes have, for the first time, made feasible the study of population genetics at the level of DNA sequence variation.

More profound reasons for the burgeoning interest in mtDNA concern its transmission genetics and rate of evolution. Metazoan mtDNA is maternally inherited and apparently does not undergo recombination (Avise and Lansman 1983, Brown 1983, Wilson et al. 1985a); hence, it is transmitted in essentially clonal fashion through maternal lineages. In many animal groups mtDNA appears to diverge at a rate of approximately 2% per million years, a rate 5-10 times the mean rate for single copy nuclear DNA (Brown et al. 1979,1982, Brown 1983, Wilson et al. 1985a). The clonal transmission and rapid evolution of mtDNA make this molecule an unequalled marker of close phylogenetic relationships. In mtDNA analyses, local populations often emerge as distinct branches of intraspecific phylogenetic trees (Avise 1986, Avise et al. 1987a).

Another important, but less widely appreciated, attribute of mtDNA concerns the way its population genetics are expected to differ from those of nuclear genes. Although numerically polyploid in the extreme (it occurs in hundreds to thousands of copies per cell) mtDNA is functionally haploid since most individuals are homoplasmic, that is, they have only a single detectable form of the molecule (Avise and Lansman 1983, Brown 1983). Assuming an equal sex ratio and equal dispersal of sexes, the haploidy and maternal inheritance of mtDNA lead to the prediction that rates of mitochondrial gene flow among populations should be one quarter those of nuclear gene flow (Birky et

al. 1983). As a consequence, populations can remain distinguishable in terms of their mitochondrial genes at levels of mixing sufficient to eliminate differences in their nuclear genes (DeSalle et al. 1987b). Hence, mtDNA analysis is likely to be a more sensitive means of probing population structures than techniques, such as allozyme electrophoresis, that examine nuclear genes.

Why American shad?

At the time this project was initiated, nearly all available data on intraspecific mtDNA polymorphism concerned either mammals or Drosophila. Studies of population-level mtDNA variation were confined to rodents (Avise et al. 1979a,b,1983, Lansman et al. 1983, Ferris et al. 1983a,b). One goal of my doctoral research, then, was to extend knowledge of mtDNA polymorphism to non-mammalian vertebrates with population structures different from those of rodents. Clearly, any number of poikilothermic vertebrates could have served this basic objective well. There were, however, a number of reasons to choose American shad.

The American shad is an anadromous fish that spawns in the spring in rivers ranging from Florida to southern Quebec. The juveniles remain in their natal rivers until autumn, then go to sea for at least four years before returning to their natal rivers to spawn (Mansueti and Kolb 1953). In the sea shad from widely separate rivers form mixed aggregations during lengthy seasonal migrations (Dadswell et al. 1987, Melvin et al. 1988).

Over their wide latitudinal range, shad exhibit a striking pattern of variation in reproductive traits (Leggett and Carscadden 1978). In

rivers south of Cape Hatteras, N.C., shad spawn once, then die; north of this point a proportion of shad survive their initial bout of spawning and return to spawn in successive years. This proportion increases with latitude to a maximum of 80% in eastern Canada. In contrast, the relative fecundity of shad exhibits a reciprocal trend, decreasing with latitude from a maximum in the southern part of its range (chapter 2, Figure 8). At their extremes, these intraspecific differences in post-spawning survival and energy allocation to gametes are of a magnitude equal to differences that characterize different genera of salmon (Leggett 1985).

These trends in reproductive traits had been assessed in the context of life history theory (Stearns 1976, 1977, 1980) and interpreted as adaptive (i.e. genetically based) (Leggett and Carscadden 1978, Glebe and Leggett 1981a,b). A problem with this interpretation, however, was the lack of corroborating evidence. If the life history variation in shad really is genetically based, then shad ascending particular rivers (or at least rivers in particular geographic areas) should constitute genetically discrete assemblages. At least two attempts to test this proposition using allozyme electrophoresis failed. Neither study uncovered sufficient genetic variation to support or reject the hypothesis that river populations of shad are genetically differentiated (Shoubridge 1978, G.D. Melvin, Ashburn and Gillis Ltd., Fredricton N.B., personal communication).

I chose to study mtDNA polymorphism in shad because the attributes of mtDNA described above offered the opportunity to answer two questions of importance to the interpretation of the life history

variation in shad: (1) Do shad form genetically discrete assemblages, either river by river, or at regional levels? (2) Are the two life history types in shad, semelparity in the south and iteroparity in the north, associated with intraspecific phylogenetic divisions?

The mtDNA paradigm

As I began to pursue the questions above, conflicts emerged between my observations of shad mtDNA and the then prevailing paradigm for mtDNA polymorphism and evolution (Avise and Lansman 1983, Brown 1983). I have already alluded to some elements of this paradigm, but below they are stated explicitly:

(1) **Individuals are homoplasmic**. Despite the extreme numerical polyploidy of mtDNA and the high frequency of polymorphisms among individuals, individuals themselves are homoplasmic, that is they bear only a single type of mtDNA.

(2) **MtDNA polymorphisms take the form of single base substitutions or very small (few bp) insertions and deletions.** Larger length variation was known in the mtDNA of Drosophila, but thought to be very rare in vertebrates.

(3) **The evolution of mtDNA is rapid.** Estimates of the rate of mtDNA divergence suggested that a rate of ca 2% per million years prevailed in vertebrates.

Structure of this thesis

In chapter 1 I describe how polymorphism in shad mtDNA differs from two elements of the paradigm above, namely that heteroplasmy (the condition in which two or more types of mtDNA occur in the same individual) and length polymorphism are common. I characterize the

molecular basis of the length polymorphism, and present evidence that the widespread heteroplasmy in shad mtDNA stems from multiple origins. In chapter 2 I characterize population-level attributes of mtDNA polymorphism in shad and explore their implications for the population structure and evolutionary genetics of shad. In chapter 3 I describe evidence of the reciprocal exchange of mtDNAs between two European species of shad. Finally, in chapter 4 I describe evidence of another departure from the paradigm above. I present data that support an estimate of mtDNA divergence rate in shad at least an order of magnitude lower than the prevailing one for vertebrates.

CHAPTER 1

LENGTH AND RESTRICTION SITE HETEROPLASMY IN THE MITOCHONDRIAL DNA OF AMERICAN SHAD (*Alosa sapidissima*)

INTRODUCTION

The mitochondrial genome of animals is compact in organization, highly variable in sequence and ranges in size from 15700 to about 23000 base pairs (bp) (Brown 1983, Kessler and Avise 1985, Moritz and Brown 1986). Numerous studies (summarized in Avise and Lansman 1983, Brown 1983, 1985, Avise 1986) based on nucleotide sequencing and restriction endonuclease analysis have led to two general conclusions regarding the variability of animal mitochondrial DNA (mtDNA): (1) Polymorphism is common among conspecifics, but individuals usually appear homoplasmic, that is, they exhibit only a single mtDNA genotype; and (2) At least among conspecifics and closely related species, most of this polymorphism takes the form of silent base substitutions or minor (few bp) insertions or deletions.

These generalizations are based primarily on data from mammalian mtDNA. Recent observations suggest that they may not be valid for at least some nonmammalian groups. Major length polymorphisms (several hundred bp or more), in some cases associated with heteroplasmy, have been reported for several species of *Drosophila* (Fauron and Wolstenholme 1980b, Reilly and Thomas 1980, Solignac et al. 1986a,b Hale and Singh 1986), *Gryllus* crickets (Harrison et al. 1985), *Cnemidophorus* lizards (Densmore et al. 1985, Moritz and Brown 1986)

Rana and Hyla frogs (Monnerot et al. 1984, Birmingham et al. 1986) and Amia calva, a fish (Birmingham et al. 1986).

In this study I report on another species, the American shad (Alosa sapidissima). I find that mtDNA variation in shad also differs markedly from the generalizations above. A. sapidissima is a widely distributed anadromous fish that spawns in rivers ranging from Florida to Quebec. The juveniles remain in their natal rivers until autumn, then undertake lengthy seasonal migrations in the sea for 4-6 yr before homing to their natal rivers to spawn (Mansueti and Kolb 1953). I have been studying mtDNA polymorphism in A. sapidissima primarily to assess the extent of genetic differentiation among river populations. I address here an unusual aspect of mtDNA variation in shad, that is the occurrence of frequent and widespread heteroplasmy. I have observed two types of heteroplasmy in samples of shad mtDNA, one involving a large length polymorphism, the other a single restriction site. Below I present data on the molecular character of the length polymorphism in shad mtDNA. I also report on the frequencies of heteroplasmy among samples collected from 14 populations spanning the range of the species. Finally, I describe restriction site data that lead to the conclusion that both forms of heteroplasmy in shad mtDNA have arisen more than once.

MATERIALS AND METHODS

Female shad were collected in 1985 and 1986 during their upstream spawning migrations in 14 rivers ranging from Florida to southern Quebec (Figure 1). Ovaries were removed from the shad at the collection sites and held on ice for 1-7 days before use. Mitochondrial DNA was isolated from 5 g of ovarian tissue (consisting almost entirely of mature or nearly mature oocytes) from each fish. The method used was a modified version of that described by Chapman and Powers (1984). Samples were homogenized in 4-5 volumes of TEK buffer (50 mM Tris, 10 mM EDTA, 1.5% KCl, pH 7.5) with a motor driven glass Teflon homogenizer. The homogenate was then transferred to a 50 ml polypropylene centrifuge tube and underlayed with a 15% sucrose-TEK solution using a long stem pasteur pipette. The homogenate was then centrifuged at 1,000 x g for 10 min. Following centrifugation a relatively sharp boundary was usually visible at the top of the sucrose-TEK layer. The upper layer was carefully drawn off the sucrose cushion and transferred to another 50 ml centrifuge tube, then centrifuged at 12,000 x g for 10 min. The supernatant was then decanted and the remaining crude mitochondrial pellet was resuspended in 10 ml of TEK, transferred to a 15 ml glass centrifuge tube, and centrifuged again at 12,000 x g for 10 min. The supernatant was poured off and the pellet was resuspended in 1.8 ml of TEK, then 0.2 ml of 10% non-idet-TEK solution was added to the sample. The sample was shaken to distribute the non-idet, then left for 5-10 min to allow complete lysis of the mitochondria. The lysate was then centrifuged at 12,000 x g for

Figure 1. Origin of shad samples. Names in parentheses, and the positions of letters on the map denote approximate locations of collection sites. A, St. John's R. (Welaka and Mayport, Florida); B, Altamaha R. (Darien, Georgia); C, Cooper R. (Bonneau Dam, South Carolina); D, Waccamaw R. (Georgetown, South Carolina); E, Pamlico R. (Washington, North Carolina); F, York R. (Virginia); G, Delaware R. (Lambertville, New Jersey); H, Hudson R. (Claverack, New York); I, Connecticut R. (Lyme, Connecticut); J, Annapolis R. (Annapolis Royal, Nova Scotia); K, St. John R. (Cambridge Narrows, New Brunswick); L, Miramichi R. (Newcastle, New Brunswick); M, St. Lawrence R. (Trois Rivieres, Quebec); N, Richelieu R. (Chambly, Quebec).


L
K
J
M
N
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A

10 min and the resulting pellet discarded. The supernatant was extracted a minimum of three times with phenol/chloroform, following which mtDNA was precipitated with the addition of two volumes of ethanol.

Mitochondrial DNA from one shad was purified by CsCl gradient centrifugation (Lansman et al. 1981). PstI fragments collectively encompassing the entire mitochondrial genome of this fish were cloned in pBR322 using techniques described by Maniatis et al. (1982). The same method was also used to clone a PstI fragment encompassing the position of a variant SalI site in two other shad.

Restriction endonuclease digestions were carried out under the conditions specified by the vendor (Bethesda Research Laboratories). Some digestions were treated with 1-2 μg of RNase A to facilitate the visualization of small (<500 bp) restriction fragments that would otherwise be obscured by RNA present in samples prepared by the Chapman and Powers (1984) protocol.

Restriction fragments were separated in 0.6 and 1% horizontal agarose gels, and visualized by ethidium bromide staining. A 1-kb DNA ladder purchased from BRL served as a weight marker. The stoichiometric relationships of some bands were evaluated by scanning 10.2 x 12.7 cm (Kodak Tri-x Pan) photographs of gels with a (Bio-Rad model 1650) densitometer and integrating absorption peaks. The intensity of each band was assumed to be directly proportional to the amount of DNA present. Corrections were made for the size of the DNA fragments in each band when evaluating the molar relationships of the different bands.

The DNA fragments in some gels were transferred to (Bio-Rad Zeta-Probe) nylon membranes using the Reed and Mann (1985) modification of the Southern (1975) technique. These were hybridized to DNA probes labeled with [^{32}P]dCTP by nick translation (Rigby et al. 1977). The hybridization probes comprised cloned PstI fragments of shad mtDNA, a 1.5-kb EcoRI fragment isolated from cloned shad mtDNA by polyacrylamide electrophoresis (Maniatis et al. 1982), and two cloned fragments of Rattus norvegicus mtDNA corresponding to known portions of the rat mitochondrial genome (Brown et al. 1986, G.G. Brown, unpublished data). Hybridizations were carried out under the conditions specified by Bio-Rad, except that for those involving the rat probes, the temperature of the final wash was dropped from 55° to 25°. The results were visualized by autoradiography.

Both whole mtDNA and cloned PstI fragments were used to map the relative positions of cleavage sites recognized by the endonucleases KpnI, ScaI, HpaI, EcoRV, PvuII, SstI, SstII, PstI, SalI and EcoRI (Figure 2). The positions of the sites were determined by the double digest method (Maniatis et al. 1982).

RESULTS

Length polymorphism and heteroplasmy

SalI restriction analysis of mtDNA samples from 244 A. sapidissima revealed two size classes of mtDNA that differed by approximately 1.5 kb. SalI cleaved the mtDNA of most shad at three locations, yielding fragments of approximately 0.47, 8.1 and 9.7 kb (Figures 2 and 3). In a

minority of shad, however, the "normal" 9.7-kb band was replaced by two clearly substoichiometric bands, one 9.7 kb as before, and another of approximately 11.2 kb (Figure 3). The relative intensities of the two substoichiometric bands varied widely among individuals.

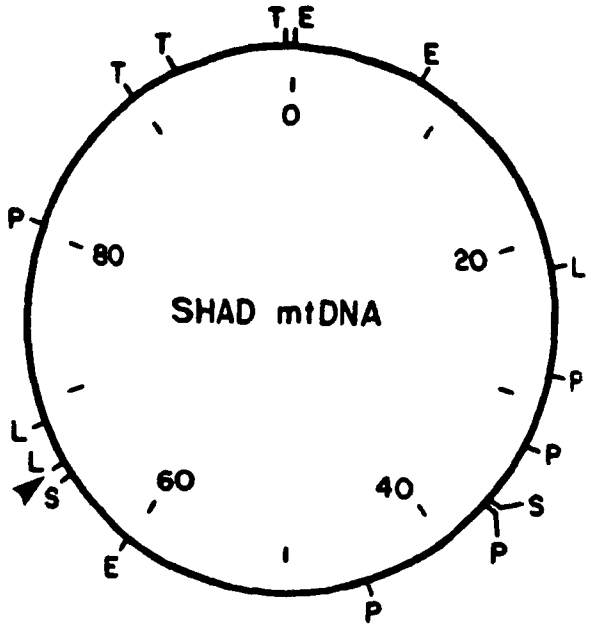
This pattern of length variation and heteroplasmy was verified with other restriction endonucleases. Concordant patterns of variation were observed when representative samples were digested with a variety of enzymes including PvuII and KpnI (Figure 3) as well as SstI, SstII and XbaI (not shown).

Only one size class of mtDNA (approximately 18.3 kb) was detected in 214 out of 244 samples of shad mtDNA digested with SalI and visualized by EtBr staining. The remaining 30 shad were heteroplasmic: these fish carried mtDNA of both 18.3 and 19.8 kb (henceforth referred to as type S and type L mtDNA, respectively). Most of the shad carrying type L mtDNA originated from rivers in the southern part of their range (Table 1). Indeed, fish bearing type L mtDNA occurred at higher frequency (55%) among the St. John's River (Florida) samples from the extreme southern end of the range of shad, than anywhere else. Only two shad (from the Annapolis River, Nova Scotia) bearing type L mtDNA originated from north of the Delaware River.

Restriction site mapping and DNA hybridization experiments revealed that types S and L mtDNA contain two and three copies, respectively, of a tandemly repeated 1.5-kb DNA sequence corresponding almost exactly to an EcoRI fragment of the same size. They also revealed that each of the tandemly repeated sequences varies by as much as 40 bp among individual shad as well as among copies of the repeat within individuals.

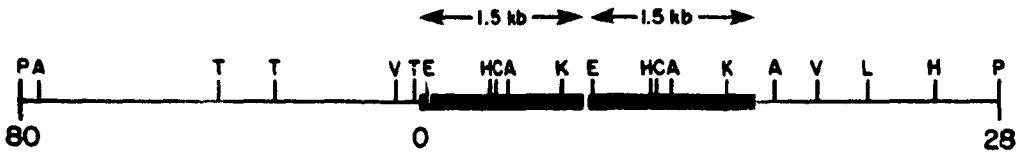
Figure 2. Restriction site map of shad mtDNA. A, ScaI; C, EcoRV; E, EcoRI; H, HpaI; K, KpnI; L, SalI; P, PstI; S, SstI; T, SstII; V, PvuII. A, Partial map of entire molecule of type S form. The arrow indicates the SalI site that is absent in Sal-B mtDNA. B, Detailed map of region (map units 80-28) containing tandem repeats (denoted by thickened segments).

A



B

TYPE "S"



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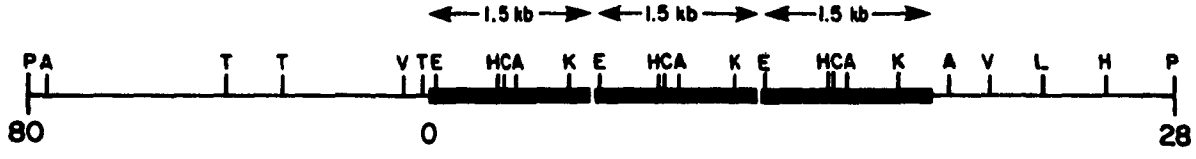


Figure 3. Length heteroplasmy and restriction site polymorphisms in shad mtDNA. Samples of shad mtDNA used in digestions are named according to river of origin. A, SalI digest on 0.6% agarose gel stained with EtBr. Individuals G1 and H1 are homoplasmic for type S mtDNA; C1 and B1 are heteroplasmic and contain both type S and type L mtDNA (visible in top band of digest). G1 and H1 are Sal-A and Sal-B genotypes, respectively. A 0.47-kb band present in the G1 digest is not visible on this gel. B, KpnI digests on same gel as A. G1 and H1 are Kpn-A and Kpn-B genotypes, respectively. B1 is Kpn-B genotype; type L mtDNA in this sample is again visible in top band on gel. C: PvuII digests on 1% gel. The type L mtDNA in C1 is visible in the 5-kb band.

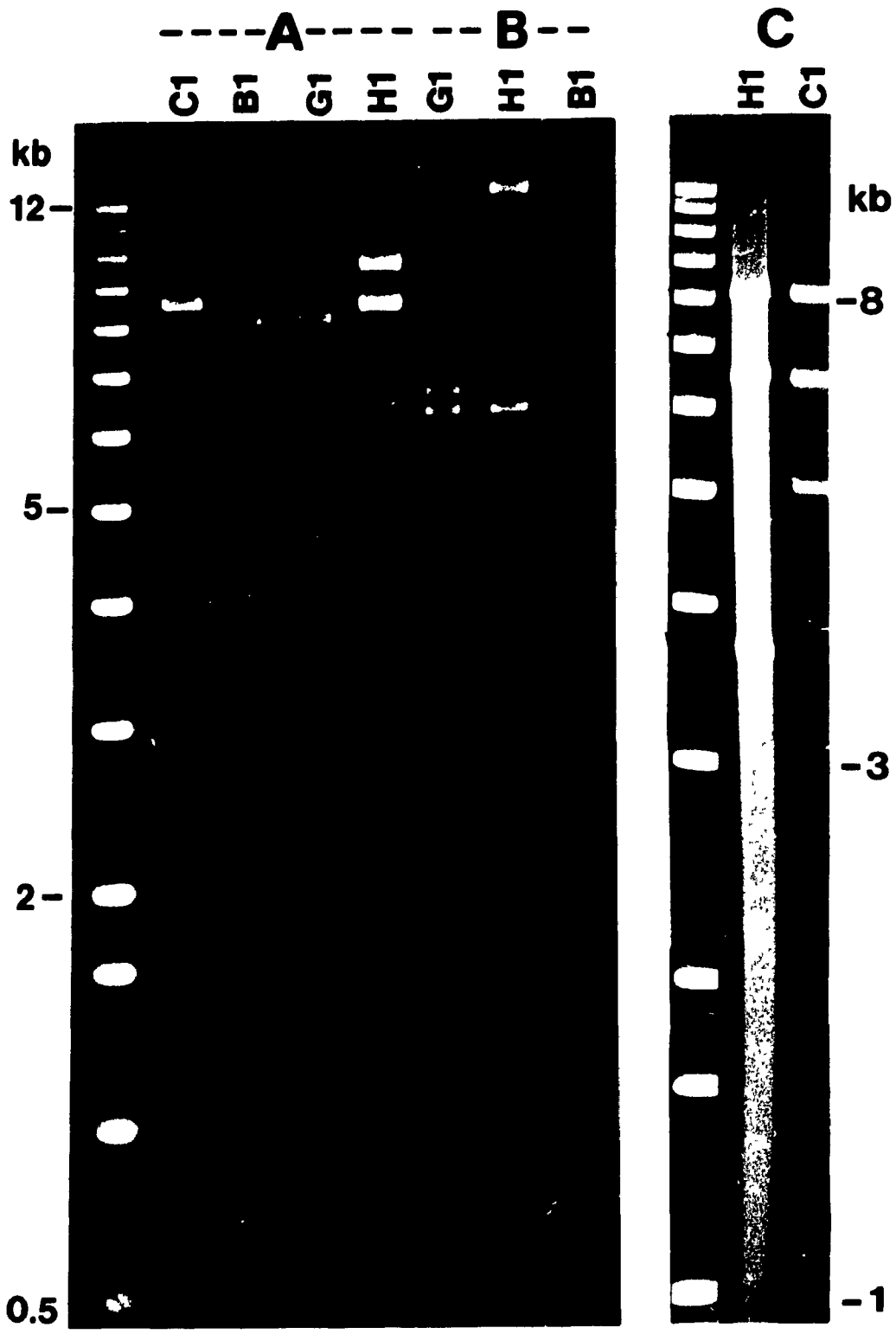

A
B
C
C1
B1
G1
H1
G1
H1
B1
H1
C1
kb
12
5
2
0.5
kb
8
3
1

TABLE 1

Geographic distributions of shad mitochondrial genotypes

River	Genotype			N
	Size	SAL	KPN	
(A) St John (Florida)	S	A	A	13
	S,L	A	A	15
	S,L	A	B	1
				29
(B) Altamaha	S	A	A	8
	S	A	B	5
	S	B	B	2
	S,L	A	B	2
	S,L	B	B	1
				18
(C) Cooper	S	A	A	8
	S	A	B	2
	S	A,B	B	2
	S,L	B	B	1
				13
(D) Waccamaw	S	A	A	11
	S	A	B	1
	S	B	B	1
	S,L	A	A	4
				17
(E) Pamlico	S	A	A	9
	S	A,B	A	1
	S	A	B	1
	S	B	B	7
	S	B	A	1
	S,L	A	A	1
				20
(F) York	S	A	A	12
	S	A	B	3
	S	A,B	B	2
	S	B	B	2
	S	A	C	1
	S,L	A	A	2
				22

River	Genotype			N
	Size	SAL	KPN	
(G) Delaware	S	A	A	11
	S	A,B	A	1
	S	A,B	B	1
	S	B	B	5
	S,L	A	A	1
				19
(H) Hudson	S	A	A	17
	S	B	B	3
	S	A,B	A	1
				21
(I) Connecticut	S	A	A	10
	S	B	B	1
				11
(J) Annapolis	S	A	A	12
	S	A	B	2
	S	B	A	1
	S	A,B	A	1
	S	A,B	B	1
	S,L	A	A	1
	S,L	A	B	1
				19
(K) St John (N B)	S	A	A	6
	S	A	B	3
	S	B	B	1
				10
(L) Miramichi	S	A	A	12
	S	A	B	2
	S	B	A	3
				17
(M) St Lawrence	S	A	A	16
	S	A	B	4
	S	B	A	1
				21
(N) Richelieu	S	A	A	7
				7

These assertions are based on the following lines of evidence: (1) Five restriction enzymes generate a single 1.5-kb fragment in type S mtDNA. The restriction sites delimiting the 1.5-kb fragments occur in a repetitive pattern in a single 3-kb stretch of DNA (Figure 2). (2) When samples containing type L mtDNA were digested with any of the five enzymes, the resulting restriction fragment patterns were similar or identical to those obtained with type S mtDNA. Either a single 1.5-kb band or else two closely spaced bands near 1.5 kb in size were observed (e.g., fish A4 and A3, respectively in Figure 4). When only a single 1.5-kb band was seen, densitometry scans revealed an excess of DNA in the band over that expected from a single fragment. (3) The 1.5-kb EcoRI fragment corresponding to the proposed repeat was isolated from cloned (type S) mtDNA and used to probe a Southern blot bearing EcoRI digests. The probe hybridized to two fragments in a sample homoplasmic for type S mtDNA, and three in samples containing type L mtDNA (fish A2 and A3, respectively, in Figure 4). (4) Similar patterns of fragment size variation were observed with several enzyme combinations, including PvuII/KpnI and PvuII/EcoRV (Figure 5) and ScaI (not shown) that divide the region containing the tandem repeats in types S and L mtDNA into two or three fragments, respectively (Figure 2).

The fact that the region encompassing the major length polymorphism maps adjacent to an SstII site suggests that it might be located in the D-loop containing region, since SstII cleaves the mtDNA of widely diverse vertebrates at two locations in the rRNA genes, close to the D-loop (Glaus et al. 1980, Brown and Simpson 1981, Moritz and Brown 1986). SstII cleaves shad mtDNA at three locations (Figure 2); two of

Figure 4. Evidence of 1.5-kb tandem repeats in shad mtDNA. A, EcoRI digests on 0.6% gel stained with EtBr. B, Autoradiograph of Southern blot of same gel probed with radioactively labeled 1.5-kb EcoRI fragment derived from cloned type S mtDNA. Individual A2 is homoplasmic for type S mtDNA; A3 and A4 are heteroplasmic and contain type L mtDNA. Due to length variation that is prevalent among copies of the repeat, the two 1.5-kb fragments formed by the digest of A3 appear as discrete bands, whereas they appear as a doublet in the digest of A4. The 1.5-kb probe has also hybridized to another copy of the repeat present in the largest fragment in each digest (see Figure 2).

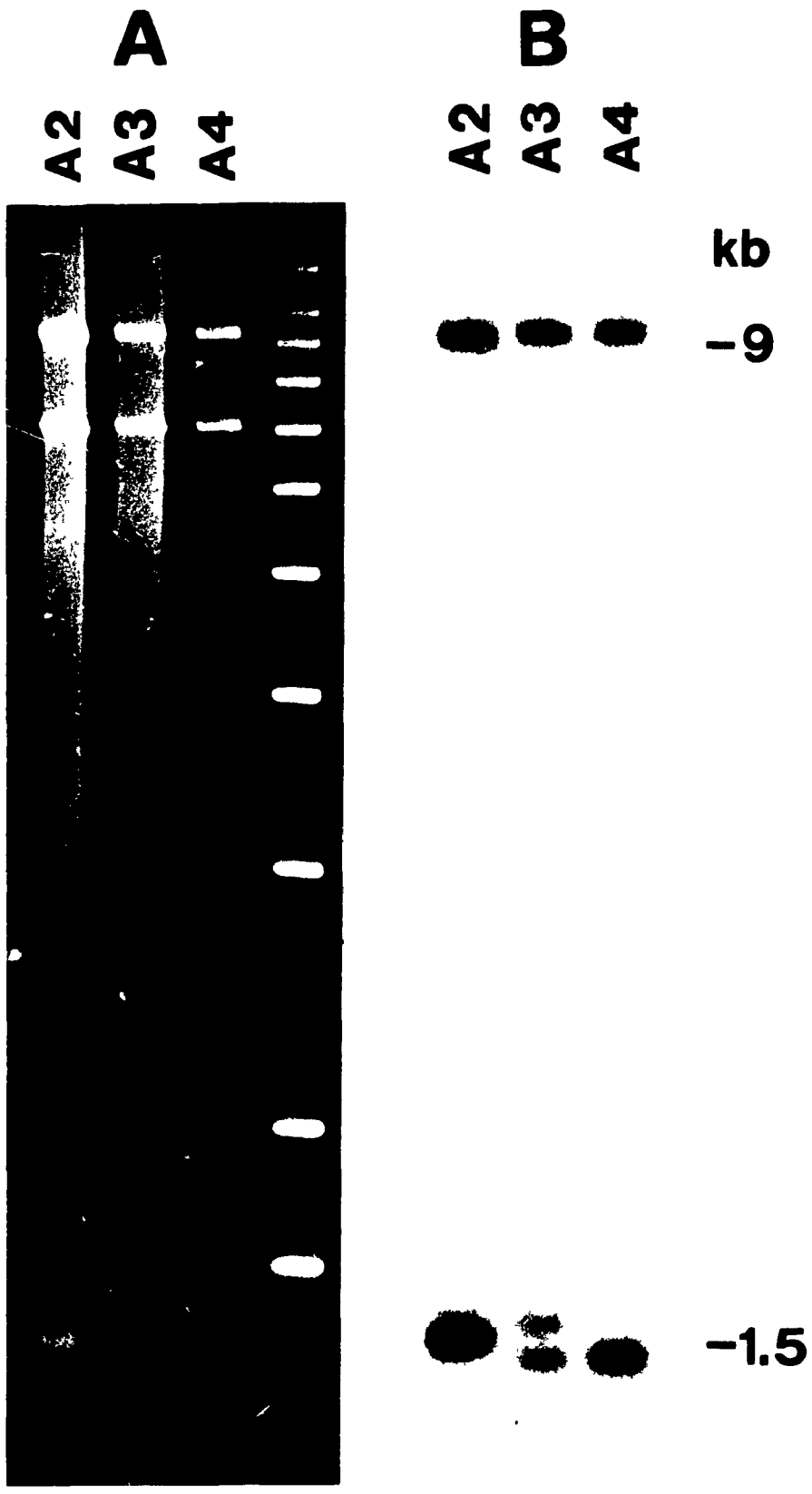
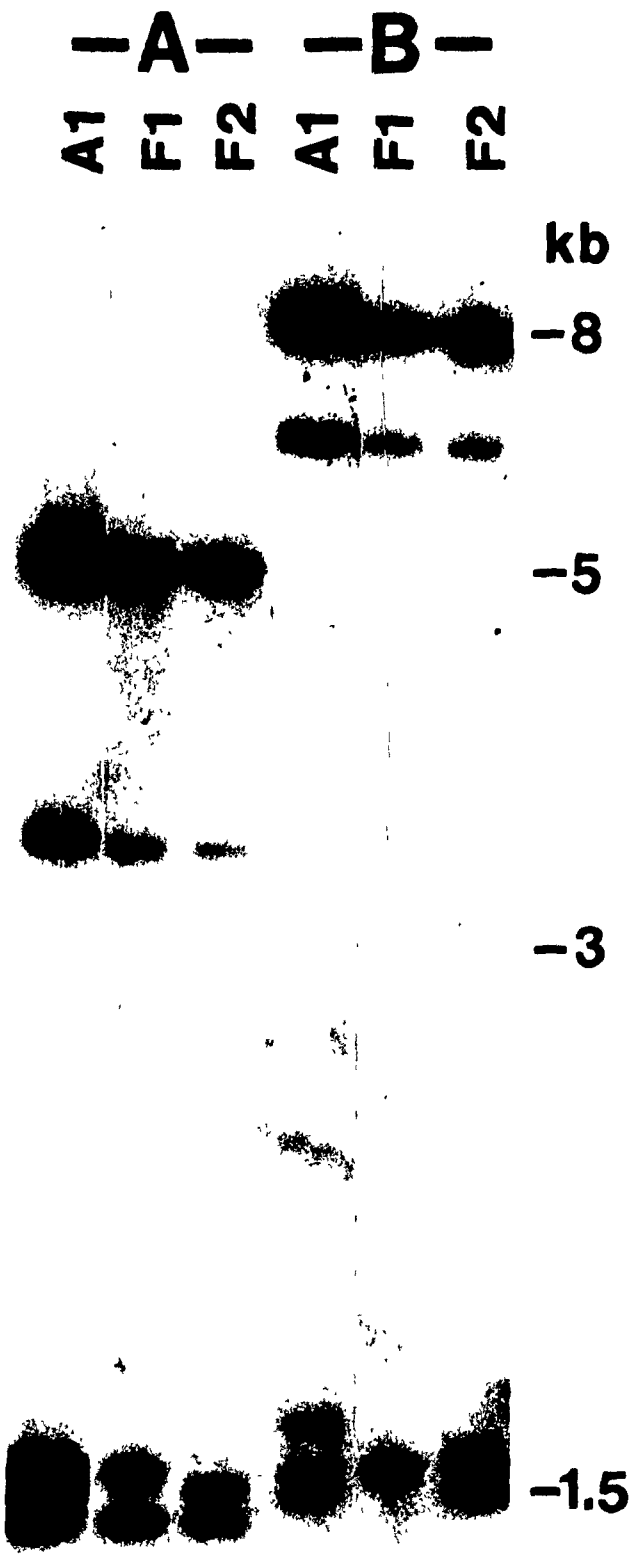

A
A2
A3
A4
B
A2
A3
A4
kb
~9
~1.5

Figure 5. Length variation among copies of the tandem repeat. Autoradiograph of Southern blot of 1% gel probed with radioactively labeled cloned PstI fragment (map units 80-28). A, PvuII/KpnI; B, PvuII/EcoRV. Individual A1 is heteroplasmic and contains type L mtDNA; F1 and F2 are homoplasmic for type S mtDNA. Each enzyme combination cleaves the region containing the tandem repeats in types S and L mtDNA into two and three segments, respectively (Figure 2). Variation in the size of homologous fragments is evident in both enzyme combinations.


A
B
A1
F1
F2
A1
F1
F2
kb
8
5
3
1.5

Two of these presumably represent the conserved rRNA sites. Two hybridization experiments were performed to test this conclusion and orient the shad mtDNA restriction map. Digests of shad mtDNA were probed with clones of rat mtDNA specific to two adjacent regions on the molecule: (1) the 16S rRNA gene and (2) the 12S rRNA gene, the D-loop containing region and part of the cytochrome b gene (Brown et al. 1986, G.G. Brown, unpublished data). The first probe hybridized strongly to a shad PstI/SstII fragment spanning map units 80-90 in Figure 2. The second probe hybridized to an SstII fragment spanning map units 93-10, as well as to an adjacent SstII/PstI fragment spanning map units 0-28. These observations indicate that the organization of shad mtDNA in the region extending from the rRNA genes into the cytochrome b gene is similar to that of mammalian mtDNA, and confirms that the major length variation occurs in the D-loop-containing region.

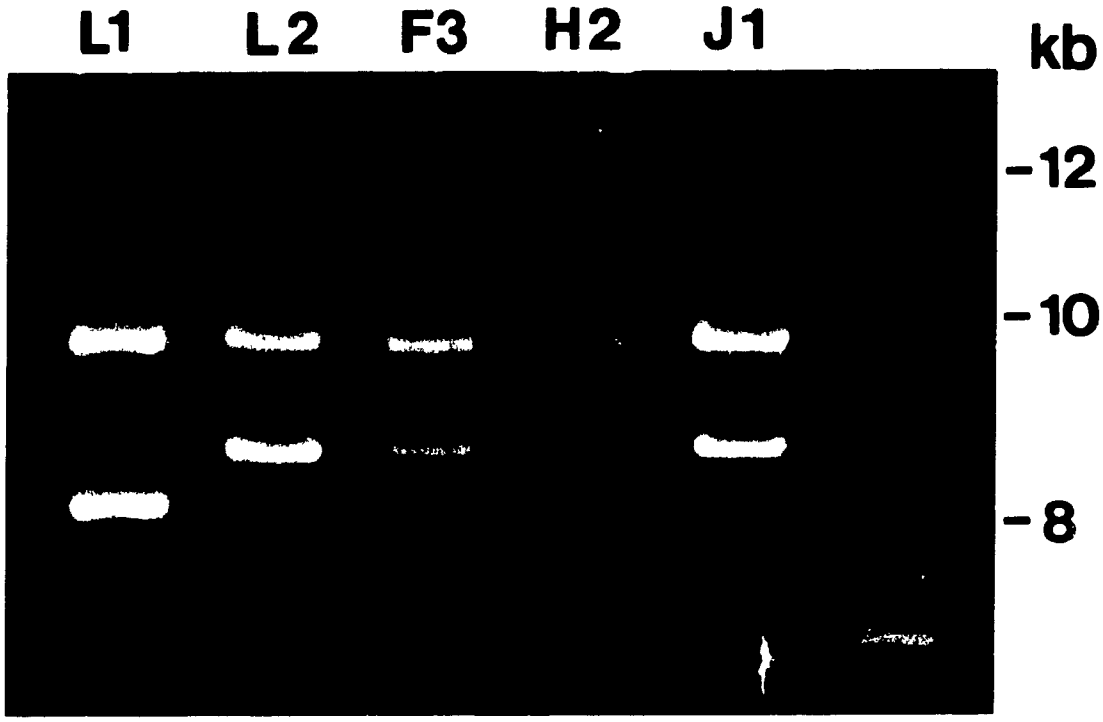
Site heteroplasmy

Although the mtDNA of most shad contained three SalI restriction sites (henceforth referred to as Sal-A mtDNA), a second mitochondrial genotype (Sal-B) was also observed. Mapping experiments indicated that Sal-B mtDNA was characterized by the loss of one SalI restriction site (Figure 2). SalI digests of Sal-B mtDNA yielded two fragments of approximately 8600 and 9700 bp (e.g., fish L2 in Figure 6). Samples that contained Sal-B mtDNA were geographically widespread in origin. They were observed in 12 of the 14 shad populations sampled, and amounted to 40 (16%) of the 244 shad assayed with SalI (Table 1).

Ten of the samples that carried Sal-B mtDNA also appeared to contain some Sal-A DNA. SalI digests of these samples yielded fragments

of both 8600 and 8100 bp in sub-stoichiometric quantities (e.g., fish F3, H2 and J1 in Figure 6). The possibility that this apparent heteroplasmy was caused by a 500-bp length polymorphism was eliminated by the observation that no sub-stoichiometric bands were seen when the same samples were digested with PstI, SstI and SstII. In addition, a Southern blot of SalI digests of these samples revealed the presence of a 470-bp fragment in substoichiometric quantities. Such electrophoretic banding patterns could have resulted from incomplete digestion of Sal-A mtDNA, but two lines of evidence countered this possibility: (1) Five of the apparently heteroplasmic samples were digested several times with increasing amounts of enzyme and increasing incubation times. Even when these samples were exposed to 50 units of SalI and incubated for sixteen hours, the resulting electrophoretic banding patterns remained unchanged from those initially observed (Figure 6). These digestion conditions represented a many-fold increase in the amount of enzyme and incubation time over that usually required to obtain complete digestion with SalI. (2) If the apparently heteroplasmic mtDNA samples were really incompletely digested Sal-A mtDNA, the 8100-bp band would be expected to be more intense than the 8600-bp band, which would then represent the residual presence of an intermediate digestion product. In fact, in all but one of the samples in question (see sample H2 in Figure 6), the 8600-bp band was more intense than the 8100-bp band. Moreover, none of the SalI digests of these samples exhibited any other evidence of incomplete digestion, such as additional restriction fragments corresponding to the sum of other fragments.

Figure 6. Restriction site heteroplasmy in shad mtDNA. Restriction fragment patterns obtained on 0.6% gel after exhaustive digestion with SalI (details in text). Individuals L1 and L2 are homoplasmic for Sal-A and Sal-B mtDNA, respectively; F3, H2 and J1 are heteroplasmic for the two genotypes. A 0.47-kb band generated by the Sal-A genotype is not visible on this gel.


L1
L2
F3
H2
J1
kb
-12
-10
-8

Finally, the possibility that the SalI site polymorphism and associated heteroplasmy might have been the product of an artifact such as postmortem methylation of the DNA was ruled out by a cloning experiment. The variant SalI site in question was observed in DNA cloned from an individual with Sal-A mtDNA, but not in corresponding DNA fragments cloned from two individuals with Sal-B mtDNA, thus indicating that the site polymorphism is the result of an alteration in the primary sequence of the DNA.

KpnI Data

Whether heteroplasmic shad are the products of persistent heteroplasmy stemming from unique mutations, or alternatively, result from recurrent mutations, can be addressed only by restriction site data that delineate separate lines of descent. For the samples heteroplasmic for type L mtDNA, this issue is partially resolved by the SalI site data, since although most samples bearing type L mtDNA were Sal-A, two were Sal-B (Table 1). Thus, the L form appears to have arisen at least twice, once in a Sal-A lineage and once in a Sal-B lineage. For the shad heteroplasmic for the SalI restriction site, however, data from at least one other informative enzyme are needed.

A preliminary survey of several shad from each population with 16 hexanucleotide restriction enzymes revealed very few polymorphisms (chapter 2). Only one enzyme, KpnI, revealed a polymorphism that was both widespread and common; hence it was used to further analyze the 244 shad mtDNA samples previously surveyed with SalI.

Three KpnI genotypes were observed. Kpn-A was characterized by four restriction sites generating fragments of approximately 1.5, 4.3, 6.1

and 6.4 kb (Figures 2 and 3). In Kpn-B both restriction sites within the tandem repeats were absent, resulting in fragments of 6.1 and 12.3 kb (Figure 3). In Kpn-C only one restriction site within the tandem repeats was missing; the remaining sites generated fragments of 7.9, 6.1 and 4.3 kb. Among the samples surveyed with KpnI, 187 were Kpn-A and 56 were Kpn-B. Only one sample from the York River, Virginia, was Kpn-C. The two common genotypes were also geographically widespread: Kpn-A was observed in all, and Kpn-B in all but one, of the 14 populations (Table 1). All four possible combinations of the SalI and KpnI genotypes were observed. Of the samples that were heteroplasmic Sal-A,B, four were were Kpn-A and six were Kpn-B. Samples heteroplasmic for type L mtDNA were divided among three composite genotypes. The majority (24) were Sal-A/Kpn-A, but four were Sal-A/Kpn-B and two were Sal-B/Kpn-B (Figure 3, Table 1). Samples containing type L mtDNA that were Kpn-B lacked the variant KpnI sites in all three copies of the tandem repeat. These observations support the view that both forms of heteroplasmy have arisen more than once in shad mtDNA (see DISCUSSION).

DISCUSSION

Length polymorphism

The mapping and hybridization data indicate that the 1.5-kb length polymorphism in shad mtDNA is generated by a 1.5-kb tandemly repeated sequence that occurs in two copies in type S and three copies in type L mtDNA. The occurrence of a sequence triplication of this scale is a

novel feature of the shad mtDNA length polymorphism. The only other major length polymorphism that has been characterized in vertebrate mtDNA involves a single tandem repeat (Moritz and Brown 1986).

Each of the copies of the 1.5-kb repeat is subject to minor (≤40 bp) length variation both within and among individuals. The tandem repeats are in the D-loop containing region and map adjacent to an SstII site located within the 12S rRNA gene. This suggests that the polymorphism may also encompass the gene for phenylalanyl tRNA, and possibly part of the 12S rRNA gene, assuming that shad mtDNA shares the gene arrangement common to other vertebrates (Brown 1983, 1985).

Apart from a single report of a major deletion involving the coding region of mouse mtDNA (Boursot et al. 1987) other examples of large-scale length variation in animal mtDNA have also implicated the D-loop containing region or its homologue in insects, the adenine + thymine (A + T) rich region. Large-scale length variation in the A + T rich region has been observed both among and within several species of Drosophila (Fauron and Wolstenholme 1976, 1980a,b, Wolstenholme et al. 1979, Reilly and Thomas 1980, Hale and Singh 1986). Much of this length variation is generated by 470-bp tandem repeats that vary in copy number among and within species (Solignac et al. 1986). The D-loop containing region is the site of length variation among species of artiodactyls and primates (Upholt and Dawid 1977, Ferris et al. 1981) as well as minor (< 15 bp) intraspecific variation in mammals (Cann and Wilson 1983, Hauswirth et al. 1984, Brown et al. 1986), and much larger variation (400-700 bp) in the frog Rana esculenta (Monnerot et al. 1984). Major intraspecific length variation in mtDNA has been

characterized in several species of Cnemidophorus lizards. In C. tesselatus and C. tigris marmoratus mtDNA, tandem repeats of a 64-bp stretch in the D-loop containing region are primarily responsible for variation of up to 370 bp among individuals (Densmore et al. 1985). In C. exsanguis mtDNA, a length polymorphism of at least 4.8 kb is caused by a single tandem repeat that incorporates minor internal length variation, and spans the D-loop containing region as well as some adjacent structural genes (Moritz and Brown 1986).

Heteroplasmy

The survey of mitochondrial genome size in shad produced evidence of restriction site heteroplasmy in ten individuals. This is noteworthy, since very little evidence of restriction site heteroplasmy has previously been reported. This may be less a consequence of the absolute rarity of the phenomenon than of two biases that affect restriction endonuclease analysis of mtDNA (Avise and Lansman 1983). First, examples of heteroplasmy involving only a single restriction site are much less likely to be detected than those associated with major length polymorphisms, since the former manifest themselves with only one enzyme whereas the latter can be detected with many enzymes. Second, cases of restriction site heteroplasmy are liable to be misinterpreted as instances of incomplete digestion, since the patterns of substoichiometric bands that result from both phenomena may be similar. Of course, this ambiguity also means that apparent cases of restriction site heteroplasmy must be viewed with caution. In this study, we addressed the possibility of incomplete digestion by considerably increasing both enzyme quantity and incubation time in

repeat digests of suspect samples. Since the substoichiometric bands evident in Figure 6 showed no change in intensity from previous digests carried out under less intense conditions, the conclusion that they represent restriction site heteroplasmy is warranted.

Prior to this study, evidence of restriction site heteroplasmy has been limited to a single maternal lineage each of cows (Hauswirth and Laipis 1985) and Drosophila melanogaster (Hale and Singh 1986). The occurrence in widely scattered locales of shad heteroplasmic for a SalI restriction site (Table 1) provides empirical evidence that site heteroplasmy may be a widespread phenomenon in at least some species.

All of the mtDNA examined in this study was isolated from oocytes. Since high rates of heteroplasmy have also been detected in frog oocytes (Monnerot et al. 1984), it is possible that heteroplasmy may be more prevalent in germ-line cells than in somatic tissues.

The heteroplasmy in shad mtDNA could have resulted from biparental inheritance, or from incomplete segregation of the variant forms of mtDNA involved. I favour the latter possibility, since all available evidence indicates that at least within limits of detection, metazoan mtDNA is inherited solely through maternal lineages (Avise and Lansman 1983, Avise et al. 1984).

The various composite SalI/KpnI genotypes and the possible mutational pathways that link them are depicted in Figure 7. The pathways indicate that if the inheritance of mtDNA in shad is strictly maternal then at least the transition between alternate SalI genotypes has occurred more than once. For example, for all instances of SalI heteroplasmy to have resulted from a single mutational event would

require that in some lines of descent the mtDNA population remained heteroplasmic for the SalI polymorphism while undergoing a complete transition between KpnI genotypes involving two restriction site changes. Almost certainly then, the transition between the alternate SalI genotypes occurred at least once in each of two lineages, one Kpn-A and the other Kpn-B. Similarly, since samples containing type L mtDNA were divided among three composite genotypes, shad bearing this form of heteroplasmy stemmed from at least three independent mutational events.

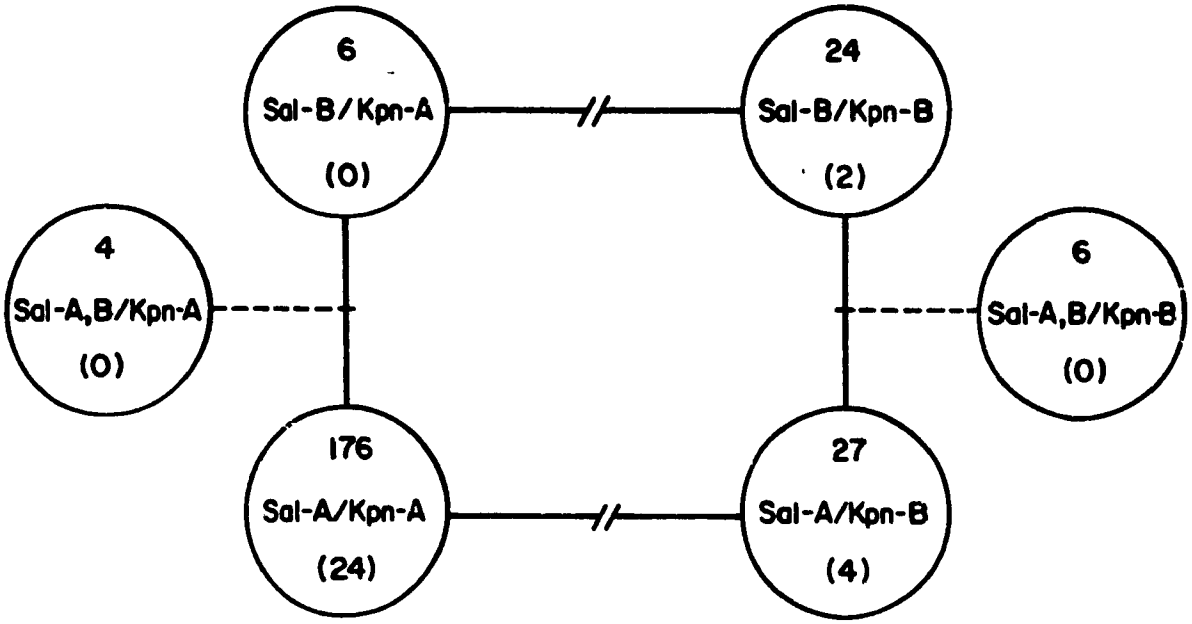
The two common KpnI genotypes were Kpn-A and Kpn-B. The transition between these genotypes entails the parallel loss (or gain) of two restriction sites, one in each copy of the tandem repeat present in type S mtDNA. A third genotype (Kpn-C), in which only one of the restriction sites within the repeat is absent, was only observed in one individual. These features suggest that the 1.5-kb repeat sequences may evolve in concert, as has been suggested for the 470-bp repeats in Drosophila mtDNA (Solignac et al. 1986a).

The occurrence of continuous length variation within the 1.5-kb repeat suggests that the gain or loss of the variant KpnI sites might result from sequence insertions or deletions. The fact that the length variation in the repeat sequence is widespread among individuals raises the additional possibility that the transition between the two common KpnI genotypes has also occurred more than once.

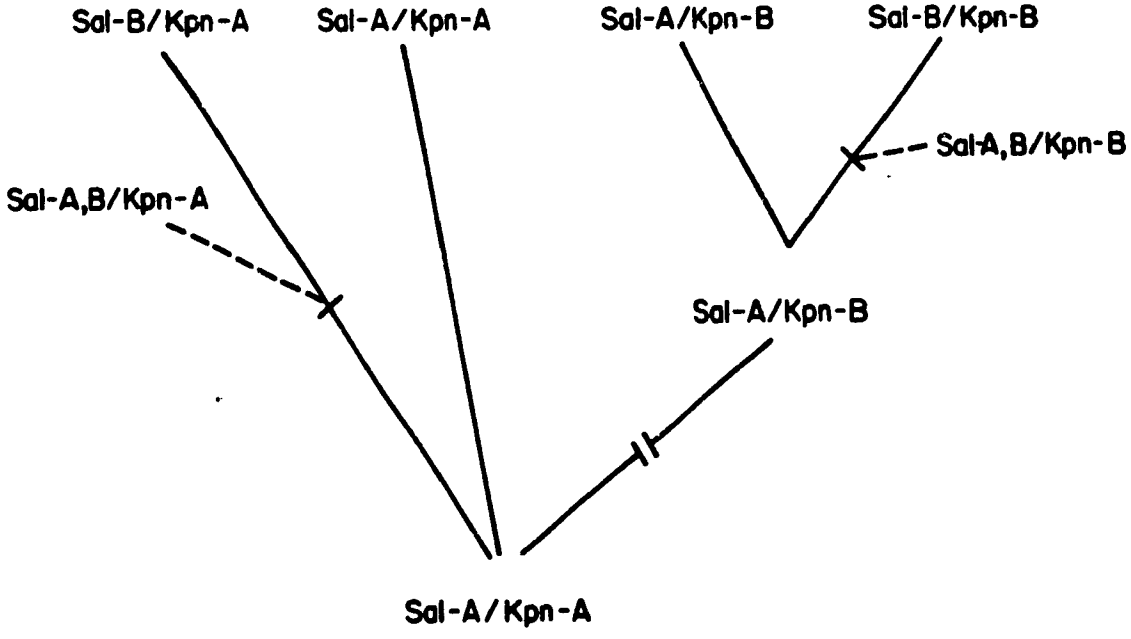
Since most animals appear homoplasmic, it has generally been assumed that mitochondrial variants sort out rapidly (Avise and Lansman 1983), a view also supported by theoretical arguments (Upholt and Dawid

Figure 7. Possible mutational pathways involved in the generation of the various composite SalI/KpnI genotypes. The solid lines crossing the branches indicate the number of restriction site changes involved in the transition from one genotype to another. A, Parsimony network linking genotypes. The total number of individuals, and the number () bearing type L mtDNA are indicated for each genotype. The transition between the two KpnI genotypes need only have occurred once, but could have occurred in inidividuals that were either Sal-A or Sal-B. B, Dendrogram depicting one of the four possible mutational pathways indicated in A. The construction emphasizes the necessity of independent origins of Sal-A,B heteroplasmy in two different lines of descent.

A


6
Sal-B/Kpn-A
(0)
24
Sal-B/Kpn-B
(2)
4
Sal-A,B/Kpn-A
(0)
6
Sal-A,B/Kpn-B
(0)
176
Sal-A/Kpn-A
(24)
27
Sal-A/Kpn-B
(4)

B


Sal-B/Kpn-A
Sal-A/Kpn-A
Sal-A/Kpn-B
Sal-B/Kpn-B
Sal-A,B/Kpn-B
Sal-A,B/Kpn-A
Sal-A/Kpn-B
Sal-A/Kpn-A

1977, Chapman et al. 1982, Birky et al. 1983). Recent breeding experiments with insects, however, indicate that heteroplasmy may persist in some species for hundreds of generations (Solignac et al. 1984, Rand and Harrison 1986).

The high incidence of heteroplasmy in shad suggests that the segregation of mitochondrial variants may occur relatively slowly in this species. It is surprising, however, that I failed to encounter any shad that appeared homoplasmic for type L mtDNA. This result contrasts with other reports of heteroplasmy in which the variant forms of mtDNA in question have also been observed in the homoplasmic state, as well as with my own observations of restriction site heteroplasmy in shad. Only a minority (25%) of the shad with Sal-B mtDNA appeared heteroplasmic. The absence of shad homoplasmic for the type L variant suggests the possibility that this form of mtDNA may be under negative selection. Nevertheless, the type L variant was clearly the predominant form of mtDNA present in some individuals (e.g. fish C1 in Figure 3).

The geographic distribution of the type L variant also points to the possibility of selective influences on the occurrence of this genotype. Unlike the Sal-B variant, which was distributed across the whole range of the species, the type L variant was largely restricted to the southern part of the range, and was particularly prevalent in the St. John's River population in Florida at the extreme southern end of the range (Table 1). It is conceivable that water temperatures or some other variable correlated with latitude might favour the ocurrence of type L mtDNA, although I am unable to propose any mechanisms for such a selective effect.

The data presented here support the suggestion made by Birmingham et al. (1986) that length polymorphisms and heteroplasmy may be more prevalent in the mtDNA of at least some lower vertebrate groups than in that of mammals or birds. They also provide the first evidence of geographically widespread forms of heteroplasmy that can be attributed to mutations that occurred independently in different mitochondrial lineages.

CHAPTER 2

MITOCHONDRIAL DNA POLYMORPHISM AND ITS IMPLICATIONS FOR THE INTERPRETATION OF POPULATION STRUCTURE AND LIFE HISTORY VARIATION

INTRODUCTION

The delineation of population structures and the interpretation of variation in morphology and life-history traits constitute problems that are often related. The existence of genetically mediated variation among localities requires the existence of discrete populations, hence the occurrence of one of these features is often regarded as evidence in support of the other (Cody 1966, Murphy 1968, Charnov and Krebs 1973, Shaffer and Elson 1975, Leggett and Carscadden 1978).

In marine fishes, however, the structures of populations and the causal basis of variation in phenotype and life-history are both frequently problematic. The occurrence of geographically restricted spawning aggregations in some species (e.g. herring: Iles and Sinclair 1982, capelin: Sharp et al. 1978, salmon: Elson 1970) suggests the existence of discrete populations; however, the potential for gene flow afforded by the passive dispersal of pelagic eggs and larvae and/or the active dispersal of highly mobile juveniles and adults (McCleave et al. 1984) suggests the contrary. Similarly, the interpretation of variation in morphology and life-history is hampered by the paucity of data on the heritabilities of such traits in marine fishes (and by the impracticality of the breeding studies needed to acquire such data).

Fishes also exhibit greater phenotypic responses to environmental variables than do homeothermic vertebrates, presumably because of their poikilothermic nature and indeterminate growth patterns (Ihssen et al. 1981). Hence, although variation in life-history traits is often assumed to be both adaptive and genetically mediated, in fact, the alternative possibility that the variation is environmental in origin can rarely be rejected.

Several attributes of mtDNA render it particularly useful in analyses of evolutionary genetics and population structure (Lansman et al. 1981, Avise and Lansman 1983, A.C. Wilson et al. 1985, Avise 1986). Mitochondrial DNA exhibits numerous, apparently selectively neutral polymorphisms that are detectable with restriction endonucleases. Because mtDNA evolves rapidly and does not undergo recombination, restriction enzyme data can be used to infer the phylogenetic relationships of lineages within species.

The mitochondrial genome is maternally inherited and functionally haploid (individuals usually have only one type of mtDNA as opposed to the diploid condition of nuclear genes). Under the assumptions of a 1:1 sex ratio and no differential migration of sexes, haploidy and maternal inheritance lead to the prediction that rates of mitochondrial gene flow among populations should be one quarter those of nuclear gene flow (Birky et al. 1983). For similar reasons, historical population bottlenecks and founder effects should manifest themselves more clearly in mtDNA variation than in nuclear DNA variation (A.C. Wilson et al. 1985). Mitochondrial DNA analysis can therefore be expected to provide

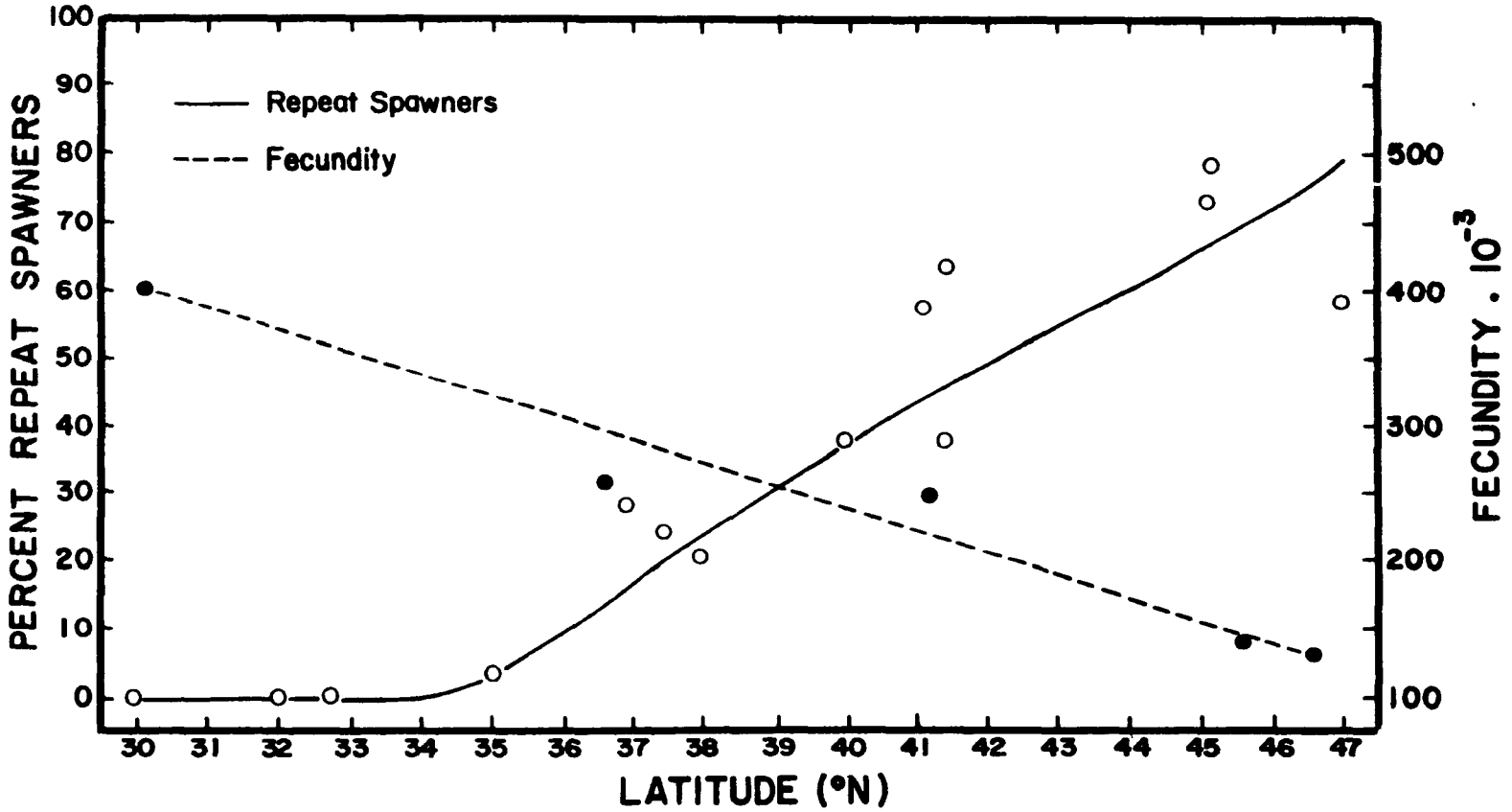
a more sensitive means of examining population structures than techniques that examine nuclear genes such as allozyme electrophoresis.

Restriction enzyme analysis of mtDNA has been widely used in phylogenetic studies (see reviews in Avise and Lansman 1983, A.C. Wilson et al. 1985, Avise 1986), but much less so in studies of population structure. In this study I employed mtDNA analysis to address both applications.

The subject of my inquiry was the American shad (Alosa sapidissima), an anadromous clupeid fish. A. sapidissima exhibits both population restriction during the breeding season and population mixing during the remainder of the year. It spawns in Atlantic coast rivers from Florida to Quebec. Juvenile and adult shad from throughout this range join in seasonally migratory aggregations in the sea (Dadswell et al. 1987, Melvin et al. 1988). Despite the intermixing of shad populations in the marine habitat, evidence that shad home to their natal rivers to spawn (Melvin et al. 1986 and references therein) suggests that adults ascending particular rivers constitute genetically discrete populations. Shad also exhibit latitudinal trends in morphological, energetic, and reproductive traits. These trends have been assessed in the context of life history theory (Stearns 1976, 1977,1980) and interpreted as adaptive (i.e. genetically based) (Leggett and Carscadden 1978, Glebe and Leggett 1981a,b). Shad can also be classified to river of origin on the basis of these traits (Shoubridge 1978, Melvin et al. 1988).

The variation in the reproductive traits of shad is particularly striking (Figure 8; Leggett and Carscadden 1978). In rivers south of

Figure 8. Changes in the frequency of repeat reproduction and fecundity with latitude among Atlantic coast populations of American shad. (Reproduced from Leggett 1985).


Repeat Spawners
Fecundity
PERCENT REPEAT SPAWNERS
FECUNDITY . 10^{-3}
LATITUDE (°N)
100
90
80
70
60
50
40
30
20
10
0
500
400
300
200
100
30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47

Cape Hatteras, N.C., all shad die after spawning; north of this point the frequency of repeat spawning increases steadily with latitude to a maximum of 80% in rivers of eastern Canada. The relative fecundity of shad exhibits a pronounced reciprocal trend, decreasing with latitude.

At least two attempts to examine population structuring in shad using allozyme data have produced equivocal results. Shoubridge (1978) examined 32 loci in shad from four rivers distributed from Florida to New Brunswick. He was unable to adequately resolve 17 loci. Fourteen of the remaining 15 loci were monomorphic. The one polymorphic locus (EST-1) exhibited a latitudinal cline. G.D. Melvin (Washburn and Gillis, 123 York St., Fredriction, N.B. E3B 2N6, pers. com.) obtained similarly inconclusive results in a study involving 14 rivers from Florida to Quebec.

I surveyed restriction site variation in shad mtDNA to answer the following question: Is the north-south split in reproductive traits in shad associated with a corresponding geographic pattern of differentiation in its mtDNA? I also examined geographic variation in shad mitochondrial genotype frequencies to test whether or not river "populations" of shad constitute genetically discrete aggregations.

MATERIALS AND METHODS

Female shad were collected from January to June of 1985 and 1986 during their upstream spawning migrations in 14 rivers spanning the full Atlantic coast range of the species (Florida to southern Quebec, Figure 1). Isolation of mtDNA, restriction endonuclease digestion of

samples, and 1% agarose gel electrophoresis were carried out as described in chapter 1.

DNA bands were usually visualized by ethidium bromide staining, but in some cases were also verified by autoradiography of Southern blots in which cloned shad mtDNA was used as the probe (see chapter 1 for details). General descriptions of the techniques involved in restriction endonuclease analysis and DNA gel electrophoresis are given in Lansman et al. (1981), Maniatis et al. (1982) and Ferris and Berg (1987).

Twenty restriction endonucleases recognizing five or six base pair sequences were initially tested on samples of shad mtDNA. Four of these enzymes, ClaI, BamHI, HindIII, and SstI cut samples of shad mtDNA either once or not at all, and will not be considered further. The remaining 16 enzymes, AvaI, BglI, BstEII, EcoRI, EcoRV, HaeII, HincII, HpaI, KpnI, PstI, PvuII, SalI, ScaI, SstII, StuI and XbaI were used on 52 samples of shad mtDNA (representing 3-5 samples from each river) in a survey of restriction site variation in shad mtDNA. DNA sequence divergences among the genotypes recognized by the 16 enzymes were estimated by the maximum likelihood method of Nei and Tajima (1983).

Four enzymes (KpnI, EcoRV, SalI and HaeII) revealed polymorphisms that were geographically widespread. These enzymes were used on an additional 191 mtDNA samples drawn from all rivers sampled to assess geographic variation in mitochondrial genotype frequencies.

As reported elsewhere (chapter 1), heteroplasmy, the condition in which two or more forms of mtDNA occur in the same individual, was observed in some samples of shad mtDNA. Two forms of heteroplasmy were

observed. One involved a variable SalI restriction site, the other a 1.5 kb length variant. In statistical analyses of genotype frequencies, individuals heteroplasmic for the SalI site (Table 5) were lumped with those homoplasmic for the SalI-B genotype (see RESULTS for further details).

RESULTS

Restriction site variation

Seven of the 16 endonucleases used to examine restriction site variation in shad mtDNA generated fragment patterns that were invariant among the 52 mtDNA samples assayed. The remaining nine enzymes exhibited 1-5 variant fragment patterns attributable to restriction site gains or losses (Table 2). These nine enzymes defined 13 composite genotypes, eight of which were each observed in only a single individual (Table 3). In contrast, the most common composite genotype (1, Table 3), which corresponded to the most common fragment pattern for each of the nine enzymes, was detected in 21 individuals distributed among all but one of the 14 rivers from which the shad were sampled.

Estimated sequence divergences among the 13 composite genotypes were generally low. None of the variant genotypes differed from the most common composite by more than 0.48 ± 0.23% (SD) (Table 4).

Geographic variation in mitochondrial genotype frequencies

In the 16 enzyme survey above, only four endonucleases revealed enough variation to merit their use in a larger survey of mitochondrial

genotype frequencies. These enzymes, SalI, KpnI, HaeII and EcoRV, were used to assay genotype frequencies among 243 mtDNA samples representing 7-23 shad from each of the 14 rivers. Each of the four enzymes recognized two common "single-enzyme" genotypes, designated "A" and "B" in order of frequency (Tables 2,5).

The most common composite genotype (AAAA) occurred in 90 shad distributed among all rivers sampled (Table 5). Although ubiquitous in distribution, the AAAA genotype varied significantly in frequency among rivers (Richelieu and St. Lawrence samples combined, chi-square = 24.87, df=12, p=0.015).

The geographic distributions of the single-enzyme "B" genotypes varied considerably among the four enzymes. The SalI-B and KpnI-B genotypes were observed in 12 and 13 rivers, respectively, and no geographic trends in frequency of occurrence were apparent for either enzyme (Figure 9a,b). In contrast, the HaeII-B genotype, which was also present across the entire geographic range sampled, occurred at higher frequency among shad taken from rivers in the Gulf of St. Lawrence drainage than from rivers elsewhere (50% and 9%, respectively; chi-square = 44.78, df=1, $p<0.001$; Figure 9c). The distribution of the EcoRV-B genotype was more limited than that of the other single-enzyme variants. It occurred in shad sampled from rivers in the middle region of the Atlantic coast range (Waccamaw to the St.John, N.B.), and was at highest frequency (40-60%) among shad taken from the Hudson, Connecticut and Annapolis rivers (Figure 9d).

Two discrete size classes of mtDNA, designated type S (18.3 kb) and type L (19.8 kb) were observed in shad (chapter 1).

Table 2. Approximate sizes (in kilobases) of fragments generated by digestion of shad mtDNA with restriction endonucleases. Fragments in parentheses were not seen on gels, but their presence was inferred from fragment changes in other genotypes.

SalI		KpnI			HaeII			
A	B	A	B	C	A	B	C	D
9.7	9.7	6.4	12.3	7.9	4.7	4.7	4.7	4.7
8.1	8.6	6.1	6.1	6.1	4.4	4.2	4.4	4.4
0.5		4.3		4.3	3.2	3.2	3.2	3.2
		1.5			2.9	2.9	2.9	1.9
					1.4	1.4	1.8	1.4
					0.6	0.6	0.6	1.0
					0.5	0.5	0.5	0.6
					0.4	0.4	(0.3)	0.5
					(0.3)	(0.3)		0.4
						(0.2)		(0.3)
18.3	18.3	18.3	18.4	18.3	18.4	18.4	18.4	18.6

Table 2 (continued)

HaeII		EcoRV		EcoRI			BstEII	
E	F	A	B	A	B	C	A	B
4.7	4.4	16.8	16.8	9.5	7.3	9.5	10.8	7.5
4.4	3.2	1.5	1.0	7.3	5.4	3.9	7.5	5.7
3.2	2.95		0.5	1.5	4.4	3.6		5.3
3.2	2.9				1.5	1.5		
1.4	1.7							
0.6	1.4							
0.5	0.6							
0.4	0.5							
	0.4							
	(0.3)							
18.4	18.35	18.3	18.3	18.3	18.6	18.5	18.3	18.5

Table 2 (continued)

HincII		ScaI		StuI		AvaI	BglI	HpaI
A	B	A	B	A	B	A	A	A
5.4	5.4	6.3	6.3	10.7	9.3	10.7	8.7	5.6
3.2	3.5	5.3	5.6	3.2	3.2	3.0	3.5	5.1
3.1	3.2	4.1	5.3	1.6	1.6	1.6	2.9	3.4
2.0	2.0	1.5	1.0	1.2	1.5	1.3	1.7	2.6
1.5	1.5	1.0		0.8	1.2	0.95	1.4	1.5
0.9	0.9			0.6	0.8	0.3		
0.6	0.6				0.6			
0.6	0.6							
0.5	0.5							
(0.4)								
18.2	18.2	18.2	18.2	18.1	18.2	17.85	18.2	18.2

PstI	PvuII	SstII	XbaI
A	A	A	A
8.7	8.3	16.5	10.0
6.5	6.6	1.3	4.7
1.5	3.6	0.5	2.6
0.85			0.9
0.7			
18.25	18.5	18.3	18.2

Table 3. Composite mitochondrial genotypes of 52 shad assayed with 16 enzymes.

--Composite Genotype-- Designation	Fragment Pattern[1]	No. of shad	River
1	AAAAAAAAA	3	St.John's
		1	Cooper
		2	Waccamaw
		2	Pamlico
		3	Delaware
		2	Hudson
		2	Connecticut
		1	Annapolis
		1	St.John
		1	Miramichi
		1	St.Lawrence
		2	Richelieu
2	BBAAAAAAA	1	Altamaha
		2	Cooper
		1	Pamlico
		1	Hudson
		1	Annapolis
		2	St.John

Table 3 (continued)

3	ABAAAAAAA	1	Altamaha
		1	Cooper
		1	York
4	BAABAAAAA	1	Annapolis
5	AABAAAAAA	1	Altamaha
		1	Waccamaw
		2	Miramichi
		2	St.Lawrence
		1	Richelieu
6	AAABAAAAA	3	Connecticut
		2	Annapolis
7	AAAABAAAA	1	St.John's
8	AAAABAABA	1	York
9	BAEACABAA	1	Hudson
10	AAAAABAAA	1	York
11	BBAAAAAAB	1	Altamaha

Table 3 (continued)

12	AAEAAAAAA	1	Waccamaw
13	ACAAAAAAA	1	York

[1]Letters correspond to fragment patterns generated by SalI, KpnI, HaeII, EcoRV, BstEII, HincII, ScaI and StuI (see Table 2).

Table 4. Estimated (%) sequence divergences (above diagonal) and standard deviations (below diagonal) among shad mitochondrial genotypes.

Genotype	1	2	3	4	5	6	7	8	9	10	11	12	13
1		0 36	0 24	0 24	0 12	0.12	0 12	0.24	0 48	0.12	0 48	0 12	0.12
2	0 20		0.12	0 36	0.48	0 48	0.48	0 61	0 62	0 48	0 12	0 49	0 37
3	0 16	0 12		0 48	0 36	0.36	0 36	0 48	0.74	0.36	0.24	0 36	0 12
4	0 16	0 20	0 23		0 35	0.12	0 35	0 47	0.48	0 35	0 48	0 36	0 36
5	0 11	0 23	0 20	0 20		0 23	0 23	0 35	0.60	0 23	0 60	0 24	0 24
6	0 11	0.23	0 20	0 11	0.16		0 23	0 35	0.60	0 23	0 60	0.24	0 24
7	0.11	0 23	0 20	0 20	0 16	0 16		0 12	0 60	0 23	0 60	0 24	0 24
8	0 16	0 26	0.23	0 23	0 20	0 20	0 11		0 72	0 35	0 73	0 36	0 36
9	0 23	0 27	0.29	0 23	0 26	0.26	0 26	0 29		0 60	0 74	0 36	0 61
10	0 11	0 23	0 20	0 20	0 16	0 16	0 16	0 20	0 26		0 60	0 24	0 24
11	0 23	0 12	0 17	0 23	0 26	0.26	0 26	0 29	0 29	0 26		0.61	0 36
12	0 11	0 24	0.20	0 20	0 16	0 16	0 16	0 20	0.20	0.16	0 26		0 24
13	0.11	0.20	0 12	0.20	0 16	0 16	0 16	0 20	0 26	0 16	0 20	0 16	

Table 5. Mitochondrial genotypes observed in four-enzyme survey.

	River	Size[1]	SalI[1]	KpnI	HaeII	EcoRV	N
A	St.John's	S,L	A	A	A	A	5
		S.L	A	A	B	A	2
		S,L	A	B	A	A	1
		S	A	A	A	A	6
		S	A	A	B	A	2
							16
B	Altamaha	S,L	A	B	A	A	2
		S,L	B	B	A	A	1
		S	A	A	A	A	4
		S	A	A	B	A	2
		S	A	B	A	A	5
		S	B	B	A	A	2
		S	A	A	C	A	1
							17
C	Cooper	S,L	B	B	A	A	1
		S	A	A	A	A	9
		S	A	B	A	A	2
		S	A	A	F	A	1
		S	A,B	B	A	A	1
							15

Table 5 (continued)

D	Waccamaw	S,L	A	A	B	A	2
		S,L	A	A	A	A	2
		S	A	A	A	A	7
		S	A	A	B	A	1
		S	A	A	A	B	3
		S	A	B	A	A	1
		S	B	B	A	A	1
							17
E	Pamlico	S,L	A	A	A	A	1
		S	A	A	A	A	6
		S	A	A	B	A	1
		S	A	A	A	B	3
		S	B	B	A	A	5
		S	A,B	A	A	A	1
		S	B	A	B	A	1
		S	A	B	A	B	1
							19
F	York	S,L	A	A	A	A	2
		S	A	A	A	A	8
		S	A	A	B	A	2
		S	B	B	A	A	2
		S	A,B	B	A	A	2
		S	A	B	A	A	3

Table 5. (continued)

F	York	S	A	C	A	A	1
		S	A	A	A	B	2
							22
G	Delaware	S,L	A	A	A	A	1
		S,L	A	A	A	B	1
		S	A	A	A	A	10
		S	B	B	A	A	4
		S	A,B	B	A	A	1
		S	B	A	A	A	1
		S	A,B	A	A	A	1
		S	A	A	C	B	1
		S	B	A	A	B	1
		S	A	A	E	A	1
		S	A	B	A	B	1
							23
H	Hudson	S	A	A	A	A	6
		S	A	A	A	B	7
		S	B	B	A	A	3
		S	A	A	B	A	1
		S	A	A	D	A	1
		S	A	A	D	B	1
		S	A,B	A	E	A	1
							20

Table 5 (continued)

J	Connecticut	S	A	A	A	A	4
		S	A	A	A	B	7
		S	B	B	A	A	2
							13
K	Annapolis	S,L	A	B	A	A	1
		S,L	A	A	A	B	1
		S	A	A	A	A	2
		S	A	A	A	B	11
		S	A,B	A	A	A	1
		S	A	A	B	A	2
		S	A,B	B	A	A	1
		S	A	B	A	A	1
							20
L	St.John	S	A	A	A	A	5
		S	A	A	A	B	1
		S	A	A	B	A	1
		S	B	B	A	A	4
		S	A	B	A	A	3
							14

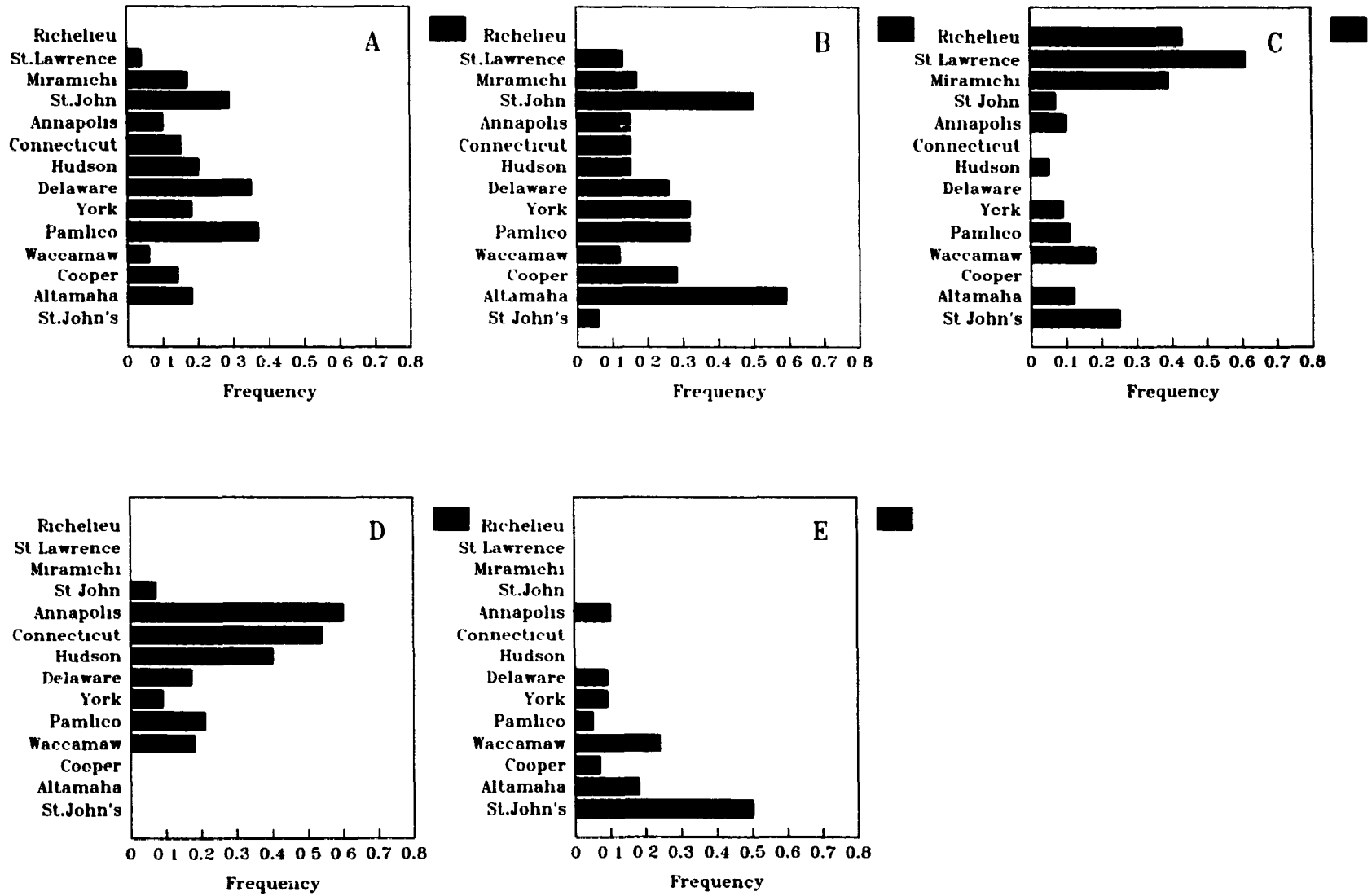
Table 5. (continued)

M	Miramichi	S	A	A	A	A	5
		S	A	A	B	A	7
		S	A	B	A	A	3
		S	B	A	A	A	3
							18
N	St.Lawrence	S	A	A	A	A	5
		S	A	A	B	A	14
		S	B	A	A	A	1
		S	A	B	A	A	3
							23
O	Richelieu	S	A	A	A	A	2
		S	A	A	A	-[2]	2
		S	A	A	B	A	1
		S	A	A	B	-[2]	2
							7

1. Where two letters are present in genotype designation, indicates that the sample was heteroplasmic for that trait.

2. EcoRV did not produce clear digestion patterns with these samples.

Figure 9. Distributions of several mitochondrial genotypes among river samples. Rivers are listed from top to bottom in latitudinal sequence from north to south. A, SalI-B genotype; B, KpnI-B genotype; C, HaeII-B genotype; D, EcoRV genotype; E, type L mtDNA.


A
Richelieu
St.Lawrence
Miramichi
St.John
Annapolis
Connecticut
Hudson
Delaware
York
Pamlico
Waccamaw
Cooper
Altamaha
St.John's
0 0 1 0.2 0 3 0.4 0.5 0 6 0.7 0.8
Frequency
B
Richelieu
St.Lawrence
Miramichi
St.John
Annapolis
Connecticut
Hudson
Delaware
York
Pamlico
Waccamaw
Cooper
Altamaha
St John's
0 0 1 0 2 0.3 0.4 0.5 0 6 0.7 0 8
Frequency
C
Richelieu
St Lawrence
Miramichi
St John
Annapolis
Connecticut
Hudson
Delaware
York
Pamlico
Waccamaw
Cooper
Altamaha
St John's
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
Frequency
D
Richelieu
St Lawrence
Miramichi
St John
Annapolis
Connecticut
Hudson
Delaware
York
Pamlico
Waccamaw
Cooper
Altamaha
St.John's
0 0 1 0.2 0.3 0.4 0 5 0 6 0.7 0.8
Frequency
E
Richelieu
St Lawrence
Miramichi
St.John
Annapolis
Connecticut
Hudson
Delaware
York
Pamlico
Waccamaw
Cooper
Altamaha
St.John's
0 0 1 0 2 0.3 0.4 0.5 0.6 0.7 0.8
Frequency

Among the 243 samples analyzed, 220 consisted solely of type S and 23 contained both type S and type L mtDNA (Table 5). The frequency of type L mtDNA was greatest in shad collected from rivers at or near the southern end of the species range (Figure 9e).

DISCUSSION

Among the features of shad mtDNA polymorphism revealed by this study the following three are especially prominent: 1) overall restriction site variability in shad mtDNA is low; 2) several of the variant genotypes found are widely distributed over the range of the species; 3) three of these genotypes exhibit clear geographical patterns in their frequency of occurrence. These and other points are addressed below.

Restriction site variation

The 16 enzyme survey of restriction site variation revealed 13 composite genotypes, only five of which occurred in more than one individual among the 52 shad examined (Table 3). These genotypes differed from each other by no more than a few restriction site gains or losses among an average of 74 restriction sites (corresponding to 420 bp) in each genotype (Table 2,3). Hence, estimated values of sequence divergence among the genotypes were relatively low (mean, 0.37%; Table 4).

All methods used to derive estimates of DNA sequence divergence from restriction enzyme data assume that restriction site gains or losses result from base substitutions rather than from insertions or

deletions (e.g. Nei and Tajima 1983). The sequence data available suggest that this is a reasonable assumption in most cases (Avise and Lansman 1983, A.C. Wilson et al. 1985). In this study, however, three of the restriction sites that accounted for the majority of the limited variation observed (those that define the "B" genotypes of KpnI and EcoRV) are located in a region of the shad mtDNA molecule (the D-loop containing region) known to exhibit extensive length variation (chapter 1). It is therefore quite possible that the changes observed at these sites result from insertions or deletions. If so, many of the sequence divergence estimates in Table 4 could be overestimates.

Regardless of whether or not the estimates in Table 4 are inflated, the extent of mtDNA sequence variation in shad is small in comparison to that reported for many other species (Avise and Lansman 1983, Avise 1986, A.C. Wilson et al. 1985). Among marine species for example, estimated intraspecific mtDNA sequence divergences reach at least 4.4% in Atlantic herring (Clupea harengus), 1.8% in Pacific herring (C. pallasi), 21% in mummichog (Fundulus heteroclitus), and 2.0% in horseshoe crabs (Limulus polyphemus) (Kornfield and Bogdanowicz 1987, Schweigert and Withler 1988, Powers et al. 1986, Saunders et al. 1986).

The low level of intraspecific mtDNA variation in shad could result from a relatively low rate of mtDNA sequence evolution, or from past population bottlenecks. The rate of mtDNA sequence divergence has been estimated to be near 2% per Myr in a number of vertebrates (Brown et al. 1979, Brown 1983, Shields and Wilson 1987, A.C. Wilson et al. 1985 and references within); however, in shad fossil data support a rate that is at least an order of magnitude lower (chapter 4). Population

bottlenecks have been invoked to account for low levels of mtDNA variation in a number of species, including humans (Brown 1980, A. C. Wilson et al. 1985, Cann et al. 1987; but see Avise et al. 1984, Latorre et al. 1986 for critiques of the the severity of the bottlenecks involved).

The low level of mtDNA variation in shad may stem at least in part from population reductions incurred during Pleistocene glaciations. During the last glacial maximum (18000 bp) glaciation extended as far south as New Jersey, and polar (oceanic) water masses as far south as 42° N., but sea temperatures around Florida were similar to those at present (CLIMAP project members 1976). Thus, the northern limit of shad must have been south of New Jersey, but the southern limit was probably little different from at present.

There is additional evidence that oceanic conditions may have been relatively unfavourable for shad within much of this reduced range. Coastal sea temperatures at least as far north as Cape Hatteras were heavily influenced by the Gulf Stream. Summer temperatures are estimated to have ranged well above 20° C (CLIMAP project members 1976). Both the current geographic distribution of shad, and marine catch data, suggest that shad avoid temperatures above 20° C (Leggett and Whitney 1972, Dadswell et al. 1987). Moreover, the response of shad to latitudinal changes in temperature, both in their seasonal distributions, and timing of spawning, is very conservative (Leggett and Whitney 1972). It is thus highly probable that American shad were confined to a constricted inshore marine feeding range along the southeastern U.S. coast during glacial maxima, much as a closely allied

form, the Alabama shad (A. alabamae) is now confined to the northern Gulf of Mexico (Burgess 1980).

I estimated the severity of the Pleistocene bottleneck required to account for the level of mtDNA variation observed in shad, under the assumption that the rate of mtDNA sequence divergence in shad is either the 2% per Myr value calibrated in other vertebrates or the 0.2% per Myr value suggested in chapter 4. I estimate the mean mtDNA sequence divergence among randomly picked shad to be approximately 0.2% (Tables 3,4). From equations (4) and (5) in A.C. Wilson et al. (1985), and assuming a mean generation time of five years (Leggett and Carscadden 1978), I calculated the long term effective population size of American shad to be 20000 or 200000 females depending on whether the high or low estimate of mtDNA divergence rate was used. Both of these values are substantially less than the current abundance of shad, which numbers in the millions (Walburg and Nichols 1967).

It is not necessary to assume that the genetic bottleneck hypothesized for shad occurred in a single step. Each of the four major Quaternary glaciations may have successively reduced population size, and hence mtDNA sequence variation, in shad. The figures quoted above should therefore be regarded as minimum estimates of effective population size in A. sapidissima during glacial maxima.

Low levels of mtDNA sequence variation appear to be a general phenomenon among North American diadromous fishes. Mitochondrial DNA sequence divergences less than 1% prevail in American eels, Anguilla rostrata (Avise et al. 1986), striped bass, Morone saxatilis and white perch, M. americana (R.W. Chapman, Johns Hopkins University, pers.

com.), steelhead trout, Salmo gairdneri (G.M. Wilson et al. 1985), and chinook salmon, Onchorhynchus tshawytscha (Wilson et al. 1987). This suggests that glaciation, and the population reductions that resulted, may have influenced them similarly. Eels, unlike the other species cited, feed and grow primarily in freshwater but spawn, and die subsequent to spawning, in mid-Atlantic (Williams and Koehn 1984). Although shad and eels have essentially opposite lifecycles they share a dual dependence on marine and freshwater habitats and occupy similar geographic ranges. Avise et al. (1986) conducted an mtDNA survey of eels collected from rivers along the east coast of North America. As in our study, they found that one composite genotype occurred over the entire range sampled. Moreover, the magnitude of mtDNA variation evident in eels was very similar to that in shad.

Species that survived glaciation in more than one refugium might be expected to preserve more mtDNA variation than those that were confined to single refugia. Thus, while mtDNA sequence variation among chinook salmon from British Columbia and Alaska is less than 1%, these northern chinook differ from conspecifics in California (which are believed to have derived from a separate refugium) by approximately 2.2% (Wilson et al. 1987).

Geographic variation

The 14 rivers from which I sampled shad are distributed over at least 4000 km of coastline and span the entire Atlantic coast range of the species. Both the 16- and 4-enzyme surveys revealed several composite genotypes that were distributed over most or all of the geographic range sampled (Tables 3,5). However, several mitochondrial

variants, the single-enzyme HaeII-B and EcoRV-B genotypes and the type L length variant, exhibited non-random, geographically clumped distributions (Figure 9).

The distributions of shad mitochondrial genotypes have probably been influenced by both founder effects and gene flow among rivers. For example, the high frequency of the HaeII-B genotype among shad from Gulf of St. Lawrence rivers (Figure 9c) may have resulted from a founder effect that occurred during the post-glacial recolonization of this region. The same argument may also account for the high frequency of the EcoRV-B genotype in the Annapolis, Connecticut and Hudson Rivers (Figure 9d).

Temporal influences on gene flow

If the type L genotype, which may be influenced by selection (chapter 1), is excluded from consideration, then the heterogeneity of genotype frequencies is greatest among rivers in the northern (glaciated) half of the range (Figure 9). The lesser heterogeneity among southern rivers could be a consequence of the longer period over which gene flow has been able to exert its homogenizing influence among these (presumably) older populations.

Geographic variation in the severity of temporal barriers to inter-river gene flow may also be an important determinant of the different patterns seen. The duration and timing of spawning in shad is cued to the occurrence of temperatures between 14° C (time of peak river entry) and 18.5° C (time of peak spawning) (Leggett and Whitney 1972). The duration of this "temperature window" varies from 2-3 months (November-February) in the south of the range to approximately three weeks (May-

June) in the north (Leggett and Whitney 1972, Provost et al. 1984, Leggett 1985). The shorter duration of temperatures suitable for upstream migration and spawning in the north may reduce the potential for interpopulation straying.

Further evidence of the importance of temporal effects on the potential for gene flow is found in the striking difference in the EcoRV-B genotype frequency between the samples from the St. John and Annapolis Rivers (7 and 60 % respectively; chi-square = 9.74, df=1, $p<0.005$). Both of these rivers are tributaries to the Bay of Fundy (Figure 1), but spring warming in the Annapolis is much more rapid than in the St. John. As a result, the spawning migration of shad in the Annapolis occurs 2-3 weeks before the migration in the St. John, and is coincident with the spawning migrations in the Connecticut and Hudson rivers located 650-720 km to the south. The frequencies of the EcoRV-B genotype in the Connecticut and Hudson samples were similar to that in the Annapolis sample (Figure 9d).

Unlike the HaeII-B and EcoRV-B genotypes, the distributions of the SalI-B and KpnI-B genotypes (and their two-enzyme composites) showed no evidence of geographic trends (Table 5, Figure 9a,b). I previously reported molecular evidence that the SalI-B and possibly the KpnI-B genotypes have evolved more than once in shad mtDNA (chapter 1). The more uniform geographic distributions of these genotypes may be a consequence of multiple evolutionary origins.

The overall extent of geographic variation and structuring of mitochondrial genotype frequencies in shad stands in marked contrast to the limited variation detected in this species with allozymes

(Shoubridge 1978). My results, and those of DeSalle et al. (1987b) and Billington and Hebert (1988) provide empirical evidence that mtDNA analysis can be a highly sensitive means of examining population structures.

Reproductive variation in shad

As mentioned above, relative fecundity and frequency of repeat spawning in shad exhibit reciprocal latitudinal trends (Figure 8; Leggett and Carscadden 1978). Shad in the south have high fecundity but die after spawning. Shad in the north have low relative fecundity but typically spawn repeatedly over several years. It has been hypothesized that this shift from semelparity in the south to a high degree of iteroparity in the north represents adaptive variation occurring among genetically discrete populations (Carscadden and Leggett 1975, Leggett and Carscadden 1978, Glebe and Leggett 1981b).

My mtDNA data indicate that northern and southern shad are not associated with any phylogenetic divisions within the species. In this respect A. sapidissima differs from another east coast fish with a similar geographic range, Fundulus heteroclitus. The latter species is differentiated into a northern and a southern form that are clearly distinct in both their nuclear and mitochondrial genotypes (Powers et al. 1986).

The absence of any phylogenetic divisions correlated with the reproductive types of shad is consistent with the possibility that the variation in reproductive traits is a result of direct environmental influences. However, my mtDNA data do demonstrate that shad spawning in particular rivers or geographic areas form discrete reproductive

units. In this respect my data provide some support for the genetic model of reproductive variation in shad. If the variation in the reproductive traits is indeed genetically mediated, the absence of common mtDNA variants unique to either reproductive type, and the low level of mtDNA variation within the entire species, suggest that the reproductive variation is likely to have evolved since the Pleistocene.

As described above, the data I have presented on mtDNA variation in American shad offer a number of implications for the evolutionary genetics of this species. These include clear evidence that river populations are reproductively discrete, as well as evidence of one or more past population reductions, founder effects, and the likely importance of temporal effects as determinants of gene flow. Although I remain unable to unequivocally reject non-genetic explanations of the reproductive variation, the mtDNA data provide substantive evidence that the variation, if genetic, is of recent evolutionary origin. In a more general context, my results demonstrate that mtDNA analysis is a powerful means of examining population structures, both in terms of their current states, and their historical roots.

CHAPTER 3

BI-DIRECTIONAL TRANSFER OF MITOCHONDRIAL DNA AMONG SPECIES OF SHAD (Alosa)

INTRODUCTION

Animal mtDNA is maternally inherited and apparently does not undergo recombination (Avise and Lansman 1983, A.C. Wilson et al. 1985). As a consequence, the mitochondrial genome can cross species boundaries unchanged. Interspecies transfer of mtDNA has been well documented among species of frogs (Spolsky and Uzzell 1984), mice (Ferris et al. 1983a), voles (Tegelstrom 1987), deer (Carr et al. 1986), and with less certainty, among species of Drosophila (Powell 1983). In these cases individuals typical of one species in terms of morphology, allozymes and/or other nuclear traits have been found to bear mitochondrial genomes typical of another congeneric species. Such cases are all believed to be the result of hybridization between two species followed by introgression of the hybrids into the paternal species in the original cross.

Here I report on a case of interspecies transfer of mtDNA among fishes. I have been studying mtDNA sequence variation among species of shad (Alosa), anadromous clupeid fishes of the Atlantic and Mediterranean basins. Two species of shad, the Allis shad (A. alosa) and the Twaite shad (A. fallax) occur in Europe and are sympatric over a broad range from the central Mediterranean to Northern Europe and the British Isles (Whitehead 1984). Although closely related (chapter 4),

alosa and fallax are morphologically distinct, and can be readily distinguished by external markings and gillraker number and morphology (Whitehead 1984).

I have analysed mtDNA isolated from samples identified as fallax from the River Severn in England, and from samples identified as alosa from the Garonne River in France. Below I describe evidence that two distinct types of mtDNA occur among these samples, but that these mtDNA genotypes are not aligned along species lines.

MATERIALS AND METHODS

A. fallax and A. alosa were collected during their spring 1986 spawning migrations in the Severn (U.K.) and Garonne (France) Rivers, respectively. Ovaries from ripe or nearly ripe females were flown on ice to Montreal. Mitochondrial DNA was isolated from the ovaries as previously described (chapter 1) within three days of the capture of the shad in Europe.

Mitochondrial DNA samples were digested with the following 16 hexanucleotide restriction endonucleases: SstI, SstII, KpnI, EcoRI, EcoRV, ClaI, SalI, PstI, XbaI, PvuII, ScaI, StuI, BclI, BglI, BstEII and HpaI. Restriction digests, 1% agarose gel electrophoresis and Southern blot analysis were performed as described in chapter 1.

RESULTS AND DISCUSSION

Restriction analysis of 10 fallax and 10 alosa mtDNA samples revealed two principal genotypes (I and II, Table 6; see also chapter 4, Figure 11). Genotype II was represented by three related genotypes that differed from each other by a single restriction site change. The two less common variants of genotype II each occurred only in a single individual (Table 6). The more common variants of the two genotypes, Ia and IIa, differed from each other by nine restriction site changes (Figure 10). This corresponded to a sequence divergence of approximately 1.2 % ± 0.45% (SD) (details in chapter 4).

Among the 20 shad assayed, type I mtDNA occurred in six fallax and one alosa, and type II mtDNA occurred in four fallax and nine alosa. This result suggests one of two possibilities: (1) The presence of shared genotypes in both species is the result of survival of mitochondrial lineages in both species that predate the divergence of the two species. (2) The two species have exchanged mitochondrial genomes through hybridization.

The first possibility is unlikely. If type I and II mtDNA were part of the intrinsic mitochondrial genetic variation of both fallax and alosa, then one would also expect the presence of a variety of less divergent genotypes in each species. Although the number of individuals examined in this study was small, the pattern of genetic variation for each species was clearly a sharply discontinuous one (Figure 10). Such sharp breaks in genetic relatedness are typical of some form of

secondary admixture, such as might be brought about by hybridization (Avise et al. 1987).

The second possibility, that alosa and fallax have hybridized, is plausible since the two species are closely related (chapter 4) and spawn in rivers at approximately the same time. This hypothesis does, however, pose at least one difficulty. Given their broad sympatry (Whitehead 1984), one might expect alosa and fallax to have well developed mechanisms for reproductive isolation.

Hybridizations between the two species could, however, be a consequence of population declines that have affected both species in different parts of their ranges. For instance alosa was historically abundant in the River Severn, but underwent a sharp decline in the latter half of the nineteenth century. It now occurs at a frequency of less than 1% among samples of shad from the Severn (M.W. Aprahamian, Welsh Water Authority, Pontypool, Wales, personal communication). The presence of small numbers of alosa among large numbers of fallax may have promoted interspecific spawning. The imbalance in numbers between the two species would also be expected to promote the introgression of the hybrids back into the fallax species, and hence the penetration of alosa mtDNA into a background of fallax nuclear genes. Relatively few generations of backcrossing to fallax following a hybridization event in which alosa was the maternal parent would be required to produce fish possessing primarily fallax nuclear genes but bearing alosa mtDNA (Gyllensten et al. 1985). A similar process may have facilitated the penetration of fallax mtDNA into alosa in the Garonne River, where

fallax is now rare (A. Belaud, Inst. Nat. Polytechnique de Toulouse, France, personal communication).

If the explanation above is correct, the hybridizations between fallax and alosa may have occurred as recently as the last century. The transfer of white-tailed deer (Odocoileus virginianus) mtDNA to mule deer (O. hemionus) is also thought to be a recent and possibly ongoing event brought about by range shifts that have brought the two species into increased contact (Carr et al. 1986). In contrast, in mice and voles the interspecific mtDNA transfers are believed to stem from hybridizations associated with founder effects that occurred thousands of years ago during post-glacial recolonization of northern habitats (Ferris et al. 1983, Tegelstrom 1987). In frogs as well, the data suggest that the hybridizations responsible for interspecies transfer of mtDNA occurred well in the past (Spolsky and Uzzell 1984).

A novel feature of the interspecies transfer of mtDNA in shad is that it has occurred in both directions. Previous cases of interspecies transfer have all been unidirectional (Ferris et al 1983, Spolsky and Uzzell 1984, Powell 1983, Carr et al. 1986, Tegelstrom 1987).

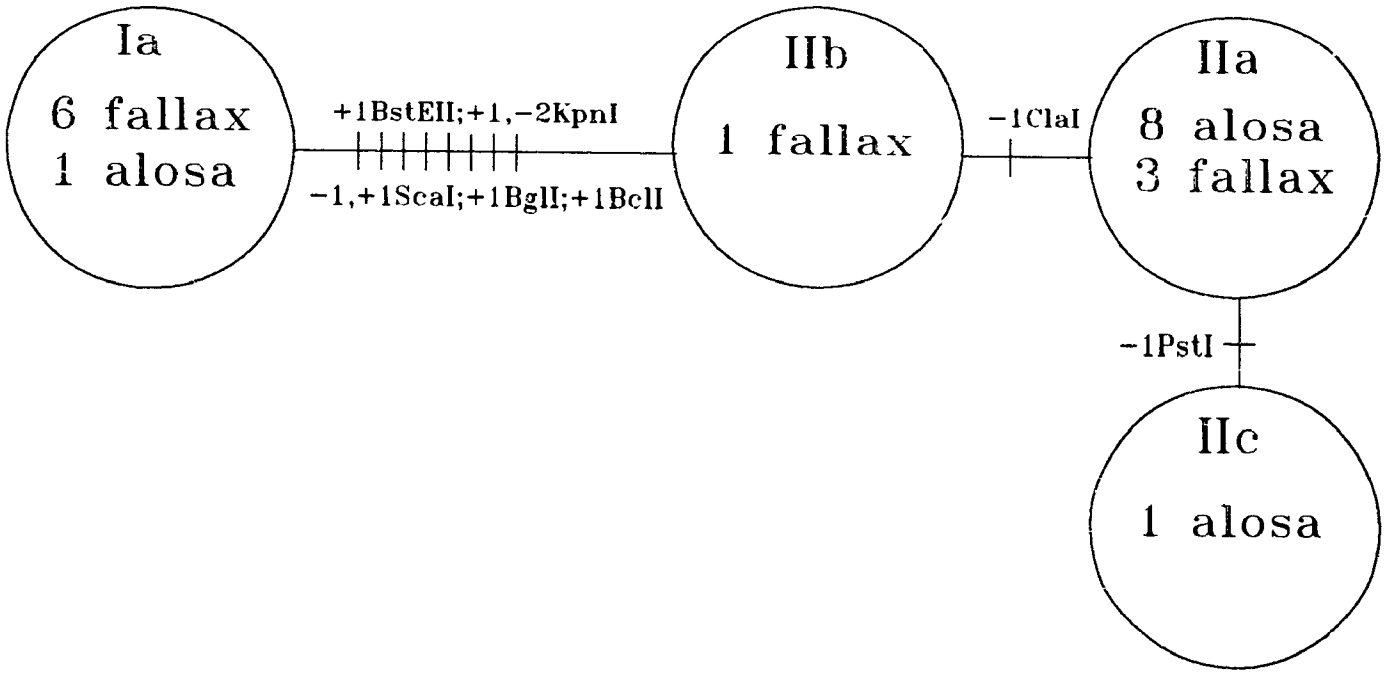
Clearly, the results reported here must be regarded as preliminary. Broader geographic surveys of both alosa and fallax will be needed to develop a firmer interpretation of the mitochondrial relationships of these species. Nevertheless, these results provide both a clear indication of the potential complexity of interspecific relationships, and strong evidence that evolution can be a reticulate as well as a branching process (Baverstock et al. 1983, Hasegawa et al. 1985).

Table 6. Composite genotypes of *fallax* and *alosa* samples.

SPECIES	--------GENOTYPE----------		N
	DESCRIPTION	DESIGNATION	
fallax	AAAAAAA	Ia	6
	BBBBBBA	)Ia	3
	ABBBBBA	IIb	1
			10
alosa	BBBBBBA	IIa	8
	BBBBBBB	IIc	1
	AAAAAAA	Ia	1
			10

The letters denote the single-enzyme genotype for each of the following enzymes that revealed variation among the samples: *Cla*I, *Bst*EII, *Kpn*I, *Bgl*I, *Sca*I, *Bcl*I, *Pst*I. For each enzyme, (A) and (B) denote the most common genotype of *fallax* and *alosa*, respectively, except for *Pst*I, for which the most common genotype (A) is the same for both species. Complete characterizations of the type Ia and IIa genotypes are given in chapter 4, Figure 11.

Figure 10. Parsimony tree indicating shortest mutational pathways between fallax and alosa genotypes. The lines crossing the branches denote the number of restriction site changes involved in the transition from one genotype to another. The actual restriction site changes (from left to right) are also shown.


Ia
6 fallax
1 alosa
+1BstEII;+1,−2KpnI
−1,+1ScaI;+1BglI;+1BclI
IIb
1 fallax
−1ClaI
IIa
8 alosa
3 fallax
−1PstI
IIc
1 alosa

CHAPTER 4

SLOW DIVERGENCE OF MITOCHONDRIAL DNA BETWEEN TWO LINEAGES OF SHAD (*Alosa*)

INTRODUCTION

A striking feature of the animal mitochondrial genome is the high and apparently uniform rate at which it evolves in a number of diverse vertebrate taxa. The mean rate of mtDNA divergence has been estimated to be near 2% per Myr in primate, rodent, perissodactyl and artiodactyl mammals, anseriform and galliform birds, and frogs (Brown et al. 1979, 1982, Higuchi et al. 1984, Ferris et al. 1983a,b, Upholt and Dawid 1977, Shields and Wilson 1987, Carr et al. 1987, also references in A.C. Wilson et al. 1985).

This apparent uniformity is consistent with evidence that mtDNA divergence among closely related lineages is largely selectively neutral (Avise and Lansman 1983, Avise 1986, Brown 1983, Moritz et al. 1987) and suggests that mtDNA sequence divergence constitutes a relatively good molecular clock. As a consequence, the 2% per Myr calibration has been widely applied to mtDNA divergences in groups outside of those in which it has been calibrated despite cautions that such usage is premature (Moritz et al. 1987).

In this chapter I describe evidence that the rate of divergence of mtDNA has been substantially slower than the 2% per Myr value among fishes of the clupeid genus *Alosa*. Members of this genus comprise two subgenera, *Alosa* and *Pomolobus* (Svetovidov 1963). The two subgenera

differ in a number of hard body parts including jaw, skull, operculum and otolith morphologies (Svetovidov 1963, Gabelaya 1975). Fossils attributed to both subgenera are common in Eurasian sediments of Miocene and Oligocene age (Romer 1966, Gabelaya 1975). Gabelaya (1975) concluded that Alosa diverged from Pomolobus during the Oligocene. Stinton (1977), however, attributed well-preserved otoliths of mid-Eocene age to species of both Alosa and Pomolobus. The divergence of these groups then, could date from either the mid-Oligocene or at least as early as the mid-Eocene, corresponding to divergence times of approximately 33 or 45 Myr, respectively (Harland et al. 1982).

I have been studying mtDNA sequence variation among five species of Alosa, including two of the Pomolobus subgenus, hickory shad (A. mediocris) and alewife (A. pseudoharengus), and three of the Alosa subgenus, American shad (A. sapidissima), twaite shad (A. fallax), and allis shad (A. alosa). Below I present estimates of sequence divergence among these species, which when combined with the fossil data described above, lead to an estimate of mtDNA divergence rate at least an order of magnitude lower than the 2% per Myr rate estimated in other vertebrates.

MATERIALS AND METHODS

Collection locales for all five species of Alosa are listed in Table 7. Mitochondrial DNA isolation, restriction digests, 1% agarose gel electrophoresis and visualization of DNA bands by EtBr staining and autoradiography of Southern blots were performed as described in chapter 1.

Table 7. Origins of shad samples on which restriction site maps were based.

SPECIES	SOURCE	DATE	N	COMMENTS
sapidissima	14 rivers Fla.-Que.	Jan-Jun 1985	21	most common genotype in 52 shad sampled over range of species (chapter 2).
fallax	R. Severn, U.K.	May 1986	5	most common genotype among 10 shad sampled (chapter 3).
alosa	Garonne R., France	Jun 1986	8	most common genotype among 10 shad sampled (chapter 3).
pseudo-harengus	Miramichi R., New Brunswick	Jun 1985	3	sole genotype observed.
mediocris	Cooper R., South Carolina	Mar 1985	3	sole genotype observed
	Savannah R., Georgia	Jan 1985	1	

Two methods, the double digest technique and selective probing of Southern blots with cloned A. sapidissima PstI mtDNA fragments (chapter 1, Maniatis et al. 1982), were used to map restriction sites recognized by the following 16 hexanucleotide restriction endonucleases in each of the five species of shad: SstII, SstI, PstI, SalI, ClaI, EcoRI, EcoRV, KpnI, XbaI, PvuII, BglI, StuI, BclI, HpaI, BstEII, and ScaI. The restriction site maps of the five species were aligned by scoring a conserved SstII site as map position 0 and a conserved EcoRI site as map position 100 (Figure 11). Note that this constitutes a reversal of the map orientation used in Figure 2, Chapter 1. This change was made to simplify comparison of mtDNAs that differed in size among species of Alosa.

Restriction sites pertaining to any given enzyme that mapped to positions within one map unit of each other in the mtDNAs of different species were considered homologous. Estimated mtDNA sequence divergences were calculated from the map data using the maximum likelihood method of Nei and Tajima (1983).

RESULTS

The mtDNAs of the five species of Alosa varied in size. Those of sapidissima, fallax and alosa were approximately 18.3 kb, whereas those of mediocris and pseudoharengus were about 16.8 kb. Mapping experiments indicated that the extra DNA present in each of the larger mitochondrial genomes consisted of a 1.5 kb tandem repeat of the type already described for sapidissima mtDNA (chapter 1). This conclusion

Figure 11. Restriction site maps of the mtDNAs of the five species of shad. The map for each species corresponds to the most common genotype observed for that species (Table 7). Abbreviated names of restriction sites are as follows: T, SstII; P, PstI; E, EcoRI; L, SalI; S, SstI; J, ClaI; C, EcoRV; X, XbaI; H, HpaI; M, BstEII; K, KpnI; V, PvuII; B, BglI; A, ScaI; U, StuI; O, BclI. Note that the scalar orientation of these maps is the reverse of that in Figure 2, chapter 1. One map unit corresponds to approximately 168 bp.

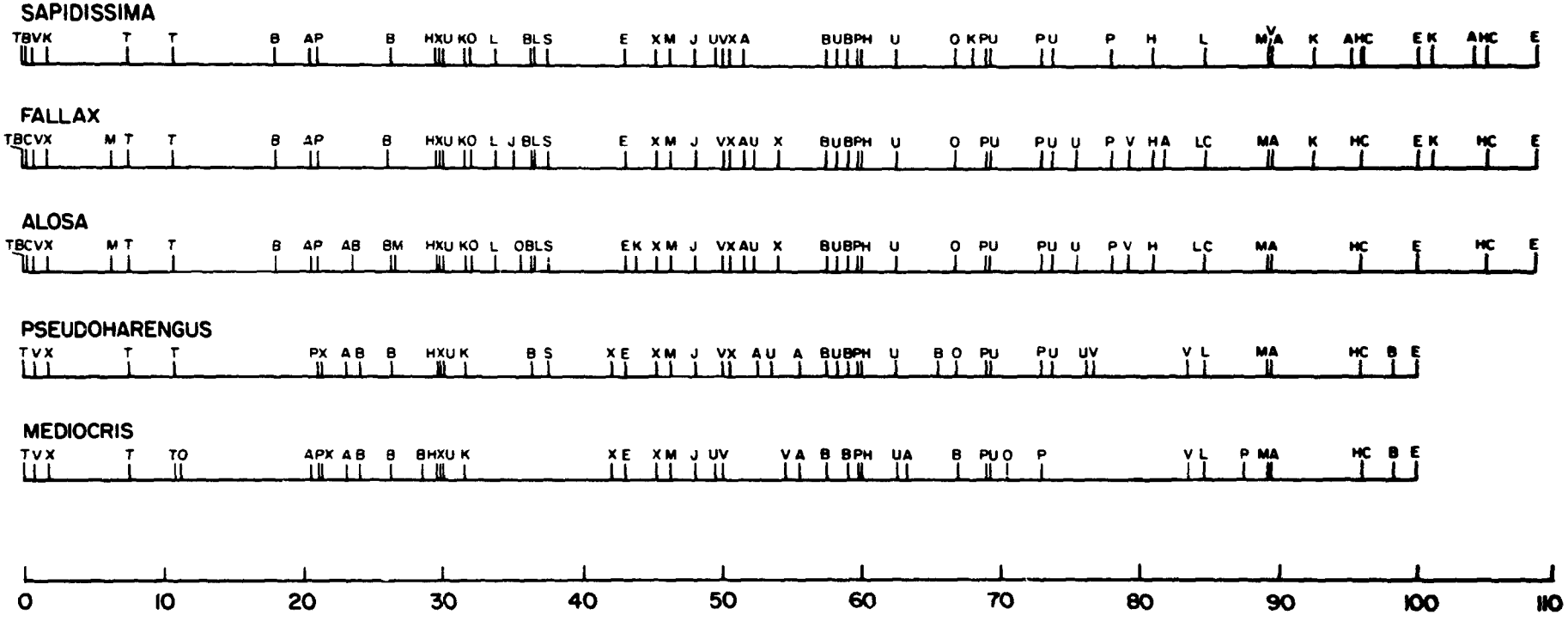

SAPIDISSIMA
FALLAX
ALOSA
PSEUDOHARENGUS
MEDIOCRIS
0
10
20
30
40
50
60
70
80
90
100
110

Table 8. Matrix of estimated percent mtDNA sequence divergences (above diagonal) and standard deviations (below diagonal) among species of Alosa. Asterisks denote comparisons between Alosa and Pomolobus subgenera.

	sapid	fallax	alosa	pseudo	mediоc
sapid		2.2	2.9	5.6*	6.1*
fallax	0.65		1.2	6.0*	7.6*
alosa	0.75	0.45		5.8*	7.9*
pseudo	1.21	1.23	1.20		4.1
mediоc	1.30	1.45	1.48	0.98	

follows from the fact that the extra DNA in fallax and alosa shared the location (between map positions 100 and 109) and a pattern of repeated EcoRI, EcoRV and HpaI sites characteristic of the tandem repeats in sapidissima mtDNA (Figure 11).

A total of 86 independent restriction sites were mapped in the mtDNAs of the five species. Five of these sites occurred in the extra 1.5 kb of DNA not shared by mediocris and pseudoharengus, and were not used in calculations of sequence divergence (Figure 11).

Estimated sequence divergences among the mtDNAs of the five species varied from 1.2% between alosa and fallax to 7.9% between alosa and mediocris (Table 8). The mean sequence divergence between members of the Alosa and Pomolobus subgenera was 6.5% (Figure 12a).

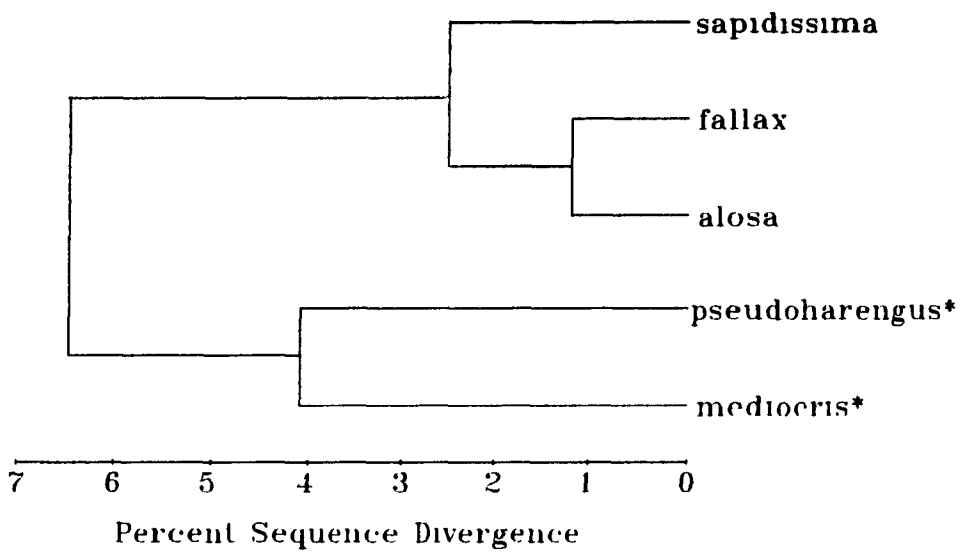
DISCUSSION

The mean mtDNA sequence divergence of 6.5% between the Alosa and Pomolobus subgenera leads to a comparatively low estimate of mtDNA divergence rate in shad regardless of which of the divergence times justified above are used. This estimate of the mean rate of divergence of shad mtDNA is 0.2 or 0.14 % per Myr based on either a mid-Oligocene (ca 33 myr BP) or a mid Eocene (ca 45 myr BP) split between the two groups. The higher of these possible divergence rates is one tenth of the mtDNA divergence rate estimated in other vertebrates (A.C. Wilson et al. 1985).

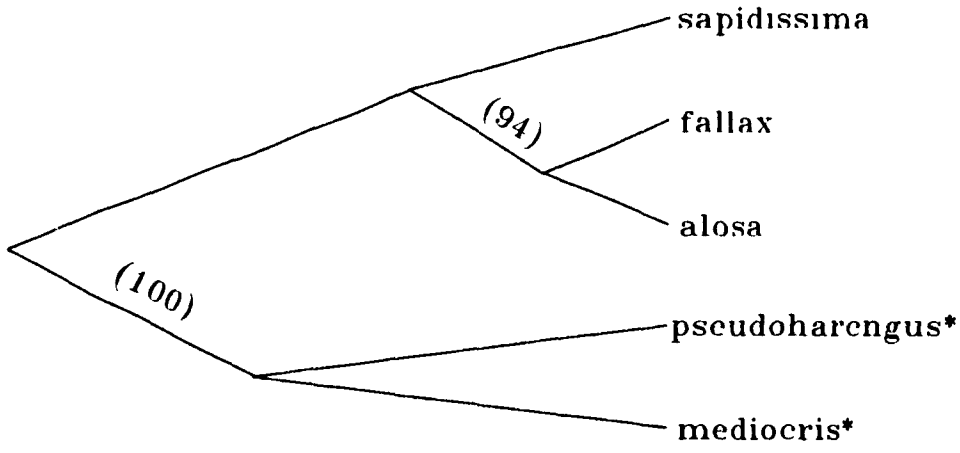
This estimate is subject to a number of uncertainties, including the possibility of errors in the restriction site maps on which the

Figure 12. The relationships of the five species of shad. A, UPGMA phenogram (Sneath and Sokal 1973) based on sequence divergence estimates in Table 8. B, Parsimony tree based on the phylogenetically informative restriction sites in Table 9. Numbers indicate the number of bootstrap replicates out of 100 that support the indicated clades. The tree and bootstrap estimates were generated using the MIX and BOOT algorithms in PHYLIP (Felsenstein 1986).

A


sapidissima
fallax
alosa
pseudoharengus*
mediocris*
7
6
5
4
3
2
1
0
Percent Sequence Divergence

B


sapidissima
(94)
fallax
alosa
(100)
pseudoharengus*
mediocris*

sequence divergence estimates were based. Although the restriction site maps (Figure 11) are the product of a large data set (on average more than 10 single and double digestions were performed for each of the 86 sites mapped) the methods I used are subject to various limitations. For instance, EtBr staining and Southern blot analyses do not permit the visualization of DNA fragments smaller than 250 bp; hence, no sites delineating such fragments appear in the maps in Figure 11. Relatively few such small fragments, however, would be expected with the hexanucleotide enzymes that I used in the mapping study, since such enzymes typically produce less than five fragments when used to cleave 17 kb mtDNA molecules (Avise and Lansman 1983, Lansman et al. 1981). Moreover, comparisons between sequence divergence estimates based on map data obtained with techniques comparable to those used in this study, and actual sequence data demonstrate that such estimates tend to be slightly higher than the actual sequence divergences (Avise and Lansman 1983, Ferris and Berg 1987).

The values in Table 8 are likely to be high rather than low estimates of interspecific mtDNA divergence for another reason as well. These estimates are based on the single most common mtDNA genotype that I observed in each of the five species (Table 7). I did not attempt to correct the estimates for intraspecific mtDNA sequence variation. Had I done so, the effect would have been to lower the divergence estimates (Nei and Li 1979, Wilson et al. 1985).

The interspecific mtDNA sequence divergence values presented here for species of shad are thus best regarded as high estimates. Accordingly, the rate estimates based on them are also high, provided

that the interpretation of the fossil record that the Pomolobus and Alosa subgenera represent distinct clades dating from the Oligocene or Eocene is correct. This last uncertainty cannot be totally eliminated with the data presently available. It is possible, however, to test the proposition that the extant members, at least, of the Alosa and Pomolobus subgenera represent distinct clades.

I tested this proposition by using a bootstrap program (Felsenstein 1986, 1985) on a presence-absence matrix of phylogenetically informative restriction sites in the mtDNAs of the five species of shad (Table 9). The bootstrap analysis supported a split between the Pomolobus species and the Alosa species in all of 100 replicates (Figure 12b).

The conclusion, then, that the mean rate of mtDNA sequence divergence in shad is substantially lower than in other vertebrates is justified. A relatively low substitution rate in shad mtDNA could be the result of a low mutation rate per round of DNA replication and/or a low fixation rate brought about by selective constraints on substitutions. At present no data are available that bear on either of these possibilities. Although evidence of non-random usage of synonymous codons suggests that even some supposedly neutral mtDNA substitutions are subject to selective pressures (Moritz et al. 1987), it is difficult to imagine that such selective constraints might be greater in shad than in mammals and other vertebrates with high rates of mtDNA evolution.

A low substitution rate in shad mtDNA might also be brought about by a relatively low rate of mtDNA turnover (Dover 1987). This

Table 9. Presence (1) or absence (0) of phylogenetically informative restriction sites in the mtDNAs of the five species of shad.

ENZYME	MAP POSITION	SAPI	FALL	ALOS	PSEU	MEDI
PstI	78.0	1	1	1	0	0
SalI	33.5	1	1	1	0	0
SalI	36.5	1	1	1	0	0
SstI	37.5	1	1	1	1	0
EcoRV	0.5	0	1	1	0	0
EcoRV	85.0	0	1	1	0	0
XbaI	54.0	0	1	1	0	0
XbaI	21.0	0	0	0	1	1
XbaI	42.5	0	0	0	1	1
HpaI	81.0	1	1	1	0	1
BstEII	7.8	0	1	1	0	0
KpnI	92.5	1	1	0	0	0
PvuII	79.5	0	1	1	0	0
PvuII	83.5	0	0	0	1	1
BglI	18.0	1	1	1	0	0
BglI	0.5	1	1	1	0	0
BglI	24.0	0	0	0	1	1
BglI	97.5	0	0	0	1	1
ScaI	20.5	1	1	1	0	1
ScaI	23.0	0	0	1	1	1
ScaI	55.5	0	0	0	1	1

Table 9. (continued)

ENZYME	MAP POSITION	SAPI	FALL	ALOS	PSEU	MEDI
StuI	52.5	0	1	1	0	0
StuI	76.0	0	1	1	1	0
StuI	49.0	1	0	0	0	1
StuI	74.0	1	1	1	1	0
BclI	67.0	1	1	1	1	0
BclI	31.5	1	1	1	0	0

possibility is supported by the fact that heteroplasmy is common in shad mtDNA (chapter 1); whereas, it is extremely rare in those vertebrates in which high rates of mtDNA divergence have been calibrated (Brown 1983, 1985, A.C. Wilson et al. 1985).

The long-term rate of fixation of divergent mtDNA types in the Pomolobus and Alosa clades might also have been slowed by lateral transfers of mtDNA from one lineage to another brought about by hybridizations between members of the two groups. This possibility is suggested by evidence of interspecific transfer of mtDNA between A. alosa and A. fallax (chapter 3). Data from other species in which such lateral transfers of mtDNA across species boundaries have been documented demonstrate that the hybridization events responsible need not leave detectable traces in nuclear genes or morphology (Ferris et al. 1983, Spolsky and Uzzell 1984, Tegelstrom 1987). Lateral transfers have also been suggested as a possible cause of anomalous divergence rates in some nuclear genes (Syvanen 1987). It seems unlikely, however, that lateral transfers of mtDNA between Pomolobus and Alosa could account for the entire ten-fold or more reduction in the apparent rate of divergence of shad mtDNA. For shad mtDNA to evolve at the "standard" rate of 2% per Myr, the most recent hybridization between Pomolobus and Alosa would have to have occurred as recently as ca 3.3 Myr BP, at least 30 Myr after the fossil record suggests that the two lineages began diverging.

Population-level processes may also have influenced the rate of evolution of shad mtDNA. Ohta (1976, 1987) predicted that, under a model of nearly neutral mutations, the rate of molecular evolution

should be inversely related to population size and the degree of environmental heterogeneity. Although mtDNA variation has generally been regarded as selectively neutral (A.C. Wilson et al. 1985), the non-random codon usage noted above (Moritz et al. 1987) as well as several other lines of evidence (MacRae and Anderson 1988, DeSalle and Templeton 1988, chapter 1) suggest that this may not invariably be the case. Moreover, Ohta's model is supported by evidence that the rate of mtDNA evolution has been three-fold higher in a lineage of Hawaiian Drosophila that has experienced repeated bottlenecks and founder effects than in another lineage of Hawaiian Drosophila that has not (DeSalle and Templeton 1988). Shad are marine fishes that occur in very large numbers (historically, at least 10^7 - 10^8 individuals, Walburg and Nichols 1967) and are capable of dispersing over large distances (chapter 2); hence, apart from possible Pleistocene bottlenecks (chapter 2), it is likely that over most of their evolution effective population sizes in shad have been large relative to those of the (primarily mammalian) vertebrates in which mtDNA evolution has been rapid.

The discrepancy between the "slow" mtDNA divergence in shad and the "fast" divergence estimated in other vertebrates can also be viewed in another light: the "fast" divergence estimates could be inflated (Fort et al. 1984). This possibility stems from the fact that although it is possible to establish relatively firm minimum estimates of divergence time from fossil or zoogeographic evidence, it is difficult, if not impossible, to establish firm maximum estimates, since the divergence

of the mitochondrial lineages involved could predate the fossil or zoogeographic events by a considerable margin.

This possibility is enhanced by differences in the magnitude of nuclear and mitochondrial gene flow. Assuming that males and females disperse equally, the rate of mitochondrial gene flow is expected to be one quarter that of nuclear gene flow (Birky et al. 1983). This bias in gene flow is increased if females disperse less than males, as is the case in many vertebrates (Lansman et al. 1981 and references therein). Empirical evidence of this bias in gene flow has been provided by surveys of a number of species in which rich patterns of geographically structured mtDNA variation involving divergences up to several percent were present, but in which geographic variation in nuclear genes was considerably less pronounced (Avise et al. 1979a,b, Avise et al. 1983, Lansman et al. 1983, Saunders et al. 1986). It is therefore possible that by the time levels of nuclear gene flow are reduced sufficiently to permit speciation, the mtDNAs of the incipient species could have been diverging for a considerable length of time (Avise et al. 1983). Evidence of such a scenario was reported by Fort et al. (1984), who found very low levels of allozyme divergence, but high levels of mtDNA divergence between two sibling species of mice.

The two possibilities raised above, that (1) population-level processes might have an important effect on the rate of molecular evolution, and (2) that mtDNA divergences might tend to predate nuclear genetic divergences could also, at least in part, account for the observation that the rate of mtDNA divergence in mammals appears to be 5-10 times that of single copy nuclear DNA (scnDNA) (Brown et al. 1979,

Brown 1983). The potential effect of the second possibility is clear, whereas that of the first possibility is less so. Under Ohta's (1976, 1987) nearly neutral model of molecular evolution the proportion of all mutations that are effectively neutral is an inverse function of effective population (N_e) size. For functionally haploid, maternally inherited mitochondrial genes, however, N_e is essentially one quarter that for diploid, bi-parentally inherited nuclear genes (Birky et al. 1983). As a consequence, the mitochondrial genome responds more strongly to bottlenecks than does the nuclear genome (A.C. Wilson et al. 1985). Hence, if evolution is accelerated by bottlenecks and low N_es in general, then this acceleration should be more marked in mitochondrial genes than in nuclear genes.

Both possible effects above would be less likely to influence real or apparent differences in the rates of mtDNA and scnDNA evolution in taxa characterized by very large effective population sizes and by high levels of both nuclear and mitochondrial gene flow. In the first case, very large population sizes would make the four-fold difference between the relative N_es of nuclear and mitochondrial genes unimportant, and in the second case, high levels of both nuclear and mitochondrial gene flow would tend to promote synchrony between the initiation of nuclear and mitochondrial genetic divergences. These propositions are supported by data for echinoids, animals that have pelagic eggs and larvae (and hence probably exhibit high levels of gene flow), and are highly abundant. In echinoids the ratios of mtDNA to scnDNA divergences are near unity (Vawter and Brown 1986, Moritz et al. 1987).

Many marine fishes, shad included, would also fit the above criteria well. In marine fishes population sizes are frequently very large, and the proportions of total genetic variation that occur between localities are generally low for nuclear genes (Gyllensten 1986), and preliminary data suggest that the same is generally true for mitochondrial genes (chapter 2, Avise et al. 1986,1987b, Graves et al. 1984, Wilson et al. 1987, Kornfield and Bogdanowicz 1987, Schweigert and Withler 1988).

There is evidence of heterogeneity in the rates of mtDNA divergence among invertebrates. Fossil based calibrations suggest mtDNA divergence rates of 0.23 - 2.2 % per Myr in echinoids (Vawter and Brown 1986, Moritz et al. 1987). Estimates of the rate of mtDNA divergence in Drosophila have ranged from as low as 0.2 % per Myr in the melanogaster group (Solignac et al. 1986b) to as high as 10 % per Myr in some Hawaiian Drosophila (Templeton 1987); however, DeSalle et al. (1987a) have criticized the validity of the low estimates, and favour an estimate of 2 % per Myr in Hawaiian Drosophila.

The processes that govern the tempo of molecular evolution are poorly understood, although they are generally believed to be stochastic in nature and hence at least roughly time-dependent (Wilson et al. 1977, Kimura 1983, 1987, Gillespie 1984, 1986). The molecular clock concept remains an attractive one, both in the realm of neutral mutation theory (Kimura 1983, 1987) and in the practical domain of inferring the age of phylogenetic events. The evidence that I have presented here, however, clearly does not support a universal molecular clock of mtDNA evolution. Among the various molecular clocks that have

been proposed, "fast" clocks such as mtDNA perhaps deserve particular caution since the sources of variance affecting estimates of time of divergence and tempo of evolution may be large relative to the absolute time scales involved. Whatever the underlying factors, my results provide evidence that the rate of mtDNA divergence in vertebrates can be at least an order of magnitude slower than previous data have suggested.

CONCLUSIONS

1) Two types of mtDNA heteroplasmy are common in American shad. One involves a major length polymorphism, the other a single restriction site. The length polymorphism is in the D-loop-containing region and consists of a tandemly repeated 1.5 kb sequence occurring in two and three copies, respectively in two principal size classes (18.3 and 19.8 kb) of shad mtDNA. Most shad are homoplasmic for the smaller size class of mtDNA, but some are heteroplasmic and contain both size classes. In addition to the major length polymorphism, minor (≤40 bp) length variation occurs within the tandemly repeated sequences, both among individuals and among copies of the repeat within individuals. These observations are in contrast to the view that was generally accepted at the start of this project, that both length polymorphism and heteroplasmy are rare in vertebrates.

2) Restriction site data indicate that each of the two types of heteroplasmy in shad mtDNA stems from multiple mutational origins. The geographic distribution of the major length polymorphism is skewed strongly toward the southern end of the range of the species, suggesting that the occurrence of the length polymorphism is influenced by selection. This is the first evidence of non-neutrality of mitochondrial variants.

3) The magnitude of intraspecific mtDNA sequence variation in American shad is low relative to many other species, but similar to that in a number of other diadromous species of fish. The low level of

mtDNA variation in shad and other diadromous species may stem at least in part from Pleistocene population reductions.

4) The distributions of two single-enzyme mtDNA genotypes in shad show clear geographic patterns. The non-random distributions of the genotypes demonstrate that river populations of shad are reproductively discrete and that shad are genetically differentiated with respect to their mtDNAs on at least a regional basis. The greater heterogeneity of genotype frequencies in the northern, glaciated, part of the range suggests that post-glacial founder effects have influenced mtDNA genotype frequencies in shad. A marked difference in genotype frequencies between two geographically close rivers that differ in the timing of the spawning migration, the Annapolis and the St. John, suggests that temporal effects may be an important determinant of mitochondrial gene flow in shad. The genetic differentiation evident in the mtDNA of shad stands in marked contrast to the absence of genetic variation in nuclear genes revealed by previous studies.

5) The evidence of genetic differentiation among populations of shad provides corroborating evidence for the hypothesis that latitudinal variation in the reproductive traits of shad may be genetically mediated. The mtDNA data also indicate, however, that southern semelparous and northern iteroparous shad populations are not phylogenetically discrete assemblages. The mtDNA data therefore suggest that the life history variation in shad, if genetically mediated, is likely to have evolved recently, i.e., since the Pleistocene.

6) Two European species of shad, A. fallax and A. alosa, have exchanged mitochondrial genomes through hybridization. Historical

trends in population numbers suggest that the transfer of *alosa* mtDNA to *fallax* at least, may have occurred during the last century. This is the first evidence of bi-directional transfer of mtDNA between species.

7) Mitochondrial DNA data support a phylogenetic split between two subgenera of shad, *Alosa* and *Pomolobus*. A fossil calibration supports an estimate of mean mtDNA sequence divergence rate between these two subgenera of 0.15-0.2% per million years. This estimate is at least an order of magnitude lower than estimates of the rate of mtDNA divergence in other vertebrates.

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