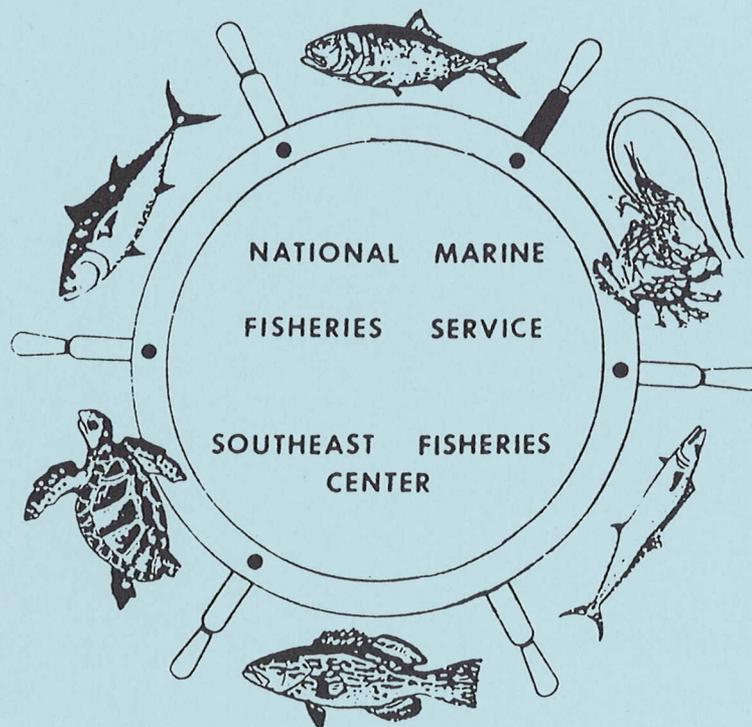


# PROCEEDINGS OF THE STOCK IDENTIFICATION WORKSHOP

NOVEMBER 5-7, 1985

PANAMA CITY BEACH, FLORIDA



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## PREFACE

The identification of discrete resource units, whether they be called stocks, populations, migratory groups or management units, is critical to the effective management of our aquatic resources. Advances in techniques and equipment have been made in recent years in the area of such identifications. A workshop to review these advances was organized by the Panama City Laboratory of the Southeast Fisheries Center, National Marine Fisheries Service. It was held at the Miracle Mile Complex, Panama City Beach, Florida, Nov. 4-6, 1985.

The major objective of the Stock Identification Workshop was to gain a more comprehensive understanding of stock identification problems and approaches to their solutions. The workshop was designed to give an opportunity to the participants to share information on methods of stock identification and to review their application to aquatic resources. The program format consisted of three sections.

Invited speakers presented critical reviews of both traditional and innovative stock identification methods. These review papers constituted the plenary session. Edited texts were submitted for external reviews for inclusion in these proceedings.

Section two contained the shorter experience presentations focusing on innovative techniques and application to fishery stock problems. In order to encourage state of the art applications and recently completed research, the contributed paper section is represented by abstracts only with a minimum of editing. The contributed paper section was moderated by the members of the Panama City Laboratory.

The final section of the workshop and these proceedings contains the discussion session. This interactive panel discussion with audience participation was moderated by Richard Shomura, who began discussion with an overview presentation. The discussion was audio and video-taped, transcribed and edited. Hopefully the editors have captured the essence of this most interesting exchange of thoughts, ideas and comments.

The editor and the organizing committee wish to acknowledge the support of the Panama City Scientific Society; American Institute of Fishery Research Biologists; Cooperative Institute for Marine and Atmospheric Studies, University of Miami; Georgia Sea Grant and Florida Sea Grant. We also wish to thank the following reviewers for their dedication and time: William Clem, Mary Fabrizio, Irving Kornfield, Michael Prager, James Shaklee, Richard Shomura, and James Zweifel.

The organizing and logistics team behind the workshop is acknowledged at the conclusion of the discussion section. We gratefully

acknowledge those involved in the development and completion of the workshop proceedings: Carol Parker for setting up the original abstract section of the contributed papers; Karen Patterson for the laborious transcription of the audio tapes from the panel discussion; Rita Bloechel for both the transcription of the panel discussion section and preparation of the initial plenary session manuscripts; last but certainly not least, Iva Walter, for preparing the bulk of the proceedings in their final form, and surviving the innumerable edits, revisions and numerous foibles of the editors.

Beyond the invaluable scientific exchange between participants, we hope that the workshop and these proceedings have raised the awareness level of scientists, fishery administrators and managers to the problems, applications and potential of stock identification research.

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## STOCK ASSESSMENT/STOCK IDENTIFICATION: AN INTERACTIVE PROCESS

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### INTRODUCTION

Stock identification is a nebulous term. When the senior author studied ichthyology and other similar courses, which at Cornell University were taught from a taxonomic and evolutionary point of view, he did not learn about stock identification. A great deal of time was spent examining the evolutionary relationships between species and higher levels of classification. Sub-species received some attention, but races, the classifications closest to stocks, were not of great concern. It was not in the courses that dealt with evolutionary and genetic relationships that the stock concept was presented, but in fishery science.

Courses on population dynamics aspects of fishery science, simplistically put, taught that one first estimated the growth rate, the natural mortality rate, the reproductive rate and the present fishing rate of a population of fish. Then by examining these rates in combination, an analyst could draw some conclusions concerning the status of that population and evaluate the effects of changes in fishing mortality on it. This simple theory, which can be expanded into some very elegant mathematics, deals with parameters that sometimes are almost impossible to estimate, but that can only be applied to a group of fish within which the estimated rates are meaningful averages. One could perform such an analysis and as a result state that, for example, if the age and the size at first entry into the fishery were increased, there would be an increased total weight of fish available for capture. However, one might find that when management implemented this advice, the fishing was concentrated on a subset of the group of fish that had a much slower growth rate than the average, and thus very few fish survived to reach the new legal size in the fishery. This would not have occurred had fishing been applied throughout the unit at a proportional rate on all components. Therefore, the

term stock and unit stock were defined as groups of fish within which these rates are meaningful averages from the standpoint of the fishery. The example above emphasizes another corollary of the stock concept -- that it is really a term that relates to the interaction between fishery stock assessment and fisheries management.

Saila and Jones (1983) summed up the state of the use of the term unit stock as follows: "Subareas of the total species range can have a characteristic single population or a set of sub-populations or groups. Either the characteristic population or the subgroups may be recognized as unit stocks in the biological sense and as preferred units for management ... The taxonomic status of the unit stock is unclear. In some cases the unit stock may be the species, in others it may be a taxonomic race within a species, and in others it may conform to no accepted taxonomic classification. However, distinct unit stocks have been perceived and labelled as such for many species. Unit stocks have been recognized and labelled as such based on physical characteristics since the turn of the century." In addition to physical characteristics, biological differences in growth and age at maturity have been considered. More recently biochemical methods have become common. In all cases some indications of significant degree of physical separation at spawning is required to support biological bases for separate stocks. Distributional observations often form the initial basis for hypotheses, but the evaluation of tagged fish is often required for more definitive conclusions to be drawn.

Reviewed in this paper are some of the major fishery assessment problems in the southeastern United States that involve stock identification concerns. Also examined are some case studies of historical stock-assessment/stock-identification interactions and the approaches required for developing a research strategy with appropriate tactics for addressing, on an ongoing basis, interactive stock- assessment/stock-identification issues.

#### STOCK IDENTIFICATION PROBLEMS IN THE SOUTHEASTERN UNITED STATES, PUERTO RICO AND THE U.S. VIRGINS ISLANDS

Some of the key species for which present or potential stock identification questions exist as presented in fishery management plans (FMPs) of the U.S. Regional Fishery Management Councils and the annual reports of the International Commission for the Conservation of Atlantic Tunas are listed in Table 1 and discussed below.

##### A. Billfishes.

Number of Stocks Recognized: The draft FMP (South Atlantic Fishery Management Council 1980) considers one western Atlantic

stock of each billfish species for management purposes, but notes the possibility of North Atlantic and South Atlantic stocks of blue and white marlins and eastern and western Atlantic stocks of sailfish. Little or no stock separation information is available for spearfish, because landings have historically been combined with sailfish. In general, the evidence for stock separation of Atlantic billfishes is inconclusive.

Management Implications of Stock Separation: If separate north/south or east/west stocks of billfish exist in the Atlantic, management of the species within the U.S. Exclusive Economic Zone (EEZ) may be somewhat more effective in maintaining or increasing catch rates by U.S. fishermen than if a single Atlantic stock exists. Even so, the highly migratory nature of these species (particularly marlins; transatlantic tag returns have occurred) (Conser 1985; Beardsley 1985) and their vulnerability to high-seas fishing make potential gains from unilateral management less certain than those for most other species managed under the Magnuson Fishery Conservation and Management Act of 1976 (MFCMA).

#### B. Swordfish.

Number of Stocks Recognized: The FMP (South Atlantic Fishery Management Council 1985) assumes that there is one northwest Atlantic stock, but notes the lack of supporting evidence. Definitive answers on stock structure are not possible at this time.

Management Implications of Stock Separation: According to the FMP, if more than one stock exists within the management unit, "It may not alter the likelihood of the variable season closure or other measures to produce benefits from delaying the harvest of small fish, but it could alter the distribution of those benefits. If there is substantial migration outside the management unit, then some of the benefits of larger fish may accrue to other countries" (South Atlantic Fishery Management Council 1985, p. 110). The Caribbean Council is particularly interested in knowing whether the fish caught in the Caribbean are from the same stock as those caught off the continental United States, because there is little historical data on which to base management and closures for that area. Fishermen have raised questions concerning observations of "different looking" fish occurring in their catches in the northeast area of the U.S. fishery. Swordfish have been caught in a continuous band across the Atlantic (Conser et al. 1985).

#### C. Sharks.

Number of Stocks Recognized: Shark stock structure is poorly known. There is insufficient information on the biology and

distribution of many species to draw conclusions about stock structure at this time.

Management Implications of Stock Separation: The preparation of a Shark FMP, or FMPs, is confounded by the fact that there are many species of sharks, many of which are difficult to identify, and for which there is little reliable information on biology and landings. Also, sharks are ecologically diverse, occupying pelagic and shallow- and deep-water benthic habitats. Species of management concern in one council area may not be the same ones of concern in another. For example, the large pelagic sharks, such as makos and blues, are of greater management interest north of Cape Hatteras than south of it, whereas some of the inshore sharks may be of greater management concern in the southeast. Stock separation within species is poorly known. Before effective management of sharks can be undertaken, some division of this very diverse assemblage of species will probably be necessary.

#### D. Atlantic Bluefin Tuna.

Number of Stocks Recognized: Like marlins, the possibility of either a single Atlantic stock or separate eastern and western Atlantic stocks exists, though some mixing of eastern and western Atlantic fish occurs. Management has been based on separate eastern and western stocks (Brown and Parrack 1985). The possibility of a separate Mediterranean stock also exists.

Management Implications of Stock Separation: Management through the International Commission for the Conservation of Atlantic Tunas is currently based on the two-stock hypothesis. Estimates of the extent of interchange are less than 10%, but variability of the interchange that occurs between the eastern and western Atlantic is not well known. Management is conservative in that separate regulations have been established for the two groups of fish. The highly migratory nature of this species, like billfishes, makes stock identification difficult and means that the species is fished well beyond the U.S. EEZ.

#### E. Coastal Migratory Pelagics (Mackerels).

##### Number of Stocks Recognized:

King Mackerel: Two "migratory groups" are recognized in the FMP (Gulf of Mexico Fishery Management Council 1985) based primarily on tagging data. These groups, the Gulf Migratory Group and Atlantic Migratory Group, are managed on the basis of a line of separation that moves seasonally along the Florida east coast. Other stock separations have been hypothesized, including east and west Gulf of Mexico stocks; these are based primarily on recent electrophoretic studies. Although king mackerel are known to

occur in the southern Gulf of Mexico, Caribbean, and off northeastern South America, the extent to which these fish interact with those within the U.S. EEZ is unknown.

**Spanish Mackerel:** The FMP notes genetic differences between Gulf and South Atlantic fish. Although there is no stock separation for management purposes at this time, the South Atlantic and Gulf of Mexico Councils are proposing that Gulf and Atlantic Spanish mackerel be treated as two separate management units in Amendment 2 to the Coastal Migratory Pelagics FMP.

**Cobia:** There is no evidence for stock separation at this time.

**Bluefish:** Although bluefish are not regulated under this FMP, Gulf of Mexico bluefish are considered a different stock from the Atlantic seaboard bluefish.

Management Implications of Stock Separation: The FMP lists stock identification as a major research need for both king and Spanish mackerels. The plan currently regulates primarily king mackerel, and imposes quotas and bag limits by area based on current stock separation into two migratory groups. The number and boundaries of these groups have major management implications because stock status and management measures vary between them. Due to recent evidence of recruitment overfishing and severe stock declines in the Gulf Group, allocations of fish in that group are very restrictive. Hence, the issues of where the line of separation between the Atlantic and Gulf Groups should be and whether there are one or more stocks within the Gulf of Mexico have major and immediate economic implications. In Amendment 1 of the FMP (Gulf of Mexico Fishery Management Council 1985), an artificial (based on socioeconomic and availability factors rather than on biological productivity) line of separation of the Gulf Group was drawn at the Alabama-Florida border to allow Louisiana fishermen more access to fish under a suballocation of the Gulf Group. Another issue is the extent to which Mexican catches of king mackerel in the gulf (which have been increasing) affect fish managed within the U.S. EEZ. Little information is available on mackerel catches from Cuba or elsewhere in the Caribbean, and the relationship of these fish to those caught within the U.S. EEZ is almost totally unknown.

#### F. Bluefish.

Number of Stocks Recognized: The draft Bluefish FMP (Mid-Atlantic Fishery Management Council 1984) proposes management based on a single Atlantic seaboard population. Gulf of Mexico bluefish are considered to be a separate stock and are excluded from this plan. Literature cited in the plan (Lund 1961; Wilk 1977; Anderson 1980) suggests that several subunits of the

Atlantic seaboard population may exist, but because separate stock assessment information and landings records are not available for each of these groups, the FMP proposes to manage them as a single population. The disjunct distribution of bluefish worldwide suggests that separate stocks exist outside U.S. waters, as well.

Management Implications of Stock Separation: Separation of Gulf and Atlantic seaboard stocks results in bluefish being managed under two different FMPs in the southeast; Gulf bluefish are included in the Coastal Migratory Pelagics FMP (Gulf of Mexico Fishery Management Council 1985). Considering the Atlantic seaboard fish as a single management unit simplifies regulations and data collection, but assumes that the subunits mentioned in the plan will be equally well managed by the proposed regulations. Because of the migratory nature of bluefish, the extent to which subunits are separated throughout the year is unknown.

#### G. Reef Fishes.

##### Number of Stocks Recognized:

**South Atlantic:** The reef fish species complex is managed as a single unit within the South Atlantic management area. Two stocks of seabasses are recognized in the Plan (South Atlantic Fishery Management Council 1983), with a break at Cape Hatteras.

**Gulf of Mexico:** The reef fish species complex is managed as a single unit within the Gulf management area (Gulf of Mexico Fishery Management Council 1981a). A possible faunal break may occur at about Mobile Bay, though insufficient data on most species precludes definitive stock separation at this time.

**Caribbean:** The Shallow-water Reef Fish Plan (Caribbean Fishery Management Council 1985) assumes that each isolated bank or island within the council's area of jurisdiction supports its own "stock" for management purposes.

Management Implications of Stock Separation: Because there is no evidence that reef fishes in the south Atlantic area are separable into discrete stocks (with the exception of seabasses), there appears to be little management implication of stock separation. In the Gulf of Mexico, the possibility of a faunal break in the northern gulf means that different management measures for eastern and western gulf reef fishes could be desirable. In the Caribbean, the question is one of recruitment (internal vs. external), with the same implications as those discussed under the Caribbean Generic Ecosystem FMP below. The Shallow-water Reef Fish Plan takes the conservative approach of treating each isolated geographic area as a separate stock for management purposes.

## H. Caribbean Generic Ecosystem Plan.

Number of Stocks Recognized: No distinction is made between stocks in the draft plan (Caribbean Fishery Management Council 1983), but the implications of internal vs. external recruitment to the fisheries of the shallow-water areas proposed for management are recognized.

Management Implications of Stock Separation: This plan is unique in that it proposes to manage stocks of multiple, diverse species as a unit based on ecological relationships, rather than setting regulations on a species-by-species basis. Under such a management regime, the separation of stocks at the species level would be less important than information as to what extent the platform ecosystem is self-sustaining. A major question is whether recruitment to the fisheries is from internal (spawned locally) or external (spawned elsewhere) sources. If recruitment is primarily external, then management of the fisheries could be based on socioeconomic grounds, rather than on concern for maintaining spawning stock. However, exploitation of the resources without regard for recruitment has broader implications, since management of the resources within the EEZ will likely affect the fishery resources in other areas. International cooperation and data sharing thus take on increased importance.

## I. Lobsters.

Numbers of Stocks Recognized: The main species in the management unit is the spiny lobster. Only one stock is recognized in the FMPs (Caribbean Fishery Management Council 1981; Gulf of Mexico Fishery Management Council 1982), but there is considerable debate over whether recruitment to areas such as the Virgin Islands, Puerto Rico, and south Florida is from "upstream" populations, from local sources, or both. Electrophoretic work (Menzies et al. 1978; Menzies and Kerrigan 1979) has indicated that at least some detectable differences in genetic makeup of Caribbean and Florida populations exist.

Management Implications of Stock Separation: The question of recruitment sources is important to management for determining whether or not local regulations will affect recruitment to that area. If not, then management might be based solely on socioeconomic considerations. If so, then management measures need to ensure adequate recruitment to the local fishery. Another issue is that of being "good neighbors" and setting a good example for fishery managers in other areas. If one area's larval production directly influences recruitment to a fishery "downstream," then there are broader questions to be considered, in addition to the biological ones. For example, if Puerto Rico's spiny lobsters were recruited from the Lesser Antilles, from Puerto Rico's standpoint there would be little biological reason

for fishermen to protect the spawning stock within Puerto Rican waters. However, if Puerto Rico's lobsters turn out to be the source of recruitment to the Dominican Republic, the Bahamas, or even the Florida Keys, then Puerto Rico's management of spiny lobsters would take on great importance to these other areas. Likewise, the management of lobsters in the Lesser Antilles would then be of concern to Puerto Rico.

#### J. Shrimp.

Number of Stocks Recognized: Due to "apparent genetic continuity, the need for a multipurpose approach to management, and the partial lack of data necessary to evaluate potential benefits derived by modifying current management practices," (Gulf of Mexico Fishery Management Council 1981b p. 4-17) the Gulf Council considers shrimp species as having gulf-wide stocks and manages the species in the fishery as a single management unit.

Management Implications of Stock Separation: The decision has been made to manage on the basis of single stocks. The annual nature of the shrimp crops means that management is primarily attempting to prevent growth overfishing. Biological stocks may exist, but because the fishery does not appear to be resource limited, this is of little management concern at present.

#### K. Stone Crabs.

Number of Stock Recognized: No stock separations are made in the FMP (Gulf of Mexico Fishery Management Council 1984), but delineation of stock units is recommended as a management need.

Management Implications of Stock Separation: At this time, no stock separations are made and the only major stone crab fishery in the United States occurs along the southwest coast of Florida. However, several small stone crab fisheries have developed along the coasts of other gulf states, such as Texas. If separate eastern and western gulf stocks were shown to exist and if the other developing fisheries became significant in size, different management measures would probably be considered for the different stock units. As long as fisheries outside southwest Florida remain small, there will be little reason to delineate stocks for management purposes.

### STOCK IDENTIFICATION/STOCK ASSESSMENT INTERACTION CASE STUDIES

Stock identification problems have been persistent throughout the history of fisheries management and stock assessment. There are, as we have seen, numerous serious questions concerning stock identification and stock intermixing in the EEZ of the

southeastern region of the United States of concern to the Southeast Fisheries Center and the Fishery Management Councils that it serves. The record of stock identification studies in addressing such problems, however, is not one that leads to a great deal of optimism for our ability to achieve simple, clearcut solutions.

### Yellowtail Flounder

Stock identification techniques that are genetically based often give results such as that for yellowtail flounder in the northeastern United States; they show a distinct difference between yellowtail flounder on the Grand Banks of Newfoundland and the Georges Bank-Southern New England area of the United States. Fish from these areas differ in growth rates, age at maturity and genetic characteristics. In addition, tagging studies have not indicated intermixing. Although these differences may be of great interest to those studying the biology and evolutionary relationships of this species, the interaction of the fish between the two areas was never in question. The topography of the ocean bottom and the distance between the areas of yellowtail flounder concentration all led to management under the hypothesis that the stocks are different.

This is not so, however, when one gets to the Georges Bank-Southern New England area. There the question of the existence of a stock on Georges Bank, and a stock off southern New England separated by a moderately deep but narrow channel, has been a real challenge to fisheries management. Small differences in growth rates between the two areas (Lux and Nichy 1969) could well be due to different environmental conditions, even with a common gene pool, or to different fishing patterns in the two areas. For example, older and larger fish have always occurred on Georges Bank and have commanded a higher price in the market, yet the historical existence of voluntary limits on the amount of fish brought in and the higher cost of fishing in the offshore areas of Georges Bank as opposed to southern New England could have led to greater selectivity for larger fish in the offshore area. Other studies did not indicate differences, but this does not in itself allow for acceptance of a one-stock hypothesis, only the conclusion that no evidence for a two-stock hypothesis has been found. Tagging studies done on these fish indicated that movement between the two areas was limited (Royce et al. 1959; Lux 1963). However, tagging data are difficult to interpret because, as is often the case in studying oceanic populations, logistical problems precluded a sound statistical design. Even though the recoveries can be interpreted against the distribution of fishing effort, accurate measurements of the distribution of fishing effort, although better than for many studies, are still somewhat crude. The questions of differential tag reporting, mortality

during tagging, and so forth, all contribute to increasing the uncertainty of the interpretation.

Since management for yellowtail flounder under a two-stock hypothesis would require more severe restrictions on one component of the fishery than would be the case under a one-stock hypothesis, this debate raged for a considerable period of time. Eventually the problem was solved, because heavy fishing effort on one component of the stock was reflected in an immediate decline in abundance in that area. The ability to follow different abundance trends in the two components after the initial decline with both fishery independent and dependent data indicated that, for a significant number of years (at least five), differential abundance continued to exist between the components. This was the strongest evidence possible, at least from a fishery standpoint, that the degree of intermixing (which everyone agreed occurred at some level) was not sufficient to eliminate the negative effects of very heavy fishing on one component of the resource. This validated the use of separate stock management though, over some period of time, the two stocks might in fact show some degree of leveling out, even if there were no regulations. In the fishery sense, however, we tend to look at periods of 5 to 10 years as being the length of time within which fishery managers strive to avoid having severe disruptions and hardships within fisheries; this length of time was not enough for the yellowtail flounder in southern New England and Georges Bank.

### Atlantic mackerel

Atlantic mackerel in the northwest Atlantic present another example of the difficulties in stock identification, stock assessment and fishery management. Sette (1950) had hypothesized two stocks, one spawning in the southern New England/mid-Atlantic area and the other in the Gulf of St. Lawrence. This was based on analysis of tagging data and on seasonal length frequency distributions. Sette also postulated that the southern contingent was by far the largest. In the 1950s, assessment and management based on a two-stock hypothesis was quite logical, since only a minor U.S. inshore fishery and a small Canadian fishery off Nova Scotia and in the Gulf of St. Lawrence were in operation. In the 1960s, the situation changed dramatically with the entrance of the distant water fleets into the offshore winter fishery off the coast of the United States. Catches rose to 435,000 metric tons in 1973 (Anderson and Paciorek 1980).

Egg and larval studies indicated the presence of mackerel eggs and larvae from the mid-Atlantic to the Gulf of St. Lawrence, but concentrations existed in southern New England and the Gulf of St. Lawrence (Berrien and Anderson 1976; MacKay 1973). Biochemical and meristic studies did not indicate separation of these two resources (MacKay 1967; MacKay and Garside 1969). There were some

indications from research cruises that juvenile fish from the two areas may be separate in the winter time, lending support to the two-stock hypothesis. The value of delineating the areas of concentration of spawning through egg and larval surveys, and the areas where juveniles are found through distributional studies, is vital so that the evidence from tagging and other studies can be used to develop stock hypotheses. Finally, tagging studies, though limited, indicated that mackerel from the Gulf of St. Lawrence area were taken in the winter fishery off the coast of the United States (Beckett et al. 1974; Moores et al. 1974; Stobo 1976). This necessitated analyzing the stock or stocks as a single unit for the purposes of fishery management.

There is still no definite answer as to whether or not the two groups maintain spawning integrity or schools from northern and southern areas stay separate during the winter time. Nevertheless, as long as fish from different breeding areas mingle during a portion of the fishery they have to be managed as a unit. It is important to realize that it is that interaction with the fishery in a mixing area that forces joint management.

#### Atlantic bluefin tuna

Atlantic bluefin tuna provide an excellent example of the interaction between stock assessment and fishery management. Atlantic bluefin tuna are managed by the International Commission for Conservation of Atlantic Tunas; this requires agreement from a number of nations based on an assessment of the status of the resource from the scientific committee of that Commission. Fisheries for Atlantic bluefin tuna initially existed in the northwest Atlantic, off Europe and North Africa, and in the Mediterranean. Fishing by Japanese longliners across the Atlantic indicated that there was at least some occurrence of fish continuously between the eastern and western areas. A decline in bluefin tuna was apparent in the western North Atlantic (Parrack 1981) but not necessarily in the eastern (Farrugio 1981). A question of a one- or two-stock management regime became critical. If all the North Atlantic bluefin were pooled together, there was no particular need for severe restrictions, but if they were separate the western North Atlantic required drastic action to allow recovery of the stock (Parrack 1982).

Parasite studies to separate bluefin stocks were inconclusive (Brunenmeister 1980). Biochemical studies strongly supported the hypothesis of a single stock (Thompson and Contin 1980). Tagging studies indicated that there was some degree of interchange, but the degree of interchange was open to question. Finally, the Commission, which had already established a regulation designed to stabilize fishing effort for the entire resource (ICCAT 1977), recognized that the most conservative position of concern for the status of the resource would be to put further restrictions on the

western stock, given that fishing effort would not increase in the eastern area. This strategy would allow the western stock to recover if the two-stock hypothesis were correct, and the total stock to have some build-up if the one-stock hypothesis were correct. This would be true so long as fishing mortality did not increase in the eastern area. Research continued on stock movements, utilizing chemical composition of vertebrae that eventually indicated that trans-Atlantic movement was probably restricted to about 10% of the fish per year (Calaprice 1986).

The assessment committees recommended advising the Commission that the movement might vary between years and that the degree of movement might also be a function of the relative abundance of the two stock components. Again, the best evidence for the two-stock hypothesis with a minimal degree of intermixing under recent conditions is that the stock in the western North Atlantic has begun to show some recovery. Research, however, must continue into the overall rates of exchange and their variability, since as the population rebuilds it may level off at a point lower than would otherwise occur if fishing effort in the eastern area were not brought under greater control.

#### DISCUSSION AND CONCLUSION

Stock assessment studies, starting with their terminology, have a considerably different orientation from the classical approach to the evolutionary genetic relationships between groups of organisms. Stock identification has implications for stock assessment needs, which are driven by management considerations. Sometimes groupings used for management may have only the very broadest genetic relationships, but are still useful in being logical entities for conservation and management actions to achieve desired goals. Sometimes the choice of which stock hypothesis to use for management purposes can be examined by sensitivity analysis, the effect of alternative stock hypothesis evaluated, and a rational decision made without the need for extensive, expensive research. This is important, because when one evaluates the gains resulting from management against the cost of management, the cost of research to provide information to that management must be considered.

A driving force in fisheries management is the availability of a resource to particular components of a larger fishery. This makes the question of stock availability and abundance a confounded one. In addition, over time, management concerns change as conditions in the fisheries change and the stock relationships initially accepted have to be reinvestigated in that light. The concept of "splitting verses lumping" must also be viewed in the light of management needs. Where there are experts in particular methodologies within a given laboratory, it is easy to become

excited about the potentials of those methodologies, particularly those that are on the cutting edge of new technology. However, it is critical that the questions being posed be examined by a variety of appropriate methods and be based on sound hypotheses. This involves first establishing the stock hypotheses to be investigated based on the available information. One should search for logical explanations for the observed data. These hypotheses should not only consider the number of stocks possible, but also whether or not they can be discriminated by the methodology proposed. It also means that statistical properties must be examined to ensure that the sample sizes and sampling design are adequate to discern the expected differences under the hypothesis of more than one stock. Individual variation must be considered, and sampling must recognize the possibility of school or small area integrity.

An important consideration that must be kept in mind when considering the interactions between stock identification research, stock assessment analysis, and fishery management actions is the shift from a strict production of information to the use of that information in a context that affects people's lives. Stock identification studies in one context can be considered a basic research emphasis. That is, analyses can be done and the results put forth, and these results, if they are in error, reflect primarily on the personal reputation of the individual researcher. There is no other complication. In fact, as in many initial scientific studies, an adequate hypothesis supported by the available evidence can result in future research that later shows that the original hypothesis was incorrect. This is perfectly proper and is the way science normally works. In fact, the researcher who does the further work that changes the hypothesis may well be the individual who put it forth in the first place. The consequences of putting these hypotheses out early in a study based on limited information may thus be sound in a scientific context and properly encourage further research.

In stock assessment analysis the purposes are different. By its very nature the assessment study does not put out information on only one piece of the puzzle concerning the status of a stock. Unfortunately, it also cannot wait until large amounts of information have been gathered. If that were done, only an elegant postmortem of the fishery could be built. Assessment analyses are similar to the work of the diagnostician in the medical profession. Assessment scientists are required to integrate all of the available information and evaluate the consequences of various hypotheses with respect to the effect on the resources of past, present, and future fishing. Assessment analysis is done for the purpose of providing information to those who have to make management decisions.

Fishery managers are often constrained by the information that is presented to them from the assessment analysis. They may choose not to act in a given way because they feel that the information presented to them is not sufficient to make a decision, but under most protocols established nationally and internationally, fishery managers have to act within the constraints of the scientific information presented to them. The consequences, therefore, of premature conclusions, be they stock identification conclusions or other aspects of the assessment process, can result in actions that restrict fishing which in turn affects people's livelihoods, the communities where they live, and in some cases, consumers. Therefore, the implications of assessment studies are very significant. An unnecessary action can cause severe social and economic disruption without any future benefit. Failure to act, on the other hand, can result in a long term reduction of the resource, causing even greater disruptions. It is important that the information from any particular analyses be correctly interpreted; to avoid confusion and inappropriate actions by management, results of such studies are best processed through analytical assessment procedures. The latter process should not be the work of any single individual, but rather a group of individuals skilled in these aspects of fishery science. This committee approach is exactly what international bodies have established and what is being set up and implemented by the Fishery Management Councils in the southeastern United States.

Finally, it is important that stock identification be recognized as a continuing process, evolving as management needs for stock assessments change, but always viewed against a background of a rational examination of all available data.

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Table 1. List of species with stock identification problems in the southeastern United States, Puerto Rico and the U.S. Virgin Islands managed under the Magnuson Fishery Conservation and Management Act of 1976 (MFCMA) and the International Commission for the Conservation of Atlantic Tunas (ICCAT).

Management Group	Management Plan	Plan Status	Councils	Quality of Stock Separation Information
<b>Billfishes</b>				
Blue Marlin ( <u>Makaira nigricans</u> )	Atlantic Billfishes	Draft FMP	South Atlantic Gulf of Mexico Caribbean Mid-Atlantic New England	Poor
White Marlin ( <u>Tetrapturus albidus</u> )				
Longbill Spearfish ( <u>Tetrapturus pfluegeri</u> )				
Sailfish ( <u>Istiophorus platypterus</u> )				
<b>Swordfish</b>				
Swordfish ( <u>Xiphias gladius</u> )	Swordfish	FMP	South Atlantic Gulf of Mexico Caribbean Mid-Atlantic New England	Poor
<b>Sharks</b>				
Sharks	Sharks	Draft FMP	Mid-Atlantic South Atlantic Gulf of Mexico New England Caribbean	Poor to none
<b>Tunas</b>				
Bluefin Tuna ( <u>Thunnus thynnus</u> )	None	None	ICCAT	Good

Table 1. (continued)

Management Group	Management Plan	Plan Status	Councils	Quality of Stock Separation Information
<b>Mackerels</b>				
King Mackerel ( <u>Scomberomorus cavalla</u> )	Coastal Migratory Pelagics	FMP	Gulf of Mexico South Atlantic	Fair to none sometimes conflicting
Spanish Mackerel ( <u>Scomberomorus maculatus</u> )				
Cobia ( <u>Rachycentron canadum</u> )				
In FMP for data collection only:				
Cero ( <u>Scomberomorus regalis</u> )				
Little Tunny ( <u>Euthynnus alletteratus</u> )				
Dolphin ( <u>Coryphaena hippurus</u> )				
Bluefish (Gulf only) ( <u>Pomatomus saltatrix</u> )				
<hr/>				
<b>Bluefish</b>				
Bluefish (Atlantic only) ( <u>Pomatomus saltatrix</u> )	Bluefish	Draft FMP	Mid-Atlantic South Atlantic New England	Fair to good
<hr/>				
<b>Reef Fish</b>				
South Atlantic: 8 families, 69 species	Snapper-Grouper	FMP	South Atlantic	Poor to none

Table 1. (continued)

Management Group	Management Plan	Plan Status	Councils	Quality of Stock Separation Information
Reef Fish (continued)				
Gulf of Mexico: 2 families, 33 species in management unit; 7 other families, 16 species included for data collection	Reef Fish	FMP	Gulf of Mexico	Poor to none
Caribbean  14 families, 64 species	Shallow-Water Reef Fish	FMP	Caribbean	Poor to none
Caribbean Generic Ecosystem Plan				
22 Approx. 110 species of fish, 26 groups of invertebrates	Fishery Resources of the Puerto Rican and Virgin Islands Geological Platforms	Draft FMP	Caribbean	Poor to none
Spiny Lobster				
Spiny Lobster ( <u>Panulirus argus</u> )	Caribbean Spiny Lobster	FMP	Caribbean	Poor
Spotted Spiny Lobster ( <u>Panulirus guttatus</u> )	Gulf of Mexico and South Atlantic Spiny Lobster	FMP	Gulf of Mexico South Atlantic	Fair
Smooth Tailed Spiny Lobster ( <u>Panulirus laeviscauda</u> )				
Spanish Lobsters ( <u>Scyllarides nodifer</u> , <u>S. aequinoctialis</u> )				

Table 1. (continued)

Management Group	Management Plan	Plan Status	Councils	Quality of Stock Separation Information
Shrimp				
Pink Shrimp ( <u>Penaeus duorarum</u> )	Gulf of Mexico Shrimp	FMP	Gulf of Mexico	Fair to none
Brown Shrimp ( <u>Penaeus aztecus</u> )	South Atlantic Shrimp	Draft FMP	South Atlantic	Poor
White Shrimp ( <u>Penaeus setiferus</u> )				
Royal Shrimp ( <u>Hymenopenaeus robustus</u> )				
Seabob ( <u>Xiphopenaeus kroyeri</u> )				
Rock Shrimp ( <u>Sicyonia brevirostris</u> )				
Stone Crabs				
Stone Crab ( <u>Menippe mercenaria</u> )	Stone Crab	FMP	Gulf of Mexico	None



USING MORPHOMETRIC AND MERISTIC CHARACTERS FOR  
IDENTIFYING STOCKS OF FISH

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INTRODUCTION

An animal species may be defined as groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups (Mayr 1963). Genetic or phenetic homogeneity among these groups over the entire distribution of the species is rarely observed due to heterogeneity and discontinuities in the environment or, simply, due to isolation by distance. Fish species are no exception to this generality and are usually subdivided into more or less distinctive groups which, in the case of commercially important species, are commonly referred to as stocks. I define stock as a self-sustaining group of individuals sharing a common and unrestricted gene pool. In terms of population genetics, a stock is a panmictic subunit of a species that is generally in Hardy-Weinberg equilibrium. Although random factors may influence geographic variability within a species, we generally assume that stock variability is important to the species for continued successful reproduction and adaptation. Therefore fish biologists have long sought to define stocks of fish, to understand the spatial and temporal dynamics of stock differentiation, and to apply these data to conserving and managing the species (see Canadian Journal of Fisheries and Aquatic Science, Volume 38(12)).

Identification of stocks of fish has long been the province of morphologists. Large data sets have been, and continue to be, collected for a diverse array of commercially important fish (see Table 1). But since the 1960s, there has been a surge of technical advances in the field of molecular biology (Ayala 1976; Nei and Koehn 1983) and the use of molecular characters in fisheries biology has increased dramatically (Ryman and Utter 1987). Theoretically, molecular data--from DNA to proteins--are superior for stock identification because of their direct and simple genetic basis. This accounts for the fact that three of the four papers in this symposium discussing character sets for identifying stocks of fish are based on molecular data. Still, in this flurry of molecular work, there have been parallel advances in the concepts and techniques of viewing, collecting, and analyzing morphological data. We may see a resurgence of morphological studies (pers. comm., J. Felsenstein, University of

Washington, Seattle, WA). Given the large number of molecular studies of fish, the best recourse for the morphologist is to continue to study morphological variability but to view it in light of its relationship with other molecular character sets. A better understanding of morphological characters will inevitably result. As Lewontin (1984) observed, "It often happens that the observed morphological differentiation is clear and statistically significant, while the differences in gene frequency are less powerful in discriminating populations and species."

My objective is to present some of the newest developments in the collection and use of morphological data for the identification of stocks of fish. I will focus on three areas: 1) types of characters, 2) data collection procedures, and 3) statistical analyses. Illustrations of these areas will be provided using data from my studies of milkfish (Chanos chanos) and chinook salmon (Oncorhynchus tshawytscha). I will also discuss several ways in which morphological data may be applied in concert with electrophoretic characters in management of fish stocks. This work is restricted to morphometric and meristic characters.

#### TYPES OF MORPHOLOGICAL CHARACTERS

A biologist studying morphological variation will make counts of elements in or along specific body parts and measure distances between distinguishing landmarks. In other words, he/she may collect meristic data and morphometric data. Standardized techniques for investigating these characters are described in Hubbs and Lagler (1947).

The most frequently used meristic characters are scales and fin rays. Scale counts described by Hubbs and Lagler (1947) include lateral-line scales, scales above and scales below the lateral line, circumferential scale count at the caudal peduncle and anterior of the dorsal fin, cheek scales, and scales before the dorsal fin. Counts of fin rays are taken for the median and paired fins. Other characters that are frequently used are counts of vertebrae, branchiostegal rays, pyloric caeca, gill rakers, and teeth.

For over 30 years, most morphometric investigations have based the selection of characters on the set of measurements described by Hubbs and Lagler (1947). These traditional morphometric characters measure length, depth, and width of fish shape, primarily in the head and tail regions. For example, in my study of milkfish, I collected data for the 16 characters depicted in Figure 1. These characters are not unlike those used in every study of morphometric variability listed in Table 1 (e.g., see Figure 1, Meng and Stocker 1984).

Recently, the use of these traditional morphometric characters has been questioned (Humphries et al. 1981; Strauss and Bookstein 1982; Bookstein et al. 1985). These workers argue that the conventional-type characters concentrate along the anterior-posterior body axis and in the head and caudal region and therefore produce uneven and biased areal coverage of the entire body form (see Strauss and Bookstein (1982) for details). Localized changes in body shape may go undetected, they argue, amidst the long distance measures across regions of the fish body. Their suggestion is to cover the shape or outline of a fish uniformly with a network of distance measures. This crisscross pattern along the body form is called a truss network (Humphries et al. 1981). Theoretically, this systematic characterization of the geometry of a fish form will increase the likelihood of extracting morphometric differences with biological meaning within and between species.

To establish a truss network pattern, morphological landmarks are identified along the outline (or surface) of a fish. Good landmarks are not identified by extremities, like the narrowest portion of the caudal peduncle, but by anatomical features. "Anatomical landmarks are true homologous points identified by some consistent feature of the local morphology" (Strauss and Bookstein 1982). For example, I used the 12 landmarks illustrated in Figure 2. There are numerous uses of conventional landmarks in a truss network (e.g., the origin and insertion of fins). I also found good results with points like #1 (Figure 2), the posteriormost point of the maxillary, at the closest point to the body on a line perpendicular to the horizontal axis of the specimen; point #4, posterior aspect of the neurocranium; and points #11 and #12, anterior attachment of the membrane from the caudal fin. Ideally, an equal number of dorsal and ventral landmarks are used. Six distance measures connect a set of two dorsal and two ventral landmarks, producing four peripheral distances and two diagonals. A set of six measurements for four landmarks is considered a cell. Five cells have been constructed across the form in Figure 2. In this case, with 12 landmarks, five cells are constructed yielding 26 distance measurements. Cells and truss characters may be referenced according to the scheme of Strauss and Bookstein (1982). For example, the distance between landmarks 1 and 2 is truss character 1-2 in cell 1, the distance between landmarks 9 and 11 is 9-11 in cell 5, and so on.

Will truss characters help in resolving stock differences? Humphries et al. (1981) and Strauss and Bookstein (1982) present several examples illustrating how truss network characters are better for discriminating between two species than are conventional characters. I have seen similar results working with chinook salmon. Namely, I collected juvenile chinook salmon in three locations and took 11 conventional and 33 truss measurements

on each individual (Figure 3). The results of a discriminant function analysis of the two data sets are presented in Figure 4. A scatterplot of scores from the analysis of conventional measures indicated minor between-sample differences (Figure 4A). In contrast, the analysis of the truss characters indicated there was essentially complete discrimination among the three samples (Figure 4B). The details of a similar comparison of groups of Pacific salmon have been reported (Winans 1984).

An apparent drawback to truss characters is making the larger number of measurements crisscrossing over the fish form--a caliper nightmare. New developments in electronic devices discussed below, however, have solved this problem.

#### COLLECTION OF DATA

"If you want to count scales or gill rakers... go ahead." (Anon.)

"Dividers or a dial-reading caliper should be used for measurements." (Hubbs and Lagler 1947)

Many meristic counts can be made by eye, depending on the specimen size and the character. In some cases for certain scale or ray counts, dissecting microscopes are necessary. In other cases, characters like vertebrae are counted from negatives of x-ray photographs. Frequently, a red dye may help identify special characters, like mandibular pores in salmonids (pers. comm., R. Leary, U. Montana, Missoula, MT). I am aware of one attempt to automate the collection of meristic data. McAllister and Planck (1981) describe an automatic counting probe, which can be attached to a computer or data-recording device. Their description:

"The automatic counter consists of a pen-like touch-sensitive probe, whose spring-loaded tip adds one to the count each time it is lightly pressed against a series of images or objects. Countable items might include fish scales, vertebrae on radiographs, or fishes in photos of schools. When the last item is counted, the SEND button at the top of the probe is pressed, transmitting the count to the computer and resetting the display counter to zero ready for the next count."

My reservations, without actually trying out the device, concern the mechanical sensitivity and facility of using the device on small elements such as gill rakers viewed under a microscope. Experimenting with this type of data-collection device may eventually lead to a versatile and time saving tool.

The collection of morphometric measurements can still involve hand-held calipers, meter sticks, and/or a measuring board. Once the data are taken in this fashion, they are generally keypunched into a computer for analysis. Several new electronic devices with computer orientation are available which speed up the process and eliminate some of the errors involved in collecting morphometric data. I will discuss in detail the use of a digitizing board because of my experience with it, and will briefly mention several other new devices.

Distances between morphological features on a fish can quickly and precisely be determined in the laboratory with an X-Y coordinate digitizing pad. The researcher first records the positions of certain morphological features or landmarks around the outline of a specimen using a set of X-Y axes on a digitizing pad. Distances between landmarks are then calculated from the X-Y data. I have described a 3-step process (Winans 1984) for collecting distance measures in this fashion. The following is a summary of the procedure.

1. Positioning

Specimens are placed on water-resistant paper, and body posture and fin positions are teased into a natural position. Positioning of specimens in this fashion is a precise process, as evidenced by low measurement error (see Winans 1984, Table 1).

2. Pinning

Distinctive and homologous landmarks are selected around the outline of the fish form. Each landmark is indicated and recorded by making a hole with a dissecting needle in the water resistant paper alongside its respective location. Data such as specimen number, body weight, and color are recorded alongside each specimen and added to the computer file when the landmark holes for that specimen are digitized.

3. Digitizing

After the landmark information from a set of specimens has been recorded (pinned), the paper is placed on an X-Y coordinate digitizing pad to establish a reference set of X and Y axes to view interlandmark distances. The X-Y coordinate values ( $\pm 0.01$  mm) for positions of the landmarks are indicated and recorded on a computer by depressing an attached digitizing stylus into each hole. Landmarks are digitized in the same sequence for each fish. Specimen identifying data are added via a computer terminal/keyboard and also stored with the digitizing information. The Euclidean or

morphometric distances between pairs of landmarks are then calculated by computer (using the Pythagorean theorem.)

This digitizing procedure speeds up and adds flexibility to the process of collecting morphometric data. As an example, I pin 80-100 specimens in an 8-hour day and later can digitize and calculate morphometric distances for 100 fish in about 90 minutes. This is much faster than measuring specimens with hand-held calipers. And importantly, the faster data-collection process facilitates collecting synoptic data (e.g., morphological and electrophoretic data) from the same specimens.

The use of a digitizing pad leads to procedural and analytical flexibility. Recording X-Y data for relevant landmarks on a fish outline provides the morphometrician with the capability of selecting traits for analysis without the need to remeasure specimens. For example, both a truss network and a conventional data set can be calculated from the same set of digitized landmarks and compared. Since the landmarks are essentially in a 2-dimensional plane, statistical adjustments for folded or twisted fish are not necessary (see unfolding statistics in Strauss and Bookstein 1982). Also, because of the 2-dimensional nature of this setup, digitizing fish shapes from photographs is possible with a high degree of precision (unpublished data).

Several other electronic devices should be noted. McAllister and Planck (1981) describe automatic calipers which are designed to transmit measurements to a computer, pocket calculator, or data-recording device. Although the selected calipers will limit the size of fish that can be measured, the procedure is accurate and fast. With a battery power supply, the setup becomes portable for field work.

Recent advances have been made in image processing that might also be applicable to the study of body outlines of fish (Ferson et al. 1985 and references therein). Basically, the procedure involves placing an object on a screen and tracing its silhouette by digitizing. The resultant closed curve is analyzed by fitting a set of mathematical functions (e.g., elliptic Fourier approximations). The Fourier coefficients are then analyzed with conventional multivariate statistics to view between-group differences. Notably, this image processing is done without homologous landmarks. Could it work with fish? Ferson et al. (1985) write,

"Because the present elliptic Fourier methods do not need continuous traces for input and work for any sequence of two-dimensional points, when landmark data are endowed with a natural or arbitrary order, elliptic Fourier description should be adequate to capture variation in relative landmark positions."

This procedure is currently being tested on fish (pers. comm., S. Ferson, State Univ. New York, Stony Brook, NY). Some authors question the usefulness and biological meaning of describing closed shapes with Fourier descriptors (Bookstein et al. 1982). As Ehrlich et al. (1983) point out, there is need for more empirical studies where the efficacy and interpretability of one technique can be compared with another. Image processing may prove to be a powerful technique for describing shapes in some instances.

### STATISTICAL ANALYSES

There is a basic difference between meristic and morphometric characters. Meristic characters are discrete, and can assume only integer values. In contrast, morphometric characters are continuous and assume the values of real numbers. Therefore, meristic and morphometric characters should not be considered in the same statistical analysis (Seal 1964). Moreover, the data should be transformed differently. It is frequently observed that measurement means and variances are correlated, the largest characters like fork length or lateral-line scales having the largest associated variances within their respective data sets. To decrease the effect of this correlation, the raw data are transformed. Sokal and Rohlf (1981) recommend transforming meristic characters to square roots and a  $\log_{10}$ -transformation of morphometric data. The latter transformation preserves allometric relationships among the characters (Jolicoeur 1963). For a multivariate analysis, such as principal component analysis, an alternative solution for meristic characters is to use a correlation matrix instead of a variance-covariance matrix.

With the collection of large sets of morphological data, how should the data be analyzed: univariately, bivariately, or multivariately? The answer depends on how we view morphological adaptation and evolution. I concur with Sokal and Rinkel's (1963) multivariate perspective:

"Geographic variation is not likely to be due to adaptation of a few characters to a single environmental variable, but is doubtless a multidimensional process involving the adaptation of many characters to a variety of interdependent environmental factors..."

Thus, a correct understanding of morphological variation is multivariate (Gould and Johnston 1972). We ought to strive to examine thoroughly the patterns of variance and covariance among all characters in a data set using multivariate statistics.

Multivariate analyses of morphometric data sets usually identify size and shape differences among individuals and groups. In compliance with current morphometric work, size and shape are considered factors--linear combinations of variables. Size is defined here not as a single character, but a factor that can predict any distance measurement (Humphries et al. 1981). Shape is defined as a specific relationship among characters as described by specific correlations, +, -, or 0, between the characters--a measure of geometry. For most stock identification work, shape discriminators are desired, as we can usually sort fish to size quite readily just by eye. Unfortunately, shape measures are not independent of size because of allometric relationships, and size-free shape estimators are difficult to obtain. This problem is discussed in detail in Humphries et al. (1981). I will present an overview of their arguments and recommendations.

There are three general approaches for removal of size influences in analyses of shape: ratios, regressions, and multivariate analysis. Simply stated, it is believed that the division of a character by a measure of size, say, fork length, will produce a size-free measure of that character. Similarly, if a measure is regressed against say, fork length, replacing the original measurement by its residual after regression will produce a size-free measurement (Thorpe 1976). The principal argument of Humphries et al. (1981) is that these ratios or regressions only remove the effect of the one variable, e.g., fork length, from the measurement. The third possibility for producing size-free shape components is through multivariate analyses such as discriminant function and principal component analyses. Humphries et al (1981) reject discriminant function analysis as a descriptive tool because of the difficulty in interpreting the coefficients in a biological context. For example, the interpretation of shape components is based on the coefficients in the discriminant function vectors. However, as Humphries et al. (1981) point out:

"From within a set of correlated characters only the variable with the highest F-statistic will be weighted heavily. Within that set, variables that do not contribute added discrimination will have low coefficients even though they contain nearly as much information about shape as the variable with the high F-statistic."

Campbell and Atchley (1981) and Williams (1983) likewise question the interpretability and stability of discriminant function coefficients. The recommendation of Humphries et al. (1981) is to use principal component analysis to view multivariable data sets. In principal component analysis individuals are not assigned a priori to groups, thus permitting "group differences to be

discovered." Moreover, principal component coefficients are essentially the covariance of the measurement on the component axis, and are thus amenable to biological interpretation. Before describing their new approach to making size-free shape components, I will briefly describe principal component analysis.

Principal component analysis computes a set of uncorrelated composite variables called principal components (hereafter PCs) from a variance-covariance (or correlation) matrix (Dunn and Everitt 1982). The first principal component (referred to as PC I) explains the most variance in the data set. Geometrically, PC I is thought to lie parallel with the largest axis in the hyperdimensional cloud of data (see Campbell and Atchley 1981; Green 1976). PC II is independent of PC I, that is, it lies perpendicular to the axis of PC I, and explains the second largest component of variation in the data set. PC III is independent of the other PCs and explains the third most variation, and so on for the other PCs. Each PC is a linear combination of the variables and is defined by a vector (an eigenvector) of coefficients and an eigenvalue. The coefficients are essentially a measure of covariance of the character on that PC. The eigenvalue is a measure of variability explained by a particular PC; the sum of the eigenvalues equals the total variability in a data set. Since on any component only a few characters have large coefficients, the biological interpretation of a component is based on the magnitude and signs of these so-called important characters. Examples of this are given below.

What about size-related problems? PC I characteristically has + signed coefficients for all measurements and is interpreted as a size vector. Samples and individuals sort by overall size on PC I. Subsequent components describe specific covariability relations or shape, as variables have + or - signed coefficients or are zero. Frequently, though, residual size effects are observed in these shape components. For example, in a plot of PC scores for a particular component, say, PC II onto the PC I axis, the ellipse of points for a sample is at a diagonal to the PC I axis instead of parallel to it. In other words, values of PC II are not independent of the size axis, PC I. Humphries et al. (1981) describe and illustrate a multivariable method called shear analysis for removing size from PC scores and vectors. It is a modified principal component analysis and uses scores from a second principal component analysis of centered (mean-adjusted) data by group to remove size influences (see p. 300, Humphries et al. 1981, for the six steps in shearing data; or Bookstein et al. 1985).

To illustrate visually how shearing works, I will use results from an analysis of morphometric data from chinook salmon. Judging from the eigenvectors (data not presented here), PC I was a size-related axis and PC II was a shape axis. A plot of scores

on PC I and PC II is presented in Figure 5A. Clearly the scatter of points for each of the two samples is oriented at a diagonal with PC I. Namely, the larger the fish, the smaller the PC II value. Following a shear analysis, the orientation of the scatter of points in each group is parallel with PC I (Figure 5B). Shape variability along the PC II axis is now independent of size. Any component other than PC I, whether from a meristic or morphometric data set, can be sheared in this way to eliminate size effects.

Presumably, we have arrived at a set of techniques which view character variance and covariance in large data sets to produce multivariate size and size-free shape descriptors. In conjunction with shear analysis, principal component analysis provides a set of rules, defined by shape eigenvectors, that define new shape variables. Scores on these shape components can then be evaluated for significant between-group differences in routine analyses, such as analysis of variance or multiple range tests.

#### MODEL FOR STUDYING TEMPORAL STABILITY

One of the principal problems in the use of morphological characters for stock identification is that morphological phenotypes are labile to environmental variability (discussed below). Therefore, before implementing size-free shape components in stock identification programs, we must examine the temporal stability of the multivariate relationships. It is important to know if between-year variability is less than between-stock variability for a given shape discriminator. I have outlined in Figure 6 a simple model for examining temporal stability in these characters. It requires a minimum of two years of data collection. It is applicable to either meristic or morphometric data, although in the figure and text I refer to the set of important characters in a component as a shape descriptor. For two years of data, there are two steps: Step 1 is a search for a size-free combination of morphological variables that is a good stock discriminator, and Step 2 is to determine the temporal stability of the discriminator and the respective differences among locales.

In the first year, data are collected from specimens from various locales, preferably while the fish are segregated onto spawning locations. A principal component analysis produces a size factor, PC I. Subsequent components are sheared (when necessary) to produce size-free components. Analyses are conducted on the scores from the sheared components to test if significant between-locale (stock) differences exist in the data and in what pattern.

Step two is essentially a repeat of Step one, except that the researcher tests the temporal nature of the results first revealed in year 1. Namely, he/she can first examine the correlation of eigenvectors, i.e., the correlation of the coefficients of sheared PC II from years 1 and 2; and secondly, examine the pattern of mean values by locale from Year 1 and 2. Nonsignificant differences between years for both of these tests add a great deal of confidence in the use of the respective PCs as stock discriminators. As illustrated in the bottom portion of Figure 6, the data from the two years may be pooled, and the principal component scores on the sheared axis (or axes) examined in a 2-way analysis of variance. In this analysis, the researcher can quantify differences in shape due to geography (between stocks) and to time (between years within locales). The most useful results with respect to stock identification are when stock shape values do not vary significantly from year to year. This does not mean that the morphological variability is primarily genetically determined, only that the influence of yearly environmental changes is less than the geographic differences. Note that this model is applied separately for meristic and morphometric characters. I will demonstrate the use of the model in the following example.

Example 1. Morphometric variation in juvenile chinook salmon.

I am interested in evaluating morphometric variability among stocks of juvenile chinook salmon for use in identifying the origin of fish while in mixtures in an estuarine or nearshore marine environment. In 1982 and 1983 fish were collected in estuaries and rivers along the Oregon coast (Figure 7A). They were frozen and taken to the laboratory for electrophoretic and morphometric evaluation. For simplicity, I report here the results of analyses on the four most geographically separated samples: Nehalem, Tillamook, Coquille, and Sixes.

Descriptive statistics of the samples are given in Table 2. Twenty-six truss network measurements were made on each fish using 20 digitized landmarks. The first principal component, PC I, explained 88% of the total variance and was a size-related component. Coefficients were roughly equal and positively signed for all variables on this component (Table 3). PC II and III explained 3 and 2% of the total variance, respectively. Other components explained less than 1% of the variance and are not considered further. The second and third components were sheared, producing the size-free shape components, sheared PC II (SPC II) and sheared PC III (SPC III). Important characters in both of these components were located in the tail, involving landmarks 9-12. An analysis of variance of SPC II and III scores indicated significant between-locale differences. Results of a Duncan's multiple range test of PC scores are displayed in Table 4. Fish

from Location #4 in the south (Sixes River) were significantly different from the other three locations on both SPC II and SPC III. In accord with Step 1 of the model (Figure 6), tail shape differences exist among four samples collected in 1982.

The 1983 samples, described in Table 2, were analyzed in the same fashion. Again, PC I was a size-related component and explained 89% of the total variance. Components II and III each explained 2% of the variation; they were sheared to produce size-free components. The correspondence between the eigenvectors of 1982 and 1983 was high, for example, characters 9-11 and 10-12 had the largest coefficients for SPC II and SPC III, respectively, in both years. The correlation of coefficients for 1982 and 1983 was 0.86 for SPC II and 0.76 for SPC III. However, the correspondence of the sample means on these two components between 1982 and 1983 was low, as shown in Table 4. In fact, the pattern of geographic variation was reversed from that seen in 1982. Although the three northern samples were still not significantly different at SPC II, the Sixes River sample now had the highest value of SPC II in 1983.

The 1982 and 1983 data were pooled for a principal component analysis to assess further geographic variability in light of annual variability. The eigenvectors were similar to those from the independent analyses (Table 3). The results of a multiple range test in Table 5 highlight the heterogeneous nature of the results seen in Table 4. For instance, Sample #3 (Coquille River) had the largest SPC II value in 1982 and the smallest in 1983. The results of a 2-way analysis of variance confirmed these findings. For scores on SPC II and III, the amount of between-year variance, as measured by F-values, was from 3 to 30 times greater than the between-locale variance. Clearly there is no temporal stability to the pattern of geographic variation in these samples.

A consideration of some preliminary growth studies of chinook salmon aids the interpretation of these results. The early life history of Pacific salmon is marked by a smoltification period during which considerable physiological, biochemical, and behavioral changes occur as the young fish prepare for the transition from freshwater to seawater (Folmar and Dickhoff 1980). I have studied body shape changes during early development in chinook salmon reared in hatcheries and reported a dramatic change in the shape of the caudal peduncle presumably associated with smoltification (Winans 1984). The pattern of change seen in a sample of hatchery fish along the SPC II axis is illustrated in Figure 8A. The important characters associated with these changes are illustrated in Figure 9A. Interestingly enough, the same characters are contrasted in the SPC II component in the above

study of wild chinook salmon (Figure 9B). For a comparison, mean SPC II scores for the eight Oregon samples are plotted in Figure 8B. A similar pattern of shape change with growth is seen. Apparently, discrimination among these samples is more a function of the degree of smoltification than true geographic differentiation.

With respect to the model in Figure 6, my conclusions are that in Step 1, tail shape characters were identified and inter-locality differences noted, and in Step 2, tail shape variability was again detected, but yearly variability was greater than between-locality differences. Other studies indicate that shape differences are related to ontogenetic differences.

I present the following study to demonstrate the use of a principal component analysis to describe a simple rule for identifying fish to group.

#### Example 2: Meristic and morphometric variability in milkfish.

I have investigated morphological and electrophoretic variability in milkfish from 15 locations in the Pacific Ocean (Figure 7B). One of the major observations is that fish from the Philippines differed electrophoretically and morphometrically from neighboring samples along the equatorial Pacific Ocean (Winans 1980; Winans 1985). In this example, I use the two southern Philippine samples P1 and P2 and the nearest sample from the equatorial Pacific island group, Palau (PAL), to demonstrate a particular use of principal component analysis.

I examined 6 meristic characters and 19 traditional morphometric characters on each fish. A principal component analysis of the meristic data transformed to square roots indicated extensive overlap among the samples and was not considered suitable for stock identification (see Figure 5, Winans 1985). A principal component analysis of log-transformed morphometric data revealed size differences along PC I, and considerable variation along the sheared PC II axis. There was a basic dichotomy in SPC II scores, viz., Philippine samples, differed from the non-Philippine samples, with one Philippine sample, Tahiti, and Christmas Island samples adding heterogeneity to this general pattern (Figure 10). Although a shape change associated with size was apparent in Hawaii, the magnitude of this change was not greater than, or overlapped with, the SPC II dichotomy discussed here. The difference between the Palau sample and the two samples from the Philippines is illustrated in a histogram of the SPC II scores (Figure 11A). The two groups do not overlap on this character axis.

Six characters had relatively large coefficients for SPC II (Table 6). Several head characters had large, positively signed

coefficients (orbital, snout, and premaxilla lengths), contrasted with three tail characters with negatively signed coefficients (caudal depth, body depth at anus, and length of anal fin base). My biological interpretation of this shape component based on the eigenvector is that the Philippine samples have smaller heads and larger tails in comparison to the Palau sample. For practical reasons, I wanted to see if I could go one step further than just identifying the important characters on a vector. I wanted to know whether this smaller set of important measurements by itself could be used to discriminate these samples. To test this, new SPC II scores were calculated for each fish using data from only these six variables. As an example of the calculations, the calculation of a SPC II score for fish  $i$  is:

$$\begin{aligned} \text{SPC II}_i &= \text{snout length}_i (0.317) + \text{orbital length}_i (0.397) \\ &+ \text{caudal depth}_i (-0.350) + \text{body depth}_i (-0.515) \\ &+ \text{anal fin base}_i (-0.253) + \text{premaxillary length}_i (0.258). \end{aligned}$$

(Note that these values will differ from Winans (1985) because overall character means were not subtracted first from each variable, i.e., absolute values differ but the relative values do not.) The SPC II scores, calculated from these six variables are plotted in Figure 11B. The difference between the two Philippine samples and Palau sample did not decrease, but in fact, increased slightly. I conclude that a principal component analysis of 19 morphometric characters identifies shape differences associated with the head and tail regions of the fish. There is no loss of discriminatory power when only the six most important characters are used to calculate a SPC II score.

## DISCUSSION

One of the most important recent developments in evaluating morphometric variability in fish is the truss network character set. It clearly is an objective procedure for uniformly covering the outline of a fish with distance measures for shape analysis. First, albeit few, applications of this technique indicate truss characterization of shape is more sensitive for detecting differences among species and, as is relevant here, among stocks. It could be argued that enhanced discrimination with truss data is due simply to the increase in absolute number of characters presented for analyzing. Whereas  $n$  characters will generally provide better discrimination than  $n-1$  characters (Speilman and Smouse 1976), I think in this case shape discrimination also increases due to the addition of more information about local changes in body shape. The generality of this technique will be tested as more traditional and truss character sets are compared.

Considerable advances have been made recently in the development of electronic equipment suitable for morphological investigations of fish. My focus here has been on digitizing the X-Y coordinates of morphological landmarks using an X-Y digitizing board. The digitizing procedure is fast, produces data sets amenable to the calculation of various types of distance measures, and it is precise. I routinely collect morphometric and electrophoretic data from the same individuals. I can quickly thaw specimens (frozen to preserve proteins), identify the relevant landmark positions by pinning, and then refreeze the specimens at a rate of 80-100 specimens per 8 hours. Landmark information for the 80-100 fish can then be digitized in about 90 minutes. Importantly, because of the fast pinning process, tissue preparations from the refrozen specimens can later be subjected to electrophoresis without any detectable change or deterioration in electrophoretic banding patterns. Moreover, measurement error in this process is small, less than 0.5 mm for most measurements (Winans 1984). A greater use of digitizing equipment in this area of research will eventually lead to an increase in the quality and quantity of information that can be gleaned from fish shapes.

Other technical developments also look promising. Technical advances in the field of image analysis will be followed closely by morphometricians (Rohlf and Felsen 1983). When a structure or outline is free of landmarks, e.g., bones or otoliths, perhaps the best approach will be to apply image analysis in conjunction with Fourier descriptors. But further investigations into the application of Fourier analysis of closed shapes are encouraged to resolve any questions and problems associated with this potentially powerful technique (see Bookstein et al. 1982; Ehrlich et al. 1983). Developments in the field of ultrasound digitizing, including 3-dimensional viewers, are also being examined as tools for fish morphometrics (pers. comm., A. Johnson, NMFS, Panama City, Florida). As a greater variety of techniques for collecting and viewing morphological data become available, I foresee a need for more comparative studies to determine which techniques will yield the best, most reliable discrimination. As Ehrlich et al. (1983) state: "There is no reason to expect that one morphological technique will yield equally good information for all investigators," or for all species.

Principal component analysis was presented here as a useful multivariate statistical procedure for viewing multicharacter variability within and among groups of fish. Principal components describe the major axes of character variability in simple character space; typically the first few components contain most of the variability in the data set. Clearly, understanding variation at a few composite PC variates is easier than trying to understand greater than  $n$  patterns of variability at  $n$  variables. The relative contribution of a variable to a PC variate is determined by the relative size of its PC coefficient. Thus, PC

analysis can be used to identify the important variables. If a large number of variables are examined in a pilot project, the number of variables which must be measured or counted in a subsequent, large scale study may be reduced. This was demonstrated in a simple case here for milkfish (Example 2). By interpreting the PC results in Table 6 as I did, I eliminated about 2/3 of the morphometric characters without a loss of stock discrimination (Figure 11).

Determining the number of PC components for analysis can be subjective. Frequently, components are dismissed if their associated eigenvalues are less than 1.0 (Tatsuoka 1971). In Example 1, I dismissed components after PC III, because the amount of variation explained in each of these components was 1% or less. To reduce this source of subjectivity, Gibson et al. (1984) have applied the jackknife procedure to principal component analysis. This technique provides estimates of variance of the coefficients in the eigenvectors and of the eigenvalues. This is done by iteratively removing data for one individual and redoing the component analysis. They demonstrate how it is used to identify stable, interpretable coefficients, and feel that this approach "should restrain a general tendency to over-interpret." The jackknife procedure, as a method to improve the statistical robustness of principal component analysis in stock identification, should be explored further.

The primary frustration in the use of morphological variation for stock identification is that the variability is not simply or directly inherited. It is generally assumed that substantial, but usually unknown, amounts of environmental influence may be involved with patterns of morphological variability. Some meristic characters are quite heritable (e.g., Leary et al. 1985a reviewed in Kirpichnikov 1981), but we know almost nothing of the genetic basis for the multivariate meristic or morphometric characters as defined by principal component analysis. The genetic basis of, say, head length for milkfish is unknown at the present time, not to mention the genetic basis for the composite variable SPC II discussed here. It is encouraging that recent work with rodents has shown that multivariate shape characters defined by multivariate analyses have a substantial heritable component (Atchley et al. 1981; Leamy and Atchley 1984; and Leamy and Thorpe 1984). Obtaining comparable estimates for most fishes is technically unrealistic now. It is more practical to evaluate the temporal stability of multivariate morphological components to determine their reliability as practical stock descriptors.

A model is presented for examining temporal stability of morphological variation among samples (Figure 6). It is an intuitively simple program for analyzing morphological data that has been collected for a minimum of two years. The model is based

on a multivariate analysis to describe multicharacter complexes as defined by specific eigenvectors. Each fish has a single value on each component. Therefore, it is the eigenvectors and their respective component scores which require evaluation. Suggested analyses are correlation studies of the eigenvectors and analysis of variance of the component scores. Although a few workers have collected two years of data in a study, none have completed any of the informative analyses presented here (e.g., Todd et al. 1981; Riddell et al. 1981). Studying quantitative characters (like fish shape) "is a difficult and somewhat slippery affair" (Lewontin 1974). Sound sampling and statistical analyses as suggested here will give satisfactory evaluation of morphological characters. Further understanding of the forces that direct morphological characters, be they environmental or genetic, may be gained in multicharacter studies of changes in the environment and genetic structure.

Since the advent of various molecular techniques in the 1960s, considerable expertise and extensive data bases have developed with respect to population genetics of fish. Provided with readily available genotypes, fish biologists interested in morphological variation can gain new and valuable perspectives of morphological variability. Information of the association of morphological phenotypes and biochemical genotypes may be useful in fish management and conservation programs (see Soule 1980). I present two examples, one at the individual level and one at the stock level, illustrating the complementary use of morphological and molecular (in this case electrophoretic) characters.

Asymmetry of bilateral meristic characters. Bilateral characters in organisms are usually not perfectly symmetrical. The number of rays in the left pectoral fin of a fish may not equal the number of rays in the right pectoral fin. Numerous studies of fish have looked at levels of asymmetry (e.g., Felley 1980; Graham and Felley 1985; Angus 1982). The findings of Leary et al. (1984) are perhaps most pertinent to the management of fish stocks. Leary and his colleagues have examined electrophoretic variability (measured as heterozygosity) and asymmetry in several salmonids. Their general conclusion is that there is a negative relationship between heterozygosity and asymmetry between individuals within a population. That is, individuals with the most heterozygous loci are likely to have the fewest number of asymmetrical characters. Moreover, they have noted that obviously deformed rainbow and cutthroat trout are more asymmetrical in their bilateral characters than are normal individuals (see Figure 1, Leary et al. 1984). They conclude that asymmetry may be negatively correlated with biological fitness. Concerning measurements of asymmetry of meristic characters, they write (Leary et al. 1985b):

"We envision the most valuable use of this technique to be the monitoring of populations through time. A progressive increase in average asymmetry would indicate a loss of genetic variation through inbreeding or an increase in environmental stress. The ideal monitoring program would combine an examination of allele frequency changes at isozyme loci and changes in fluctuating asymmetry. Such a program would be able to both detect the loss of genetic variation and simultaneously evaluate the effects of such loss on the population."

As a potential tool for use in fisheries, as Leary et al. envision, it is important to determine the relationship, if any, between bilateral meristic asymmetry and electrophoretic variation in other commercially important fish.

Characters used in mixed stock fisheries. A common practice in fisheries science is to estimate proportions of various stocks in a mixed-stock fishery, when samples and baseline data are available from all contributing stocks (Milner et al. 1985). An important issue in problems of mixed stock fisheries is the selection of characters. Ideally characters are discrete, expressed independently of environmental variation, temporally stable, and cost effective. Allele frequency differences at protein-coding loci detected by protein gel electrophoresis generally fulfill these requirements (e.g., Grant et al. 1980; Beacham et al. 1985a and 1985b).

The work of Fournier and his colleagues has expanded the statistical model for mixed stock fisheries in two important ways. First, they have extended the model to include the use of several types of continuous and/or discrete characters simultaneously (Fournier et al. 1984). Conceivably the model can accept principal component scores of morphological data that are shown to be temporally stable. Furthermore, Fournier and his colleagues are working on another version of the model which may also accommodate so-called nonstationary characters (pers. comm., C. Woods, Fisheries and Oceans, Nanaimo, Canada). Nonstationary characters are characters that vary from year to year, and in one year, may or may not be helpful discriminators, and/or are characters which can not be measured for a database, and their stock specificity is unknown. In the proposed mixed fisheries model, each iterative step of the maximum likelihood analysis makes estimates of the stock proportions and the proportions of the nonstationary characters in the contributing stocks. The latter estimates are then reapplied to the next stock estimates. If nonstationary characters vary sufficiently among stocks, their inclusion will help stock estimates, otherwise, nonstationary characters will not affect the process. This means then that

characters such as meristics and morphometrics, as well as scale patterns, parasites and egg size will only positively affect stock estimates. The concept of this model epitomizes the use of multicharacter data for solving a fisheries problem.

In summary, I have presented some of the recent developments associated with collecting and applying morphological data in identifying and managing stocks of fish. It has been observed that many of the disciplines in biology that once were the exclusive domain of morphology have been assumed and, in some instances, taken over by molecular-oriented technology (e.g., Lewin 1985). Therefore, I have concluded this paper by presenting examples of how combinations of morphological data and molecular data (i.e., electrophoretic) can be potentially more useful than either character set alone in both genetic conservation programs and management programs. We have a lot to learn about fish genotypes and phenotypes; examining the association of different character sets at the individual and population levels is an important first step in this field of research. I feel we should continue our research of morphological variability in fish, especially in coordination with research of other character sets, testing and using as many new ideas and technologies as seems necessary and appropriate.

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Table 1. Representative morphological studies of stock structure in commercial fishes.

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<u>FRESHWATER</u>	
Bluegill	Felley 1980
Brook trout	McGlade and MacCrimmon 1979
Lake whitefish	Casselman et al. 1981 Ihssen et al. 1981
<u>DIADROMOUS</u>	
Atlantic salmon	Riddell et al. 1981
Pacific salmon	Beacham 1985 Hjort and Schreck 1982 Winans 1984
Smelt	Copeman 1977
Shad (meristics only)	Gabriel et al. 1976
<u>MARINE</u>	
Herring	Meng and Stocker 1984
Milkfish	Winans 1985
Capelin	Sharp et al. 1978
Flounder	Wilk et al. 1980

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Table 2. Descriptive statistics of juvenile chinook salmon collected in August-September of 1982 and 1983. Sample locations illustrated in Figure 7.

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	Sample size	1982			1983			
		minimum	mean	maximum	Sample size	minimum	mean	maximum
1. Nehalem	42	109	120	143	11	127	138	152
2. Tillamook	56	97	121	147	26	104	127	165
3. Coquille	51	92	110	132	24	98	106	122
4. Sixes	50	82	96	112	25	92	114	126

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Table 3. Variable coefficients on principal components I through III. Components II and III were sheared by method in Humphries et al. (1981) to produce size independent components. Coefficients are X 100. Refer to Figure 2 for characters.

Character	PC I			Sheared PC II			Sheared PC III		
	1982	1983	Pooled 82 & 83	1982	1983	Pooled 82 & 83	1982	1983	Pooled 82 & 83
1-2	14	15	15	-11	-5	-12	7	5	3
1-3	20	18	20	-9	-20	-7	-16	-5	-15
2-3	17	17	17	-9	-12	-10	-3	2	-4
1-4	16	15	16	-6	0	-6	-2	40	2
2-4	14	14	14	-8	-2	-7	-2	8	0
3-4	19	19	19	-5	-8	-10	0	3	4
3-5	20	21	20	-5	-13	0	-4	-14	-17
4-5	21	21	21	-6	-11	-4	-2	-8	-8
3-6	19	21	20	-7	-1	-4	-2	-14	-5
4-6	21	21	21	-4	-5	-2	-2	-22	-9
5-6	20	22	21	-11	-1	-12	-1	-1	2
5-7	21	20	20	-13	6	-2	-2	-1	-15
6-7	20	21	21	-10	-1	-6	-2	3	-7
5-8	20	22	21	-10	2	-11	-1	-6	3
6-8	18	19	17	-7	-16	-2	-4	43	-11
7-8	22	21	22	-10	4	-7	-2	-12	-3
7-9	19	17	19	-4	-20	-7	-6	1	-4
8-9	21	20	21	-3	-3	1	-6	-13	-10
7-10	20	20	21	-6	-5	-5	-13	-8	-8
8-10	24	21	22	3	-2	19	-35	-43	-41
9-10	19	21	20	-9	3	-16	20	20	22
9-11	24	23	23	80	85	87	-39	-9	0
10-11	19	19	19	18	23	8	22	27	34
9-12	21	21	21	30	11	23	11	13	18
10-12	18	17	19	32	7	5	74	56	70
11-12	19	19	19	-8	-20	-16	13	15	12
% of total variance explained	88	89	88	3	2	3	2	2	2

Table 4. Results of Duncan's Multiple Range Test of sheared PC scores of chinook salmon. Solid horizontal lines indicate samples which are not significantly different. Component scores were calculated from two independent principal component analyses of 1982 and 1983 data. From north to south, sample codes are 1 = Nehalem, 2 = Tillamook, 3 = Coquille, and 4 = Sixes. Mean component scores (X 10,000) are presented below sample codes.

	Sheared PC II				Sheared PC III			
1982	4 (-345)	1 (74)	2 (76)	3 (184)	1 (-163)	2 (-75)	3 (-6)	4 (300)
1983	3 (-334)	1 (-56)	2 (95)	4 (247)	3 (-167)	4 (-102)	1 (5)	2 (247)

Table 5. Results of Duncan's Multiple Range Test of sheared PC scores of chinook salmon. Solid horizontal lines indicate samples which are not significantly different. Component scores were calculated from a principal component analysis of the covariance matrix of pooled data from 1982 and 1983. PC scores were sheared by method of Humphries et al. (1981). From north to south, sample codes are 1 = Nehalem, 2 = Tillamook, 3 = Coquille, and 4 = Sixes; collection dates are 1982 (= 82) and 1983 (= 83).

1982 + 1983 data							
Sheared PC II							
3-83	4-82	1-83	2-83	4-83	2-82	1-82	3-82
Sheared PC III							
1-82	2-82	3-82	4-82	3-83	1-83	4-83	2-83

Table 6. Variable coefficients on principal components I and II from an analysis of morphometric characters in milkfish. PC II was adjusted or sheared by algorithm in Humphries et al. (1981).

Morphometric character	PC I	Sheared PC II
Fork length	0.222	-0.131
Length snout-anal fin origin	0.222	-0.132
Length snout-pelvic fin origin	0.210	-0.101
Length snout-pectoral fin origin	0.212	0.177
Length snout-dorsal fin origin	0.223	-0.107
Head length	0.214	0.232
Snout length	0.249	0.317
Postorbital length	0.222	0.089
Orbital length	0.199	0.397
Caudal depth	0.215	-0.350
Body depth at anus	0.228	-0.515
Length dorsal fin base	0.223	-0.117
Length anal fin base	0.221	-0.253
Length pectoral fin base	0.252	0.025
Pectoral fin length	0.239	0.215
Head width	0.247	-0.095
Nares width	0.267	0.067
Bony interorbital width	0.256	0.018
Premaxilla length	0.222	0.258
% of total variance	97	1.0

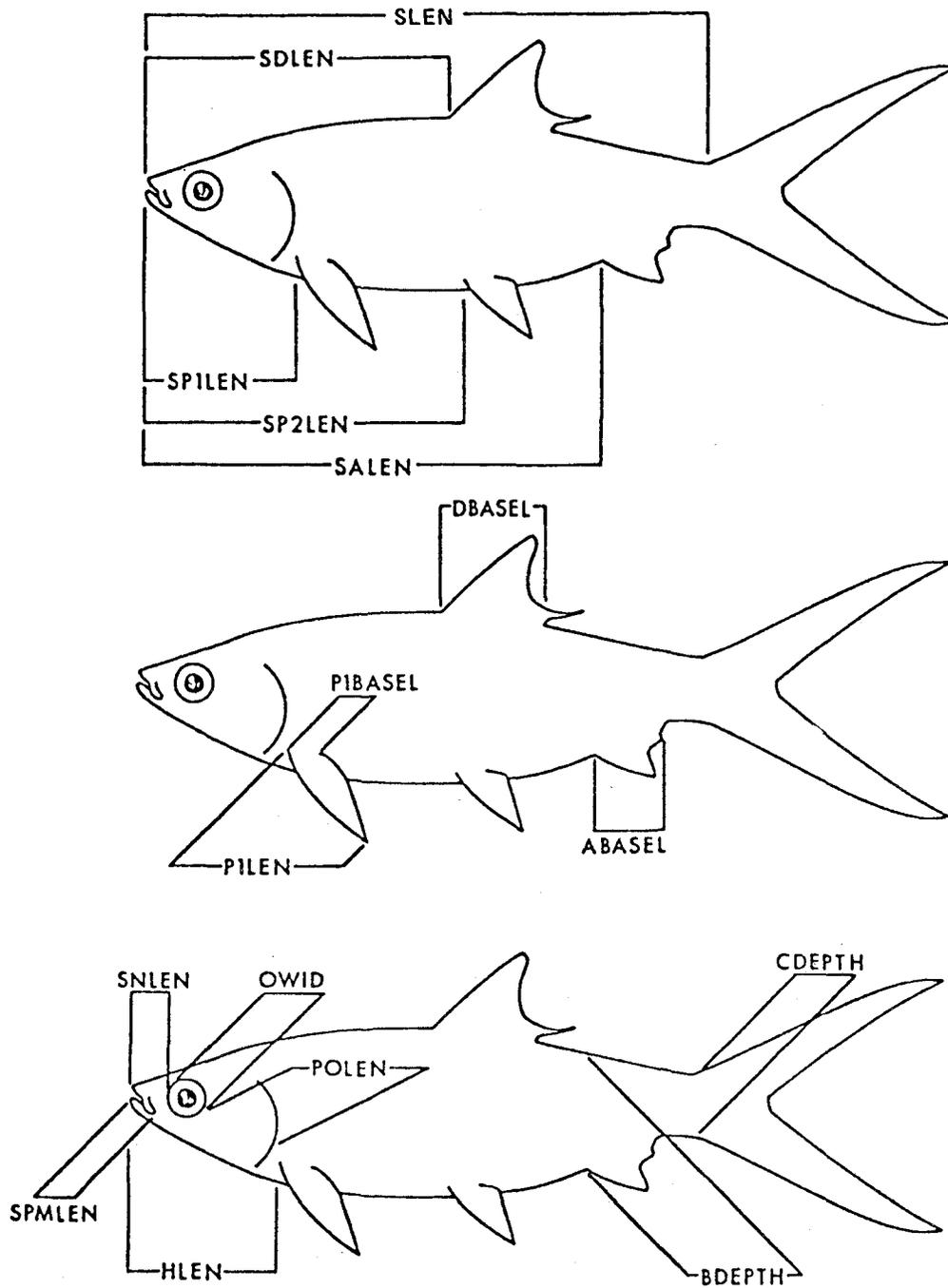


Figure 1. Example of conventional morphometric characters in milkfish. Descriptions are given in Table 6.

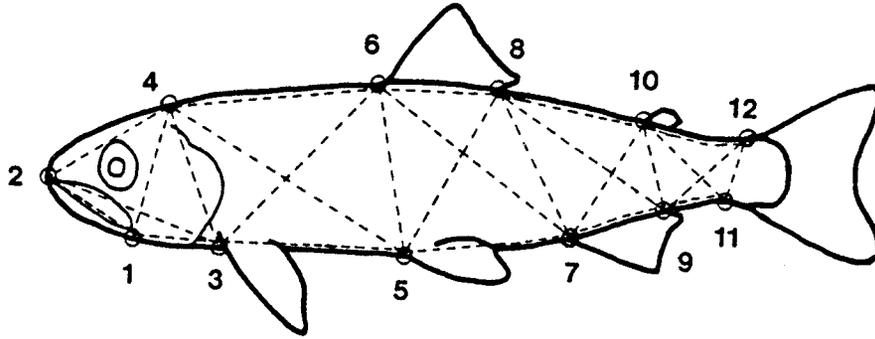


Figure 2. Example of truss network characters. Morphological landmarks are numbered and morphometric distances between landmarks are dashed lines.

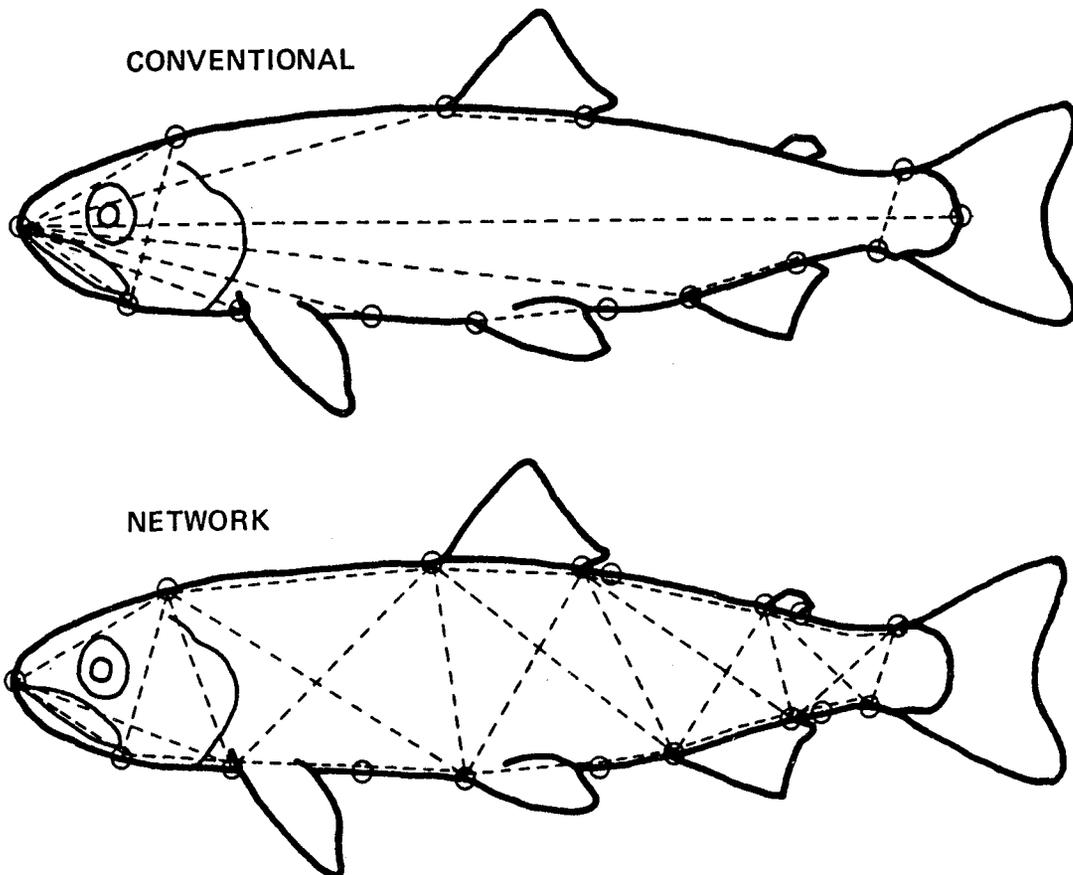


Figure 3. Truss network and conventional characters for chinook salmon.

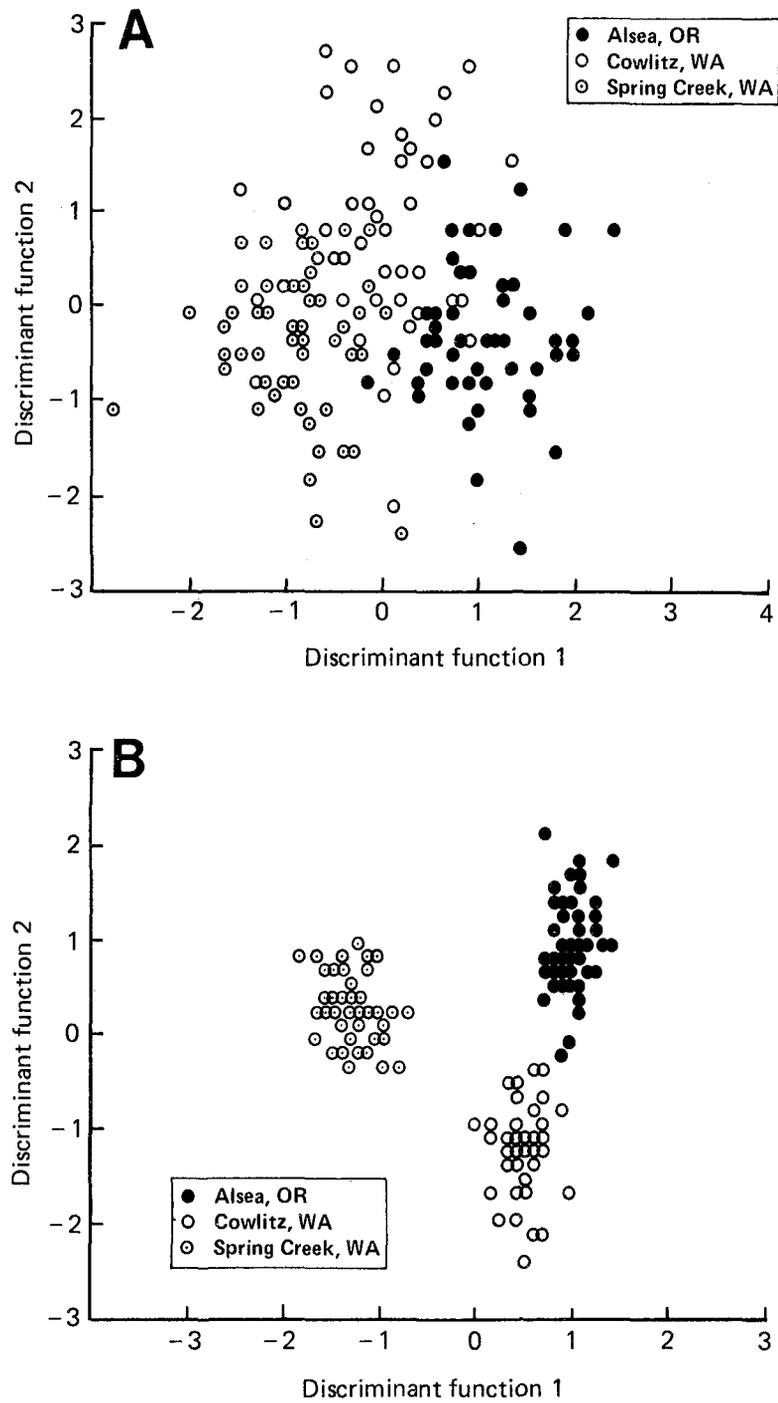


Figure 4. Results of discriminant function analyses of chinook salmon. Analyses are based on conventional measurements (A) or truss network characters (B) illustrated in Figure 3.

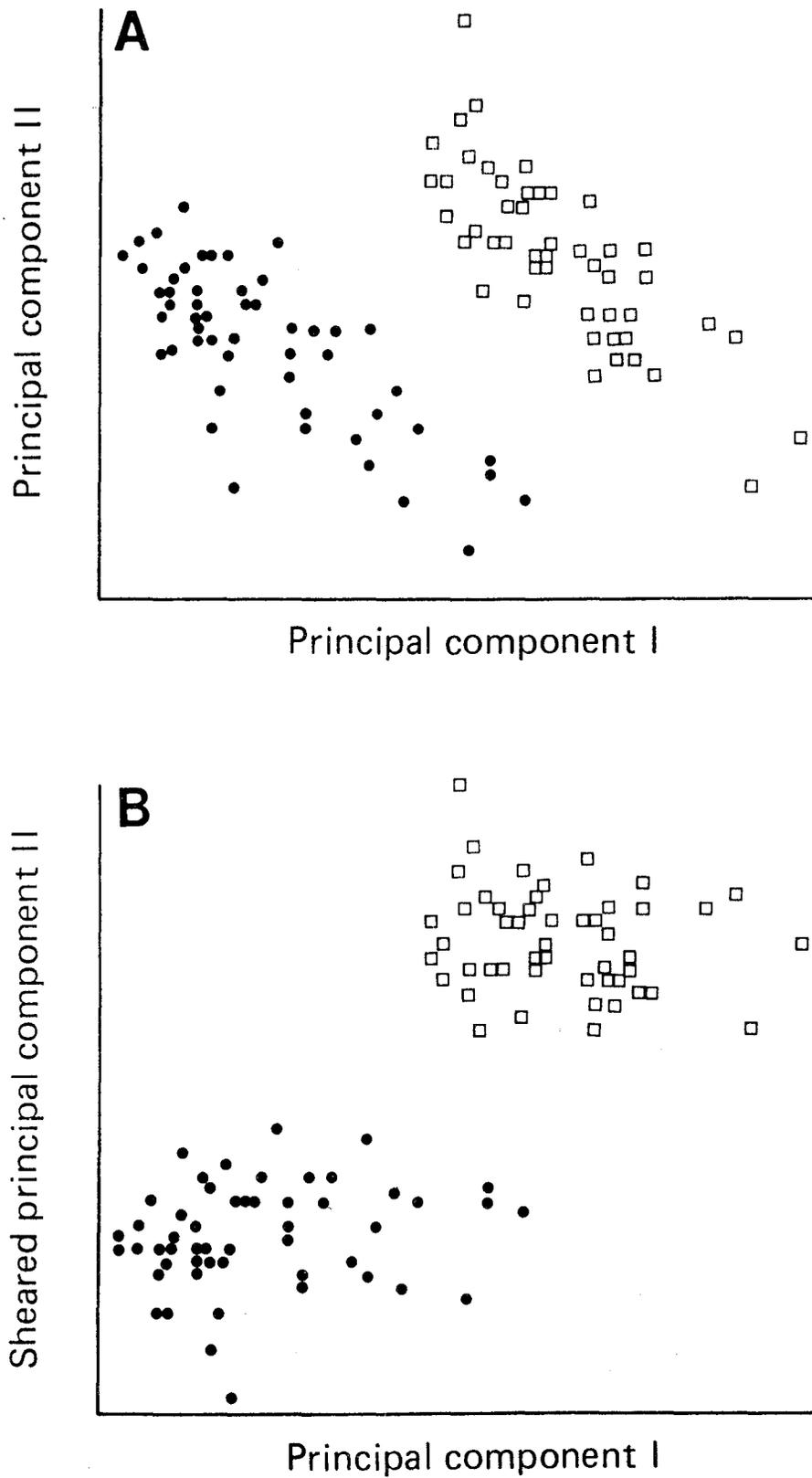


Figure 5. Example of the effects of shear analysis on two samples of chinook salmon. Scatter of points on the first PC axes before (A) and after (B) a shear analysis (Bookstein et al. 1985).

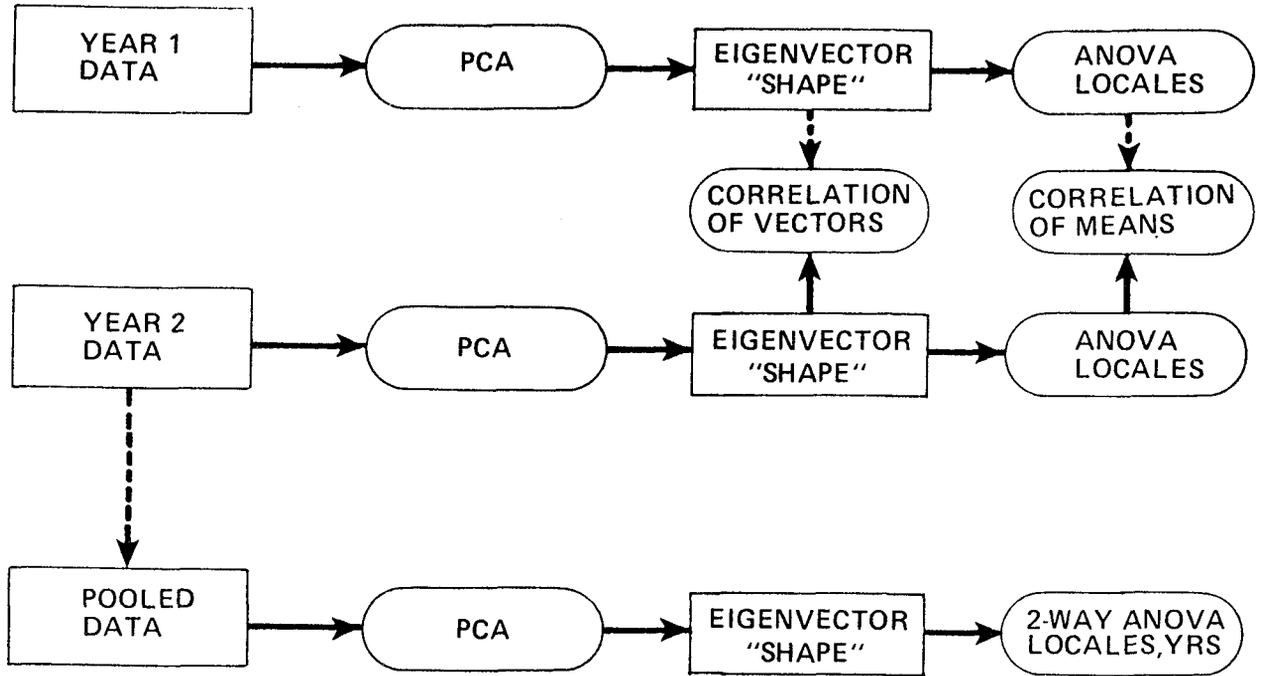


Figure 6. Model for examining temporal stability of morphological variability. PCA = principal component analysis. ANOVA = analysis of variance.

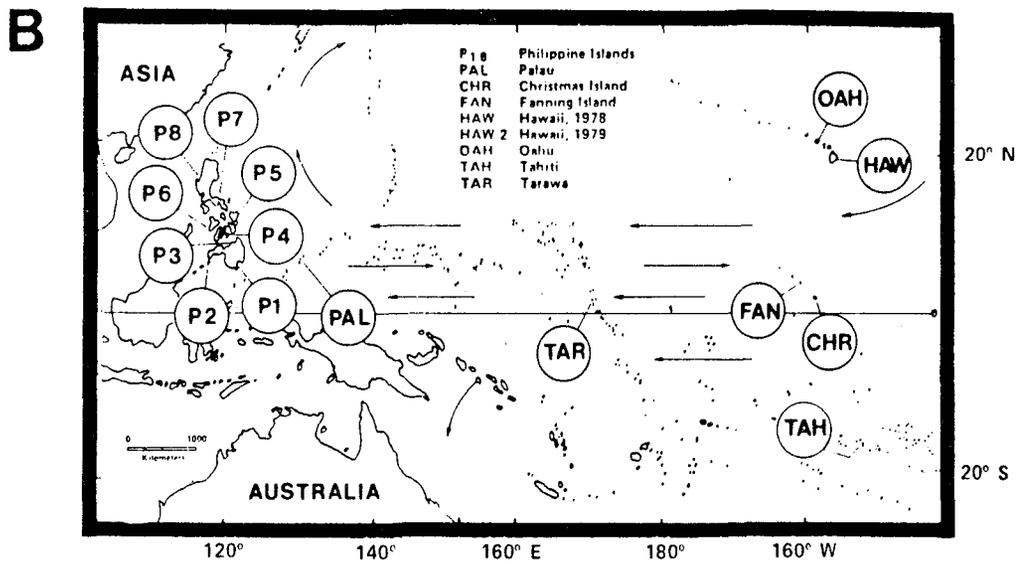
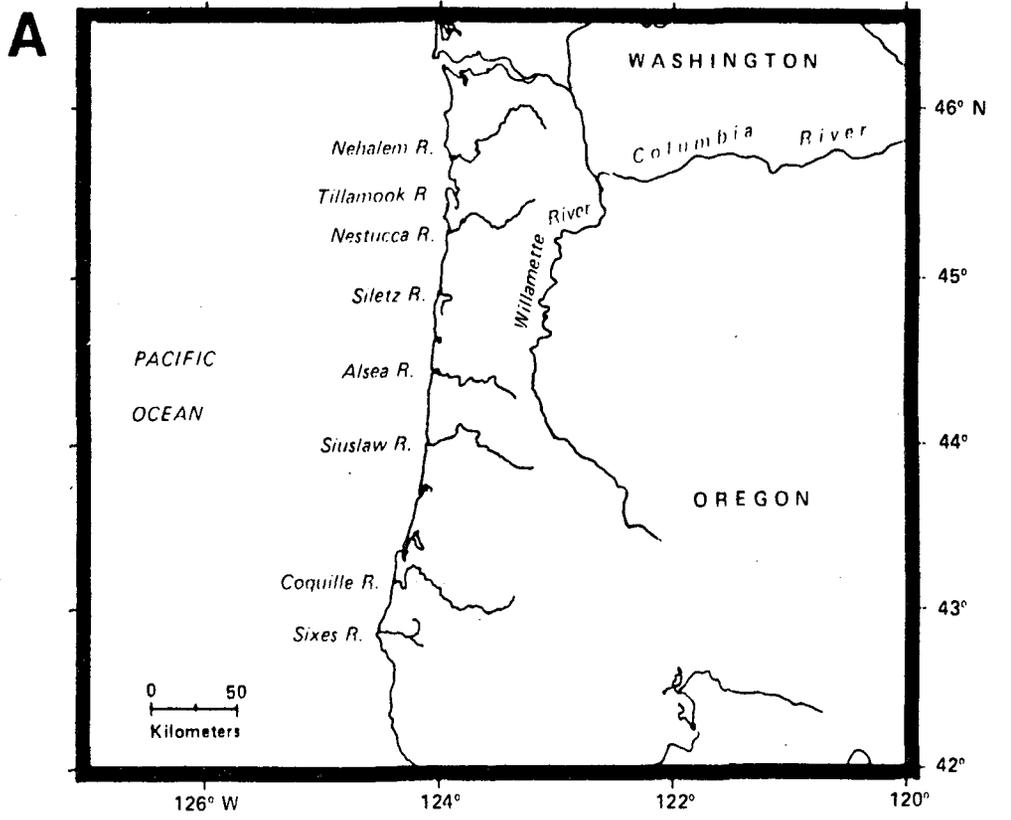


Figure 7. Sampling locations. A. Chinook salmon sampled along the coast of Oregon. B. Milkfish collected from 15 locations in the Pacific Ocean.

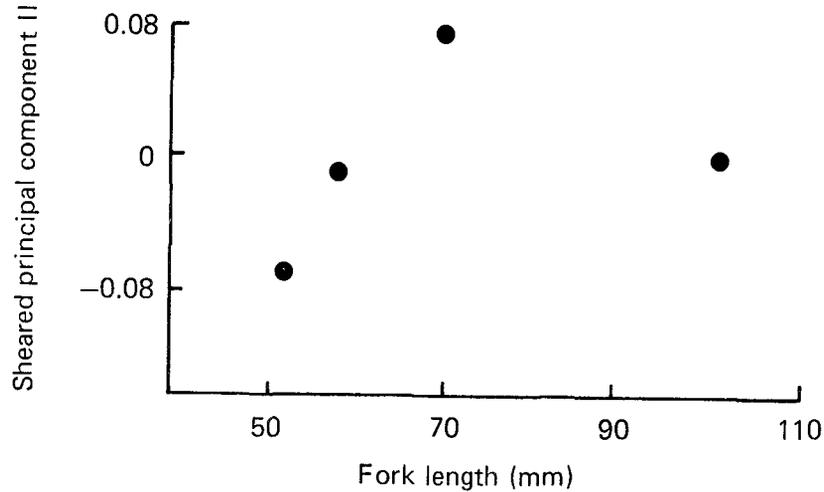
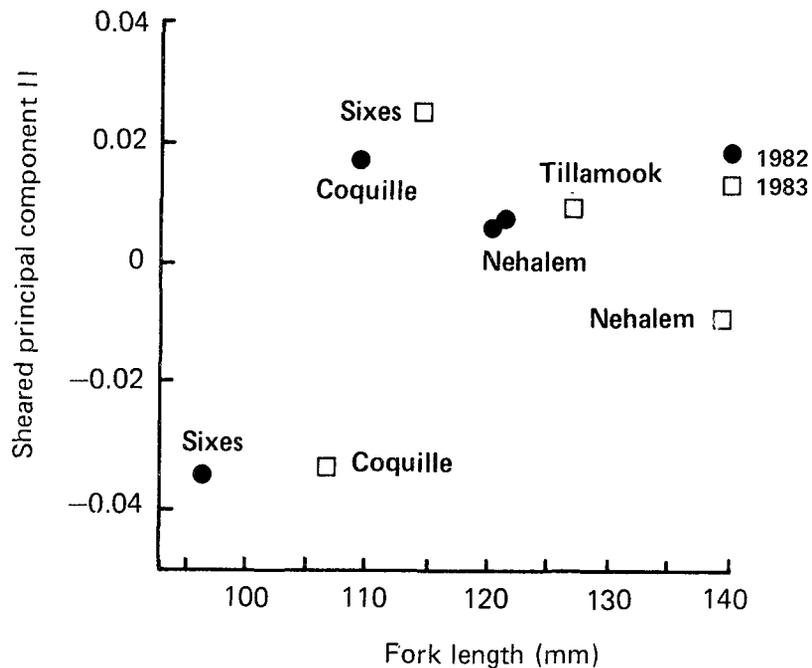
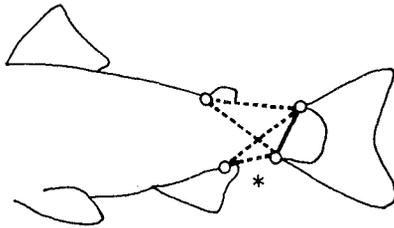
**A****GARRISON SPRINGS  
HATCHERY****B****COASTAL POPULATIONS  
OF OREGON**

Figure 8. Mean values per sample of chinook salmon for fork length and sheared PC II. Details of the study of chinook salmon from Garrison Springs State Salmon Hatchery, Fort Steilacoom, WA, are given in Winans (1984).

A

GARRISON SPRINGS  
SALMON HATCHERY: SPC II



B

COASTAL POPULATIONS  
OF OREGON: SPC II

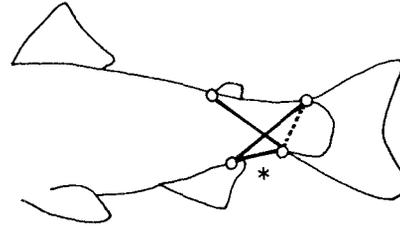


Figure 9. Important characters on SPC II axis. The asterisk indicates the most heavily-weighted character. Negatively signed characters have dashed lines; positively signed characters have solid lines.

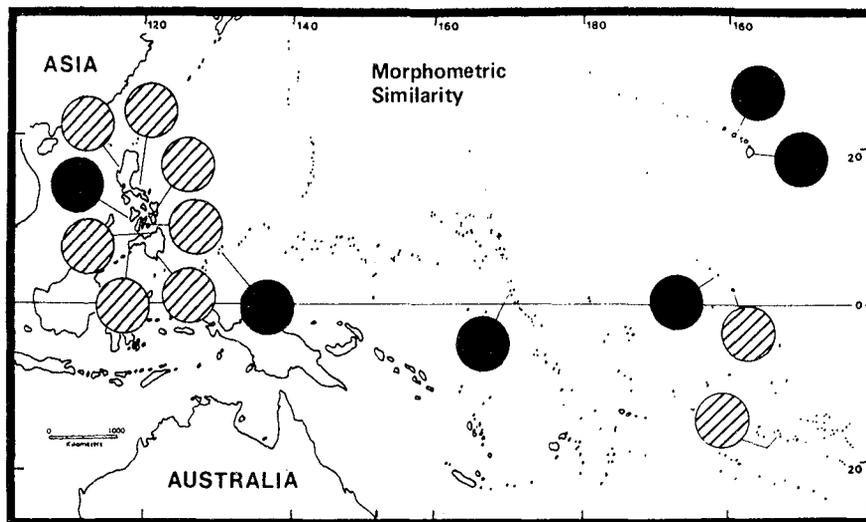


Figure 10. Morphometric similarity of milkfish samples. Solid circles indicate a positive SPC II value and lined circles indicate a negative SPC II value (see Winans 1985).

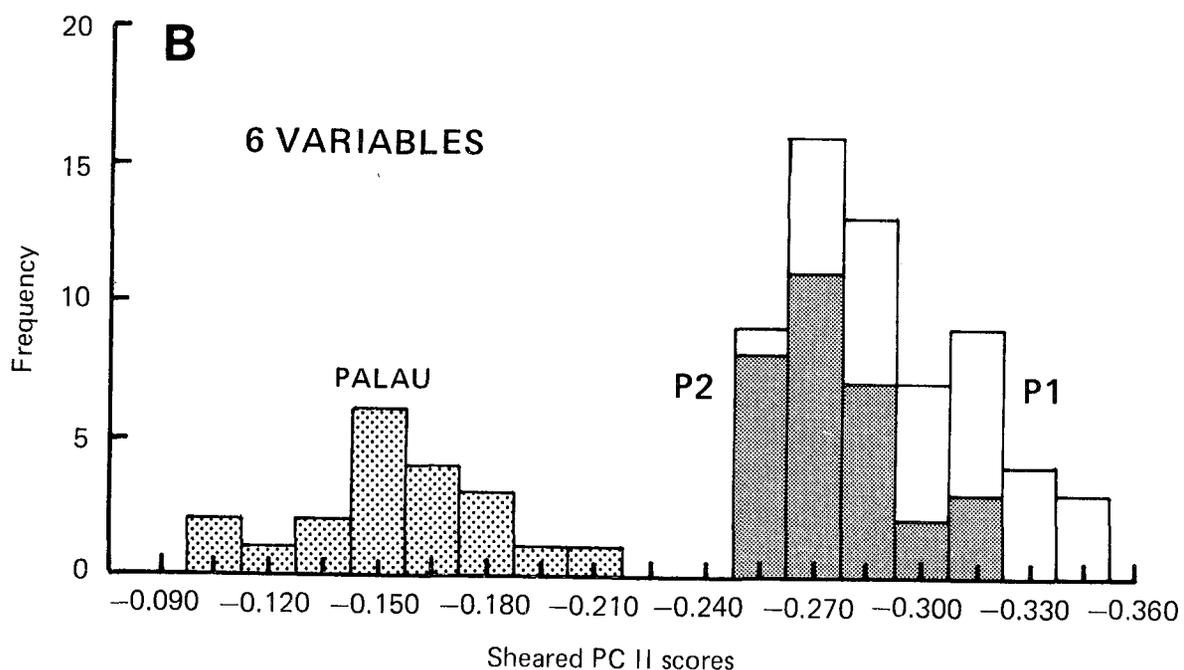
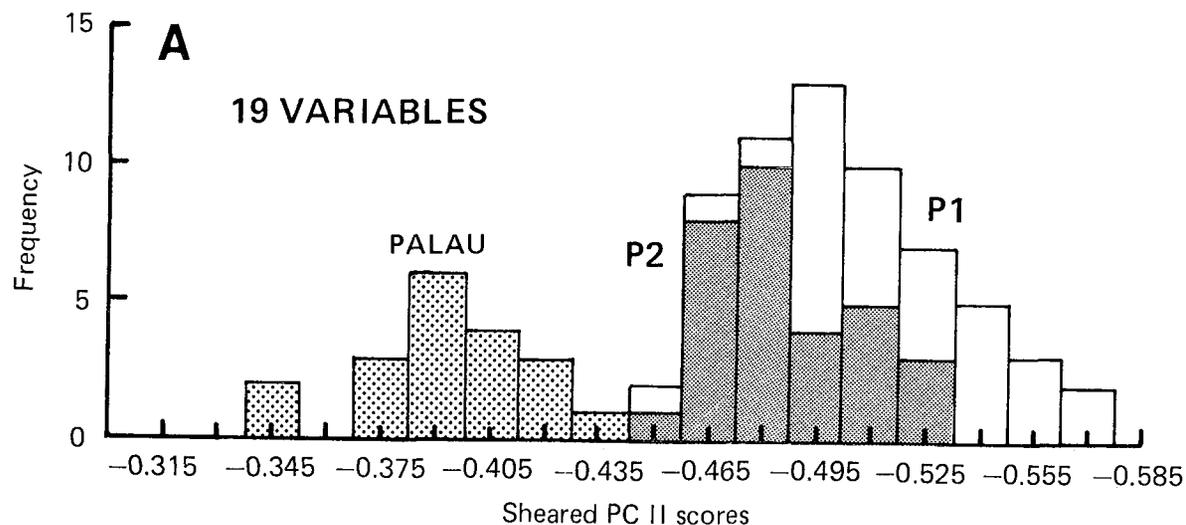


Figure 11. Frequency histogram of SPC II values. SPC II values were calculated with 19 morphometric variables (A) and with the 6 most important variables (B). Samples P1 and P2 are from the Philippines (see Figure 7B).

# PROTEIN ELECTROPHORESIS AND STOCK IDENTIFICATION IN FISHES

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## INTRODUCTION

It is well established in fishery management that harvest regulation should be based on the population dynamics of individual breeding units (summarized in Larkin 1981). Following this principle prevents the inevitable depletion of weaker units that are harvested at an optimal rate for stronger ones. Such management requires an adequate understanding of the genetic population structure within the species that constitute the resource in question.

However, as discussed in Allendorf et al. (1987) there are many reasons why genetic units of fishes are less apparent than those of other groups of intensely managed vertebrates. For instance, the aquatic habitat makes locations and numbers of fishes difficult to identify. Indeterminate growth permits unusually large variations in size among individuals within a breeding unit. Exothermy magnifies the influences of temperature variations. Such attributes have sometimes tended either to mask genetically distinct groups (Shaklee and Tamaru 1981; Grant and Utter 1984) or to give unwarranted taxonomic distinction to apparent differences (Wishard et al. 1984; Hindar et al. 1986). These difficulties in adequately identifying distinct genetic units of fishes have led to a search for purely genetic characters that could serve as markers for genetically distinct groups.

Presently, genetic characters detected by protein electrophoresis are those that are most widely used to identify genetically distinct groups of fishes. Indeed, protein electrophoresis has emerged during the past 20 years from largely a clinical procedure to the primary method for detecting single gene (i.e., Mendelian) variation in all kinds of living organisms (Lewontin and Hubby 1966; Selander 1976; Nevo et al. 1984). A previously unknown (and undetectable) reservoir of Mendelian variation has been amply demonstrated in fishes (see de Ligny 1969, 1972 for early reviews). Genotypic and allelic data from protein coding loci obtained by electrophoretic methods have provided new and valuable insights concerning the population structure of many fish species (Allendorf and Utter 1979; Winans 1980; Shaklee 1983; Ferguson and Mason 1981; Ryman 1983). However, electrophoretic data remain

largely underused among fishery biologists and managers in spite of their potential to identify genetically distinct groups (discussed in Allendorf et al. 1987).

This paper outlines the applications of genotypic and allelic data obtained by electrophoresis in stock identification of fishes. The purpose is to give a basic understanding and appreciation of Mendelian data collected by electrophoresis to potential users having inadequate familiarity with electrophoretic procedures and data. It is intended to provide such workers with a minimal background for applying such data. An overview of underlying molecular and genetic principles is followed by stepwise descriptions of data collection and analyses, with examples from the literature. The citations given are not intended as an exhaustive review of the field, but rather as appropriate examples of the particular situation under consideration. An appendix provides details of calculations carried out in analyses of a hypothetical set of electrophoretic data.

#### WHY IS INFORMATION ABOUT GENOTYPES AND ALLELES IMPORTANT FOR STUDYING POPULATION STRUCTURES OF FISHES?

This workshop reflects an underlying recognition among fishery biologists of the need to identify distinct breeding units as a basis for proper management of fishery resources. As pointed out above, special attributes of fishes have often resulted in the failure to fulfill this need using criteria that tend to be reliable in other organisms. However, regardless of such attributes, the genetic structure of a species can become apparent when adequate information is available concerning the distribution of genotypes and alleles.

First, what is Mendelian inheritance and variation? Mendel's (1866) classical experiments with peas demonstrated a basis for inheritance that has subsequently proven applicable to most higher organisms including man and most fishes. An individual inherits single, or haploid, sets of genes from each parent. Consequently, each gene occurs in a paired, or diploid, state in most cells. One or the other of each of the paired genes occurs randomly in haploid germ cells (gametes) for transmission to the next generation: such transmission is Mendelian inheritance. Genes occur linearly on bodies called chromosomes. The location of a particular gene on a chromosome is called its locus (plural = loci). Different genes at the same locus are called alleles. Allelic differences are therefore Mendelian variants, and a locus is polymorphic when such variants occur (contrasted with monomorphic in the absence of allelic variation). The diploid allelic constitution of an individual is its genotype. An

individual is homozygous at a locus when both genes are the same, and heterozygous when the respective genes are different alleles.

Next, what can be determined from Mendelian data about genetic population structures that cannot be measured by quantitative genetic characters that typically involve an unknown number of loci plus a variable, and usually substantial, environmental influence? Heritable variations in traits such as size, age at maturity, and time of spawning provide useful information about stock fitnesses in specific environments, as well as guidelines for breeding programs. However, studies of such quantitative traits yield no information on distinct alleles at particular loci. Mendelian data are the fundamental building blocks of empirical population genetics. They provide distinct markers for genetic characterization and monitoring of populations, and for estimating degrees of divergence and evolutionary relationships among populations (Crow and Kimura 1970; Lewontin 1974; Nei 1975; Wright 1978; Hartl 1980; Hedrick 1983).

#### WHAT IS THE BASIS FOR OBTAINING INFORMATION ABOUT GENOTYPES AND ALLELES FROM ELECTROPHORETIC PATTERNS?

##### The Relationship of Proteins to Genes

Most genes studied electrophoretically are templates for the synthesis of proteins. The linear arrangement of four different bases (adenine, cytosine, guanine, thymine) in deoxyribonucleic acid (DNA), which is the chemical of the gene, determines the linear arrangement of different amino acids polymerized in polypeptide chains. Active proteins are made up of polypeptide chains (subunits) alone or in aggregate, depending upon the protein. The genetic code relates specific three-base sequences in the DNA to each of the 20 amino acids commonly occurring in nature, thereby assuring that individual protein molecules are direct reflections of individual genes. Further details of this relationship are outlined in Figure 1.

A change in the base sequence (i.e., a mutation) within a segment of DNA encoding a particular subunit (i.e., a locus) may result in a change of amino acids in the subunit. Some amino acids are charged negatively or positively, giving each protein molecule a characteristic net charge. A change of amino acids of different charges can result in a different net charge for the protein. Alleles are DNA segments for the same locus that differ for one or more bases.

Gel electrophoresis separates proteins on the basis of their net charges. Therefore, proteins made up of allelic subunits

having different net charges can be distinguished electrophoretically.

### Electrophoresis

The basic procedures of gel electrophoresis are outlined in Figure 2. The process of electrophoresis includes a gel (commonly starch or polyacrylamide) in which introduced solutions of proteins are separated relative to their net charges by passage of a direct electrical current through the gel. Initially, mixtures of proteins are extracted with water (or buffered aqueous solvents) from tissues such as skeletal muscle, heart and liver) or are contained in body fluids such as vitreous humor or blood serum.

Most proteins that are studied by electrophoresis are enzymes, because it is easy to develop specific staining processes for many enzymes. The resolving power of electrophoresis has been substantially increased by applying histochemical staining procedures to visualize activities of specific enzymes (Hunter and Markert 1957). A number of sources give detailed descriptions of many procedures for visualizing enzymatic activities following electrophoresis (e.g., Harris and Hopkinson 1976; Siciliano and Shaw 1976). Each procedure uses a product of the enzyme's specific activity to locate that enzyme precisely in the gel. Specific staining for an enzyme's activity (Figure 2D) permits particular enzymes to be distinguished, one at a time, in the mixture of hundreds of proteins typically found in tissue extracts.

The term isozyme refers to different distinguishable molecules found in the same organism which catalyze the same reaction (Markert and Moller 1959; Shaw 1964; Brewer 1970). The term allozyme is commonly used to refer to allelic variants of the same protein.

The final result of electrophoresis is bands such as those of Figure 2D which identify the locations of various forms of a protein on a gel. The banding pattern of an individual contains information on that individual's genotype with respect to the locus (loci) coding for that particular protein.

### Genotypic Interpretations of Gel Banding Patterns

The relationship between DNA base sequences, protein amino acid sequences, and the electrophoretic expression of different genotypes (i.e., the gel phenotype) is most easily illustrated for a monomeric protein. Monomeric proteins are active molecules consisting of only a single protein subunit (i.e., the polypeptide

chain). Let us assume (1) a locus coding for a monomeric protein having two alleles (i.e., a polymorphic locus) designated as A and A', (2) that these alleles produce subunits (the active protein for monomers) that are distinguishable by different electrophoretic mobilities and designated a and a' respectively, and (3) that the a' protein encoded by the A' allele moves slower than the a protein encoded by the A allele.

Three different genotypes are possible at this locus - AA, AA', and A'A'. An individual with the AA homozygous genotype only produces the faster migrating protein form. This form appears at one single location on the gel as a single band. Similarly, a homozygous individual with the A'A' genotype only produces the slower migrating band at a different location on the gel. The heterozygous (AA') genotype produces both protein forms, and an extract from such a fish will express each of these two bands on the gel. Assuming that each allele encodes the production of equal amounts of protein, each band of a heterozygous individual expresses half the production (i.e., dosage) of the single band expressed by a homozygous individual. These patterns of genotypic expression (or phenotypes) of a monomeric protein encoded by a single locus with two alleles are pictured on the top of Figure 3.

Banding patterns on a gel become more complicated when the active protein is a multimer combining two or more protein subunits into an active molecule. Let us assume the above described situation except that the active protein is dimeric, i.e., consisting of two subunits. These electrophoretic expressions are pictured in the middle section of Figure 3. The electrophoretic phenotype for the AA genotype is a single band reflecting identical molecules of a subunits combined in pairs. Similarly, the phenotype A'A' genotype is another single band reflecting paired a'a' subunits at a different location on the gel. However, the phenotype for the AA' genotype consists of three bands reflecting the random combination, in pairs, of the two electrophoretically distinguishable types of subunits. Two of the bands are homomeric combinations of aa and a'a' subunits that are expressions of the respective homozygous genotypes. The third band, not expressed by either of the homozygotes, is a heteromeric band reflecting combinations of a and a' subunits (note that monomers cannot form heteromeric bands because the single subunit is the active protein.) The sum of the intensity of the three bands expressed by heterozygous genotypes is expected to equal the intensity of single banded homozygous expressions, because the same number of subunits are produced by heterozygous or homozygous individuals.

The expected electrophoretic phenotypes for a protein having four subunits (i.e., tetrameric) reflecting a single locus that is polymorphic for two electrophoretically detectable alleles are pictured in the lower portion of Figure 3 and in Figure 4. The

respective phenotypes of homozygous individuals again are single-banded because of the identity of each of the four subunits. The five-banded (heterozygous) phenotype includes three heteromeric bands in addition to the two homomeric bands, and has a total intensity equivalent to the single band of the homozygous expression.

The expected numbers of bands and their relative intensities for individuals heterozygous for protein coding loci can also be predicted from binomial expansion of the two categories of allelic subunits (a and a'). For a dimeric protein this expansion would be

$$(\underline{a} + \underline{a}')^2 = \underline{a}^2 + 2\underline{a}\underline{a}' + \underline{a}'^2.$$

In reference to the left-hand side of the binomial formula, the a and a' represent the actual protein subunits and the exponent (2) represents the number of subunits in the protein, in this case 2 for a dimer. In the expanded right-hand side of the formula, the three terms represent the number of bands and their respective coefficients (1, 2, 1) represent their relative intensities. For a tetramer, the exponent becomes a four. Following expansion then, the relative intensities of 1:4:6:4:1 would be expected from tetramers.

More complicated electrophoretic patterns arise when subunits encoded by two or more loci aggregate to form multimeric proteins. Interpretation of such patterns involve direct extensions of the principles outlined above and are described in sources including Shaw (1964) and Utter et al. (1987).

#### Strengths and Limitations of Electrophoretic Data for Studying Genetic Population Structures

The principles outlined above for directly obtaining genotypic data from electrophoretic patterns are widely applied and have resulted in electrophoresis being generally recognized as "--the most useful procedure yet devised for revealing genetic variation--" (Hartl 1980). The unmatched power of electrophoresis for detecting allelic variation is enhanced by the volumes of data that can be collected with a given amount of effort. Protein extracts can be prepared with minimal effort. In starch gels (the medium that my colleagues and I have used almost exclusively), many samples can be run on a single gel, and multiple slices of a gel can be stained for different proteins which reflect different loci. For instance, a trained worker can run six gels per day with each gel containing 50 samples for a total of 300 individuals. Data for at least six loci can be obtained from each

individual because each gel can be cut into six or more slices, and each slice can be stained for a different type of protein. Usually data from more than six loci per individual can be obtained because commonly more than one locus encodes for a particular type of protein.

Starch gel is only one of many media used for electrophoresis. Other media including paper, cellulose acetate, agar and acrylamide have also been used. Ferguson (1980) provides a review of the inherent advantages and limitations of many procedures commonly used at present. In addition to starch, acrylamide has been particularly widely used as an electrophoretic medium in population studies. With regard to acrylamide electrophoresis, Ferguson (1980) states the following:

"It is very difficult to evaluate the genetic basis of intra-specific variability from general protein patterns. For most systematic work, staining for specific enzymes is preferable, and reduces the problems of homology and interpretation of variability. In staining for specific enzymes, a maximum resolution technique may not be required, e.g., although starch gel gives poorer resolution than acrylamide for general proteins, enzyme staining is in most cases superior."

Isoelectric focusing is an alternate method to electrophoresis for separating mixtures of proteins in a gel medium. A protein solution is introduced to an acrylamide gel in which a pH gradient has been established through incorporation of synthetic polyamino polycarboxylic acids having a range of isoelectric points. The proteins move in the gel until they have reached the point in the pH gradient equivalent to their own isoelectric points. Dr. Jarle Mork (Biological Station, N-7001 Trondheim, Norway) has contributed the following statement based on starch gel electrophoresis and isoelectric focusing:

"As an analytical tool for multilocus screening of a large number of specimens, starch gel electrophoresis is probably the most efficient method available today. It is generally applicable, is supported by a very broad literature on electrophoretic and staining recipes, and is unmatched in the numbers of samples that can be examined for a given effort because of the capability for obtaining multiple slices from single gel. In special applications its resolving capacity may surpass even that of isoelectric focusing in polyacrylamide gel (IFPAG); proteins with only minor differences in isoelectric points (pI) may be separated by choosing the adequate pH for the gel buffer. In practice, however, there appears to be little need for such micro-adjustments of running conditions to resolve

most isozymes. Thus, although no electrophoretic technique produces protein bands as discrete as those obtained by IFPAG, parallel analysis of tissue enzymes in Atlantic cod showed that all alleles detected by IFPAG were also detected by starch gel electrophoresis without special efforts. IFPAG may have advantages in terms of the simplicity of sample preparation and analytical setup, but appears to be a much harsher procedure than starch gel electrophoresis for many proteins. Enzymes which are readily demonstrated on starch gels may fail to show activity after IFPAG. The reasons for such deactivations are often obscure, but may be sought among some effects inherent with the IFPAG technique. These include ampholyte chelation of metalloproteins (Galante et al. 1975), isoelectric precipitation (Rhigetti and Drysdale 1976), and focusing at unfavorable pH conditions (i.e., proteins with pI in the acidic or basic portion of the gel (cf. Mork and Heggberget 1984, Mork and Haug 1983). In practice therefore, the locus repertory is more restricted in IFPAG than in starch gel electrophoresis. Thus, although IFPAG should be welcomed as a valuable complement to existing techniques, especially when dealing with small amounts of proteins (e.g., planktonic fish eggs, Mork et al. 1983, Mork and Sundnes 1983), it does not replace starch gel electrophoresis in large scale multilocus screening investigations."

There are also limitations to the information about protein coding loci that can be obtained by electrophoresis. The information needed in population genetics relates to base sequences of DNA studied either directly or indirectly. The amino acid substitutions of proteins detected by electrophoretic data are indirect reflections of the actual differences in the base sequences. All base substitutions do not necessarily result in changes of amino acids. Furthermore, all amino acid substitutions do not result in protein changes that are electrophoretically detectable. It has been estimated that only about a third of the amino acid substitutions are detected under the conditions used to collect electrophoretic data in most laboratories (Lewontin 1974). It is apparent then, that electrophoretic identity does not necessarily mean identity of base sequences in DNA. Thus, homozygosity is often a conditional concept with electrophoretic data as it is with many other classes of genetic data where alleles are inferred by phenotypes (Allendorf 1977). Although it is useful to equate electrophoretic and genetic identity, the possibility of unrevealed genetic heterogeneity must be kept in mind.

It must also be kept in mind that even an electrophoretic sample of 100 loci still represents substantially less than 1% of the total number of genes of a particular diploid organism (Nei 1975). Thus, while electrophoretically detected differences among individuals and populations are positive indicators of genetic differences, the absence of differences cannot be equated to genetic identity at the DNA level.

Most allelic differences detected by electrophoresis also appear to have a minimal effect on the fitness of the individual (Kimura 1968; Nei 1983), although exceptions are well documented (Mork et al. 1984; DiMichele and Powers 1982). This apparent neutrality of much of the genetic variation detected by electrophoresis is a disappointment to those who had envisioned electrophoretically detected alleles as "useful genes" for breeding programs assuming that many such genes could be directly related to fitness (Robertson 1972). An investigator should nevertheless continually be aware of the possibility of differential fitness of allelic proteins, but should rigorously pursue alternate explanations when genotypic distributions suggesting selection are encountered. As Ihssen et al. (1981) observed "--a null hypothesis of neutrality rather than selection appears to be the most reasonable expectation as a first approximation of reality--."

However, the general absence of phenotypic effects on fitness of most allelic proteins enhances the value of electrophoretic variation as more or less neutral genetic markers. The primary value of such markers is for inferring the distribution and magnitude of genetic variation resulting from evolutionary processes at the vast remainder of the genome that has not been sampled electrophoretically. In this capacity, electrophoresis appears likely to remain a leading procedure for an extended time because of its capability to generate readily large volumes of reliable genotype and allele frequency data.

#### HOW ARE SETS OF GENOTYPE AND ALLELE FREQUENCY DATA USED IN STUDIES OF STOCK IDENTIFICATION OF FISHES?

A hypothetical set of electrophoretic phenotypes is used to illustrate the actual application of genotypic and allelic data collected by electrophoresis studies for stock identification in fishes. Actual studies involving similar uses are also referenced at appropriate points throughout this presentation. The set of phenotypes shown in Figure 5 contains data regarding three protein loci for each of three populations of a single species; populations 1 and 2 belong to one subspecies and population 3 belongs to a second.

The patterns of locus 1 typify the expressions of a dimeric protein having two allele variants expressed within a single population (i.e., one heterozygous and two homozygous genotypes). Note however that there are three alleles expressed in the species (based on the total sampling of 150 individuals); alleles A and A' occur in populations 1 and 2, and alleles A' and A'' occur in population 3.

Loci 2 and 3 differ from locus 1 by having no allele variation expressed within any of the populations. However, populations 1 and 2 express the homozygous genotype of a different allele (B) than is expressed in population 3 (B'). Only a single allele (C) is expressed in each of the populations for locus 3. Note that the absence of heterozygous individuals precludes inferring the number of subunits comprising the proteins synthesized by locus 2 and locus 3.

### Genetic Characterization of Samples

The first step in analyzing this set of electrophoretic data is to tabulate the individual genotypes expressed for each locus in each population (Table 1). From these genotypic frequencies, the allele frequencies can readily be determined. For instance, in population 1 for locus 1 there are 45 AA and 5 AA' genotypes in this sample of 50 individuals. Each AA individual contains two A alleles and each AA' individual one. There are, then, 100 alleles in this sample of which 95 are A and 5 are A'; and the allele frequencies are 0.95 A and 0.05 A'. The allele frequencies for the other populations and loci are calculated in the same manner. Allele frequencies are fundamentally important genetic characteristics of a particular sample.

It is useful to make further characterizations of samples before comparisons are made between them. A common statistic of genetic variability is the frequency of heterozygotes which can either be estimated directly from counting of heterozygous individuals over all loci examined, or indirectly from allele frequencies (assuming Hardy-Weinberg genotypic proportions - see below); both measures are given in Table 2. Two other estimates also are given in Table 2, the proportion of polymorphic loci and the average number of alleles per locus. The calculations for obtaining these statistics from Tables 1 and 2 are given in the Appendix. Such averages are usually made over substantially more than the three loci used in this example (e.g., 30 or more). Comparisons of these averages within a species should include the same sets of loci.

The Hardy-Weinberg Law (presented in all introductory texts of general and population genetics) is a particularly useful and broadly applied test for the expected distribution of genotypes.

This law predicts that binomial expansion of the allele frequencies of a polymorphic locus establishes the genotypic proportions of that locus under random mating. For a locus with two alleles (A and A' - not to be confused with the specifically designated alleles A and A' in Table 1) having respective frequencies of p(A) and p(A') this expansion is

$$[p(A) + p(A')]^2 = p(A)^2 + 2p(A)p(A') + p(A')^2.$$

In other words, the expected proportion of AA homozygotes in a sample is the frequency of A times itself,  $p(A)^2$ , etc. The Hardy-Weinberg Law can be extended to more than two alleles (as it is for locus 1 when the genotypes of all three populations are considered jointly - see Appendix).

The Hardy-Weinberg Law provides a valuable first approximation for expected genotypic proportions in samplings of individuals. Genotypes conform to the Hardy-Weinberg Law in large, random mating populations in the absence of migration and mutation, and where the alleles under consideration are not affected by natural selection. Genotype frequencies remain constant over successive generations when these conditions are met, but may deviate from expected Hardy-Weinberg proportions when they are not fulfilled. However, it should also be pointed out that considerable deviation from Hardy-Weinberg conditions may occur within a population but not be detected because of statistical insensitivities of the Hardy-Weinberg Law (Fairbairn and Roff 1980). The genotypic proportions within each population for locus 1 were chosen to conform closely to their binomial expectations; however, such conformance is commonly seen in samples from discrete breeding populations.

A properly genetically characterized sampling of individuals from a population is a valuable genetic snapshot. Data from a sample of at least 50 individuals and 20 loci should include estimates of allele frequencies, heterozygosity, alleles per locus, polymorphic loci, and how closely genotype frequencies conform to Hardy-Weinberg expectations. Characterization may also include equilibrium of between locus genotypes (Hartl 1980). Measurement of so-called "linkage equilibrium" is not included in this review. Adequately characterized samples give insight into the possible influence of migration, genetic drift (chance fluctuations in allele frequencies operating particularly in small populations) or selection on the population at the time of sampling.

In addition, adequate characterizations provide a basis for comparisons with other samplings of the same population. Such genetic monitorings have identified changes that indicate previously unsuspected genetic differences among groups that had

been presumed homogeneous. Allendorf and Ryman (1987) report many such changes in hatchery populations of freshwater and marine species including allelic differences from presumed source populations; allelic differences among year classes; reductions in heterozygosity, number of alleles and polymorphic loci; and deviations from Hardy-Weinberg proportions. These changes were generally interpreted as reflections of insufficient numbers of individuals in establishing and/or perpetuating the hatchery stocks. These findings are not reported as a general indictment of hatcheries. Rather, they point to a need to monitor genetic change in both hatchery and natural populations, and to use the resulting information as guidelines either for continuation of existing procedures or for possible corrective action.

### Genetic Comparisons Within Species

The data from Figure 5 and Tables 1 and 2 are now used to look into some of the many procedures that can be used to compare allelic and genotypic data from two or more populations. A contingency test is a simple and effective means to test for differences of genotypic or allelic frequencies among samples. It is obvious from visual examination of Table 1 that population 3 differs substantially from populations 1 and 2 in genotypic and allelic distributions for loci 1 and 2. However, the differences between populations 1 and 2 for locus 1 are less apparent. A contingency table based on the total number of alleles observed in the two samples can be tested with one degree of freedom by either a chi-square or a G statistic derived from a log-likelihood ratio (Zar 1974). The values of both statistics are similar (chi-square = 15.7, G = 16.9 - see Appendix for calculations) and indicate a probability of substantially less than 0.001 that these samples were drawn from the same population. Contingency tests are widely applied as measures of heterogeneity of allele frequencies among samples (Grant et al. 1980; Ryman and Stahl 1981).

A number of measures of genetic similarity or distance have been devised to quantify the amount of genetic differences among groups. The measure that is presently the most widely applied is Nei's genetic distance (D) (Nei 1975). Under certain assumptions, D identifies the average proportion of nucleotide substitutions that have occurred since two groups diverged. Genetic distance is defined as  $D = -\ln(I)$  where for a single locus

$$I = \frac{\sum x_i y_i}{\sqrt{\sum x_i^2 \sum y_i^2}}$$

Value of  $x_i$  and  $y_i$  are the frequencies of specific alleles in populations x and y, respectively. Calculations of values of D involving pairwise comparisons of populations 1, 2, and 3 are

given in the Appendix. It is convenient to display such paired genetic comparisons as a matrix when three or more groups are involved. The matrix of D values for populations 1, 2, and 3 measured over all loci is

		Population		
		1	2	3
Population	1	0.000	0.012	1.050
	2		0.000	0.876
	3			0.000

A common procedure for visualizing the patterns of genetic distance or similarity (such as the parameter (I) in Nei's measure of genetic distance) among samples is to carry out a cluster analysis on the paired values of genetic distance or similarity given in the matrix. The simplest and most widely used is the unweighted pair-group method (UPGM, Sneath and Sokal 1973 - see Appendix for computation from present data set) which produces a phenogram or tree visualizing inferred genetic relationships.

Two different values of total average heterozygosity expected under Hardy-Weinberg (i.e., binomial) genotypic proportions were calculated in Table 2 (see Appendix for calculations). The mean average heterozygosity or diversity (symbolized as H(S)) of all populations (i.e., the sum of the average heterozygosity of each population divided by the number of populations) reflects the average amount of allelic variation within populations measured over all loci and populations sampled. The heterozygosity of two populations contributing to the mean average heterozygosity may be the same although different alleles are represented (e.g., populations 2 and 3 for loci 1 and 2 in Table 1). The second value (symbolized as H(T)) is called the total diversity and reflects the amount of allele variation among populations. It differs from the first, however, by being calculated at a particular locus based on the average allele frequencies over all populations sampled (for Table 1, those allele frequencies in Total column). If no differences of allele frequency occur among populations, H(S) and H(T) are the same. However, as differences in allele frequencies increase between two or more samples at one or more loci, H(S) becomes increasingly less than H(T).

This difference is an indicator of the amount of genetic subdivision existing among the populations sampled. Because H(T) is larger than H(S) when there is genetic subdivision, a deficit of heterozygous individuals is expected with admixture of individuals from different subpopulations. Such a deficit of overall heterozygous genotypes is seen in the Total column of

Table 1 where the observed genotypes represent an admixture of 150 individuals from three subpopulations.

The difference between  $H(T)$  and  $H(S)$  may be expressed as

$$H(T) = H(S) + D(ST)$$

where  $D(ST)$  is measure of gene diversity among subpopulations (Nei 1973).

$D(ST)$  may be subdivided by different hierarchical groupings of populations (Chakraborty 1980). Such measurement of genetic differences between populations or groups of populations is called gene diversity analysis. Gene diversity analysis for the data of Table 1 is summarized in Table 3 and Figure 6 (see Appendix for calculations). First  $H(S)$  and  $H(T)$  are calculated. Next, that portion of  $D(ST)$  resulting from averaging allele frequencies between populations within subspecies is determined. The remainder of  $D(ST)$  resulting from differences between subspecies is found by subtracting  $H(S)$  plus the increase due to populations within subspecies from  $H(T)$ .

It is convenient to express the partitioning of gene diversity in a subdivided population in relative rather than absolute terms ( $G$  values). These coefficients measure the proportion or percentage of the total gene diversity contributed by the different levels of the hierarchy. Comparable values of absolute and relative gene diversities for the data of Table 1 are given in Table 2 and Figure 6A. The coefficient for total population subdivision ( $[H(T)-H(S)]/H(T)$ ) or  $G(ST)$  has been recorded from a wide variety of organisms (Hartl 1980) and is equivalent to Wright's fixation index =  $F(ST)$  (Wright 1978).

The gene diversity analysis for the data of Table 1 reflects a large amount of population heterogeneity resulting from differences between subspecies. The analysis confirms what is largely apparent from reinspection of Figure 5: only locus 1 contributes to diversity within subspecies, both loci 1 and 2 contribute to the between subspecies diversity and locus 3 proportionately reduces the absolute diversity at all levels but does not affect relative diversity. Differences between subgroups accounts for 71.3% of the total diversity (i.e.,  $G(ST) = 0.713$ ) within this hypothetical set of genetic data with by far the largest proportion resulting from differences between subspecies.

These comparative data involving three groups and loci reflect greater genetic heterogeneity than is observed in most data sets reported from conspecific populations. Usually, data are collected from 20 or more loci of which, perhaps, a third are polymorphic. The absence of shared alleles (i.e., fixed

differences) between groups, as seen for locus 2, does not usually occur at the species level, and particularly not at 50% of the polymorphic loci. Consequently, the differences between subspecies are inflated (although differences among cutthroat trout subspecies approach these levels: see Loudenslager and Gall 1980). On the other hand, the comparative data between populations 1 and 2 are more or less typical of different conspecific populations regarding levels of polymorphism and heterozygosity.

Levels of electrophoretically detected heterogeneity nevertheless vary markedly among species of fishes. Some differences appear to be generally dictated by life history variables. Gyllensten (1985) has compared gene diversities among seven marine, four anadromous, and ten freshwater species of teleosts. A higher average heterozygosity occurs in marine species than in freshwater species, and the fraction of gene diversity attributed to localities increased from marine to anadromous to freshwater species. These observations are consistent with the greater opportunity for gene flow in the marine environment. Local stock discrimination based on genetic isolation may therefore be expected to be less distinct in marine species. However, genetic heterogeneity among populations is not uncommon in marine species when sampling has involved broad geographic areas (e.g., Pacific herring, Grant and Utter 1984; yellowfin sole, Grant et al. 1983; Pacific cod, Grant et al. 1982; Atlantic cod, Mork et al. 1985; milkfish, Winans 1980; Pacific pollock, Iwata 1975; Pacific hake, Utter and Hodgins 1971). Such heterogeneity typically separates different major groups whose existences were often unsuspected prior to genetic investigations. Similar major heterogeneity within marine species undoubtedly remains to be identified on the basis of distinctive allele frequencies.

Two groups having identical allele frequencies at all detectable loci can be made distinguishable provided at least one of the groups is artificially cultured, because allele frequencies of cultured populations can be readily changed (i.e., genetically marked) when parental genotypes are known at polymorphic loci. This process has been effectively used both in short term experimental studies (Reisenbichler and McIntyre 1977; Schroder 1982) as well as in more extensive and long term investigations involving entire segments of established populations (Seeb et al. 1986; Gharrett 1985). The ease and permanence of genetic marking provides a valuable tool for measuring relative reproductive successes and rates of migration between marked and unmarked populations over multiple generations, as well as for identifying origins of individuals or stocks. Guidelines for establishing marked populations include minimizing inbreeding and involving alleles most likely to be neutral to natural selection (Allendorf and Utter 1979; Gharrett 1985).

Knowing the pattern of statistically significant differences in allele frequencies among stocks can be used to estimate compositions of population mixtures. Such use is increasing in the management of Pacific salmon which are usually harvested from complex population mixtures (Grant et al. 1980; Miller et al. 1983; Fournier et al. 1984; Beacham et al. 1985; Milner et al. 1985). Procedures for obtaining estimates (e.g., by maximum likelihood) require (1) that detectable differences in allele frequencies exist among populations potentially contributing to a particular mixture, (2) that sets of allelic data are available for representative groups of such populations, and (3) that sufficient sampling of individuals has been obtained from the mixture for adequate precision of estimates. When these conditions are met, the results to date have provided more detailed estimates than had previously been obtainable within reasonable time intervals, and at comparable or lower costs to other procedures presently used to estimate mixed stock compositions.

#### Genetic Comparisons Among Species

The primary focus of this paper is on differences within species. However, concerns for stock identification extend between species when individuals of different species cannot be readily distinguished. Electrophoretic data are particularly useful in such circumstances.

Interspecific allelic variations differ from intraspecific variation through fixed allelic differences commonly occurring at one or more loci. Such fixed differences between species are preserved by the absence of gene flow. The proportion of fixed differences generally increases as comparisons among taxonomic levels become higher. A review of many published values of genetic distances of fish at different taxonomic levels (Shaklee et al. 1982) reported average D values at the level of population, species and genus to be 0.05, 0.30 and 0.90, respectively. The relationship between genetic distance and taxonomic level has made allelic data a particularly valuable tool in systematic studies (Awise 1974; Buth 1984).

These qualitative genetic distinctions occurring between species usually preclude the requirement for detailed statistical analyses of allelic data to identify the presence of different species in a sample of a reasonable number of individuals. The presence of only homozygous individuals for different alleles at one or more loci is usually a clear indication of more than one species. This characteristic has proven useful in detecting previously unrecognized sibling species (Shaklee and Tamaru 1981) and in identifying individuals (e.g., eggs, juveniles) when species origins are uncertain (Allendorf and Utter 1979; Mork et

al. 1985). Because of qualitative species differences, we have responded to numerous requests from enforcement personnel for species identifications from fragments of muscle to resolve forensic cases. Shaklee (1983) gives a useful overview of applying qualitative species differences in fishery problems.

Fixed allelic differences between species also usually permit immediate recognition of species hybrids. First generation hybrids are characterized by heterozygous expression of alleles that are fixed in the respective parental species. Consequently, an individual heterozygous at all fixed loci that distinguish two species is readily identifiable as a first generation hybrid between these species. Identification of hybrids is less clear when subsequent hybrid generations or backcrossings are involved because distinguishing alleles may be homozygous. In such cases, as well as in instances where some common alleles are shared by the parent species, a hybrid index may be used to estimate the probability of an individual's hybrid origin (Campton and Utter 1985; Campton 1987).

#### CONCLUDING REMARKS

The presented material has established (1) that sets of Mendelian data are necessary for properly identifying and measuring genetic population units, (2) that such data were not generally available prior to the development of electrophoretic methods, and (3) that electrophoresis continues to be the primary procedure used for obtaining Mendelian data. It is understandable that new insights into genetic structures of fish species resulted from electrophoretic studies as mentioned in the Introduction. Indeed, such insights tend to be the rule rather than the exception when adequate sets of electrophoretic data are collected on a formerly unstudied species, or group of populations within a species. This point is apparent from a listing of some modified assumptions resulting from electrophoretic studies (Table 4, taken from Allendorf et al. 1987).

The insights gained from such data coupled with their relative ease of collection clearly indicate that any investigation concerned with understanding genetic structures within species as well as among closely related species should include collection of adequate sets of electrophoretic data. These data will not universally provide the biological and management insights that are sought or needed. Biological reality often fails to conform to political boundaries. Limited gene flow among partially reproductively isolated population units requiring separate management may prevent genetic divergence from being detected electrophoretically (although, as mentioned above, this limitation need not apply to cultured populations). Nevertheless, such data are a necessary starting point to determine what additional procedures may be needed to define adequately the

population units in question, and often will provide sufficient information to preclude the use of other procedures.

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Table 1. Genotypes, allele frequencies and heterozygosities from electrophoretic patterns of Figure 5. Parenthetical figures are expected proportions under binomial expansion of allele frequencies (Hardy-Weinberg equilibrium).

		POPULATION			
		1	2	3	TOTAL
LOCUS 1	<u>Genotype</u>				
	AA	45(45.1)	28(28.1)	0	73(48.2)
	AA'	5(4.8)	19(18.8)	0	24(59.5)
	A'A'	0(0.1)	3(3.1)	28(28.1)	31(18.4)
	AA''	0	0	0	0(14.1)
	A'A''	0	0	19(18.8)	19(8.7)
	A''A''	0	0	3(3.1)	3(1.0)
	<u>Allele frequency</u>				
	A	0.95	0.75	0	0.567
	A'	0.05	0.25	0.75	0.350
	A''	0	0	0.25	0.083
	<u>Heterozygosity</u>	0.10(0.09)	0.38(0.375)	0.38(0.375)	
LOCUS 2	<u>Genotype</u>				
	BB	50	50	0	100(66.7)
	BB'	0	0	0	0(66.6)
	B'B'	0	0	50	50(16.7)
	<u>Allele frequency</u>				
	B	1.0	1.0	0	0.667
	B'	0	0	1.0	0.333
<u>Heterozygosity</u>	0(0)	0(0)	0(0)		
LOCUS 3	<u>Genotype</u>				
	CC	50	50	50	150
	<u>Allele frequency</u>				
	C	1.0	1.0	1.0	1.0
<u>Heterozygosity</u>	0(0)	0(0)	0(0)		

Table 2. Average alleles per locus, proportion of loci polymorphic and average heterozygosity per locus from data of Table 1. Parenthetical heterozygosities are those expected under Hardy-Weinberg equilibrium.

	POPULATION			
	1	2	3	TOTAL
Average no. of alleles per locus	1.3	1.3	1.3	2.0
Proportion of loci polymorphic	0.333	0.333	0.333	0.667
Average heterozygosity per locus	0.033 (0.032)	0.127 (0.125)	0.127 (0.125)	
	Mean heterozygosity (H(S))		0.096	(0.095)
	Total diversity (H(T))		(0.331)	

Table 3. Components of gene diversity from allele frequencies of Table 1.

Locus	ABSOLUTE GENE DIVERSITY			
	Total	Within populations	Between populations (within subspecies)	Between subspecies
1	0.550	0.282	0.013	0.255
2	0.444	0	0	0.444
3	0	0	0	0
Average	0.331	0.095	0.004	0.232

RELATIVE GENE DIVERSITY				
Average	1.000	0.287	0.012	0.701

Table 4. Examples of biochemical genetic studies modifying previous assumptions of the genetic structure of fish species (from Allendorf et al. 1987).

	RELATIONSHIP INDICATED BY BIOCHEMICAL GENETIC DATA	REFERENCE
<b>A. <u>Identification of previously unrecognized systematic groups at the:</u></b>		
Intra-specific level	Major groups of rainbow trout corresponding to geographic region (coastal-inland) rather than drainage or life history pattern.	Allendorf & Utter 1979
	Major population units of Pacific herring on each side of the Alaska peninsula	Grant & Utter 1984
	Sharp discontinuity of populations east and west of the Apalachicola River in the southeastern U.S.A. of several freshwater species.	Bermingham & Avise 1984; Avise et al. 1984
	Reproductively isolated sympatric populations of brown trout.	Ryman et al. 1979; Ferguson & Mason 1981
Inter-specific level	Identification of previously unrecognized species of rockfish	Westrheim & Tsuyuki 1967; Seeb 1986
	Identification of previously unrecognized species of bonefish.	Shaklee & Tamaru 1981
<b>B. <u>Inconsistencies with previous assumptions of genetic divergence based on:</u></b>		
Residency vs. anadromy	Conspecificity of anadromous and landlocked forms of char of eastern North America.	Kornfield et al. 1981

Table 4. Continued

	Lack of genetic divergence between anadromous and resident populations in rainbow trout, Atlantic salmon, and brown trout (less than 0.2% and 0.5% of the total gene diversity in Atlantic salmon and brown trout, respectively).	Allendorf & Utter 1979; Ryman 1983; Ståhl 1983
Time of spawning	No apparent genetic divergence between fall and spring spawning Atlantic herring.	Ryman et al. 1984
	Major groups of chinook salmon corresponding to geographic region rather than time of spawning.	Utter et al. in prep.
Morphology	Little genetic divergence among morphologically distinct forms of cutthroat trout.	Busack & Gall 1981; Loudenslager & Kitchin 1979
	Little genetic divergence among morphologically distinct species of pupfish.	Turner 1974
	Little genetic differentiation between minnow species from two genera.	Avise et al. 1975
	Conspecificity (and local random breeding) of distinct morphological types of <u>Illyodon</u> previously considered separate species.	Turner and Grosse 1980
	Lack of apparent genetic divergence between arid adapted (redband) and anadromous (steelhead) populations of rainbow trout.	Wishard et al. 1984
	Lack of genetic divergence between two sturgeon species with overlapping geographic distribution.	Phelps & Allendorf 1983
	Conspecificity of sympatric but trophically specialized forms of Mexican cichlids.	Kornfield et al. 1982; Sage & Selander 1975

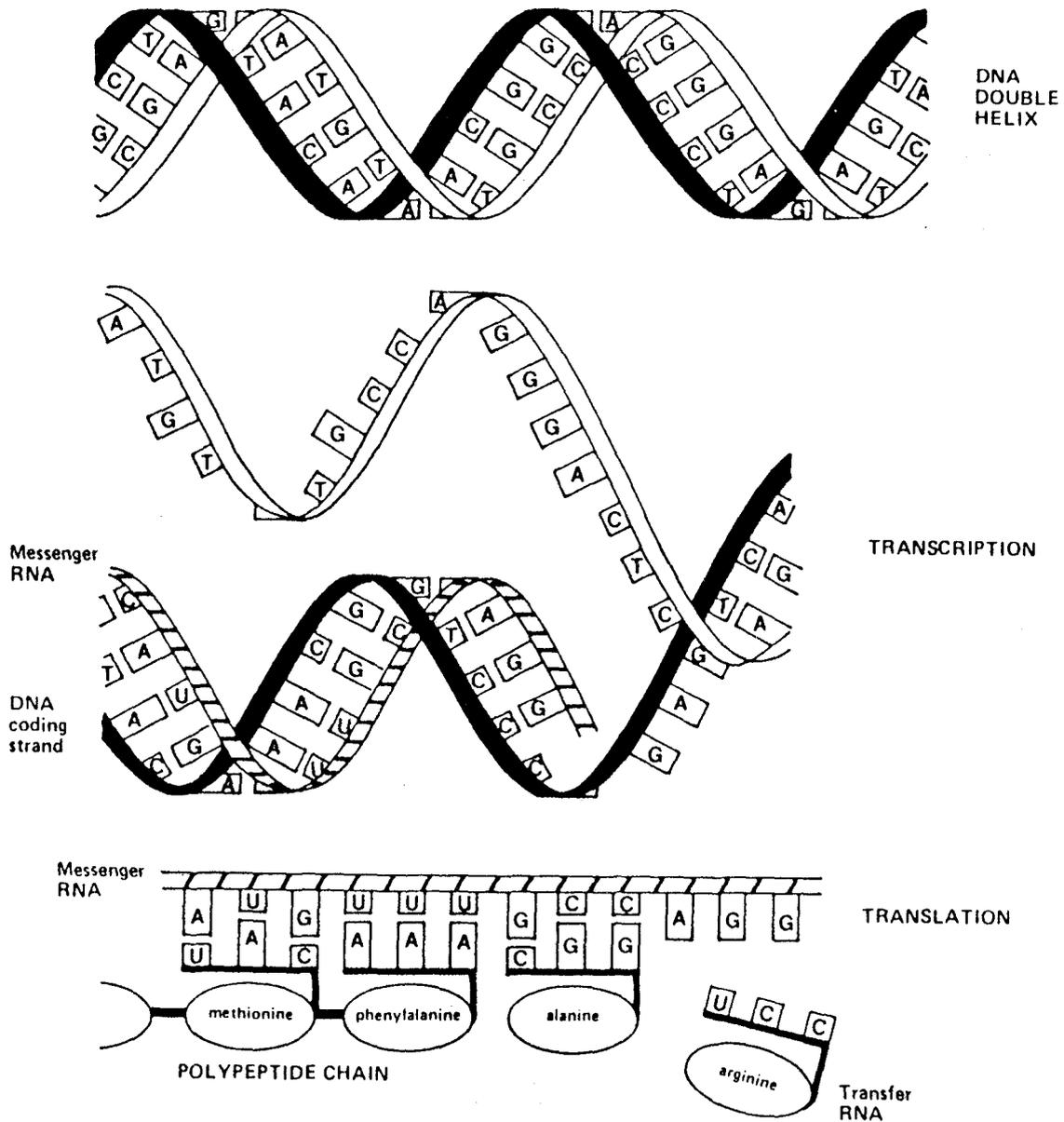


Figure 1. An outline of molecular processes relating base sequences of DNA to amino acid sequences of polypeptide chains (proteins). Messenger RNA is synthesized during transcription, and provides a template for the synthesis of the polypeptide. From Utter et al. (1987).

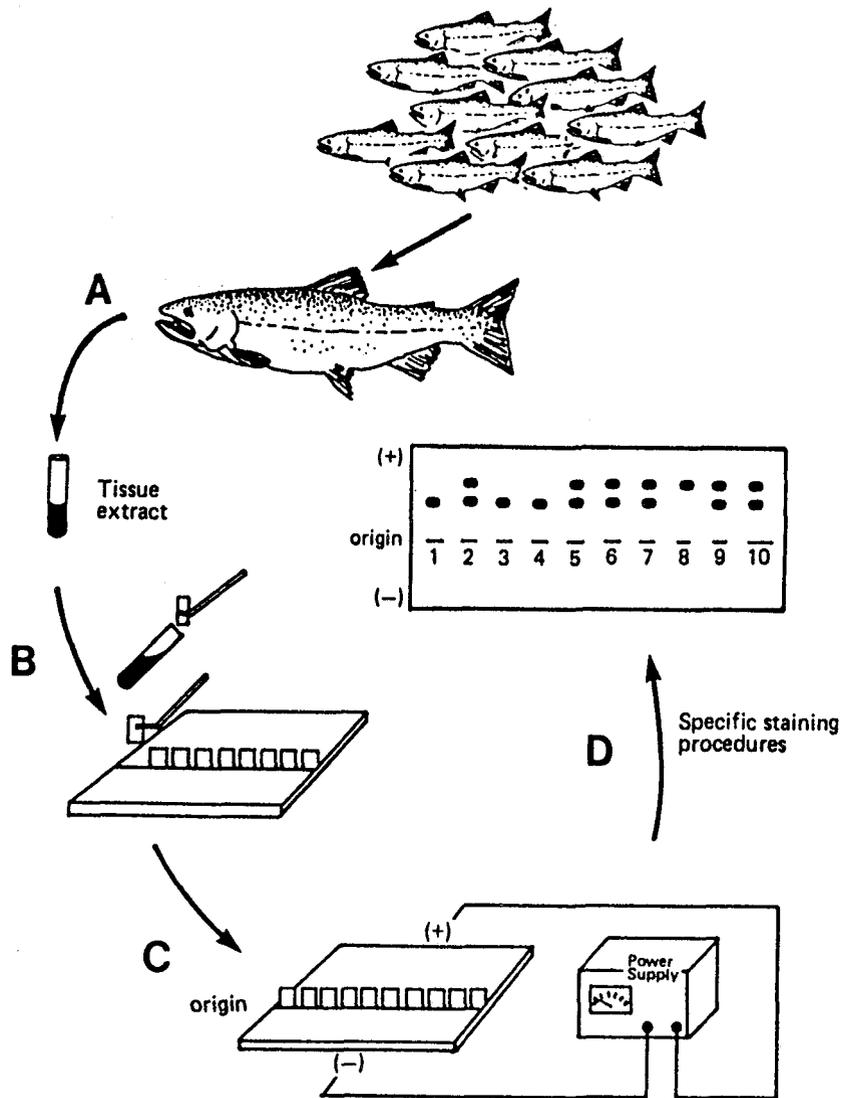


Figure 2. Standard steps for obtaining genotypic data through electrophoresis (modified from Gharrett and Utter 1982). A. Make crude protein extract from a tissue such as muscle or liver. B. Extract from each fish is introduced individually to gel by filter paper inserts. C. Different forms of a particular protein often move different distances from the point of applications when electric current (DC) is applied because of different electrical charges. D. These forms are then readily identified by a specific stain for each protein type. Specificity in staining permits identifying both the activity and the exact location of a particular protein for an individual fish from a complex mixture of proteins in each protein extract (intensities of banding patterns do not reflect differences of gene dosages in this depiction).

GENOTYPES			Subunit and subunit combinations in electrophoretic (protein) bands
AA (homozygote)	AA' (heterozygote)	A'A' (homozygote)	
<b>PHENOTYPES</b>			
Monomer	————	————	a
		————	a'
Dimer	————	————	aa
		————	aa'
		————	a'a'
Tetramer	————	————	aaaa
		————	aaaa'
		————	aaa'a'
		————	aa'a'a'
		————	a'a'a'a'

Figure 3. Electrophoretic phenotypes of two-allele polymorphisms for monomeric, dimeric and tetrameric proteins when one locus is expressed. Allelic genes at the respective loci are designated A and A'; protein subunits synthesized by these alleles are designated a and a'.

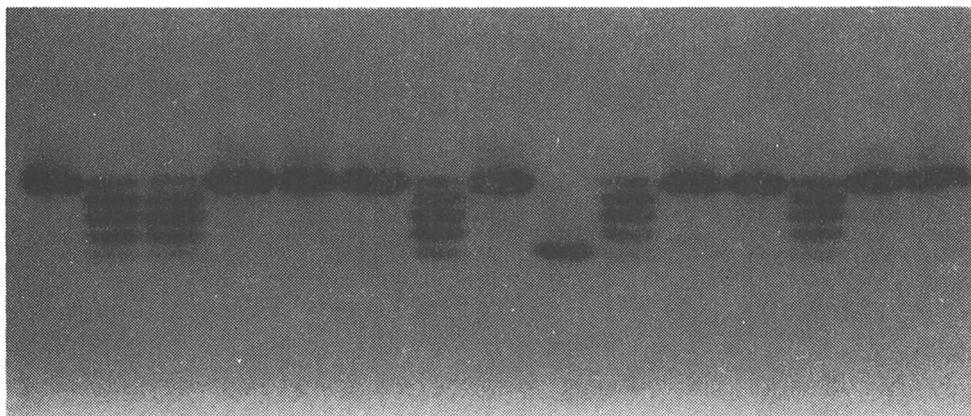
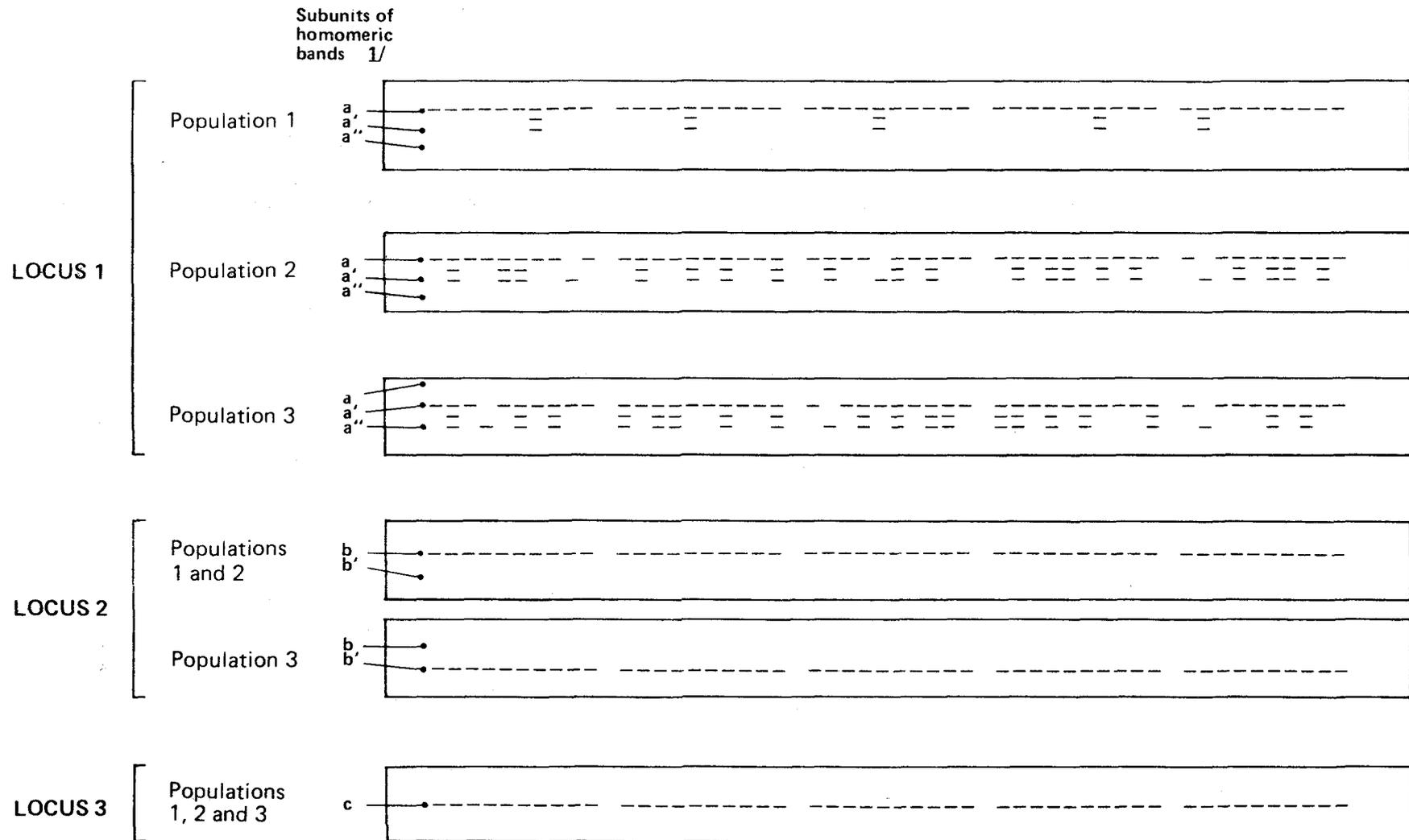


Figure 4. Actual gel patterns of single locus polymorphisms. Phenotypes of a tetrameric protein (lactate dehydrogenase) from liver extracts of rainbow trout.



1/ Subunits are the products of A, A', A'' alleles of locus 1;  
B, B' alleles of locus 2; and C allele of locus 3.

Figure 5. A hypothetical set of gel phenotypes for three loci from 50 individuals sampled from each of three populations of a species.

Source of diversity

Percentage of total

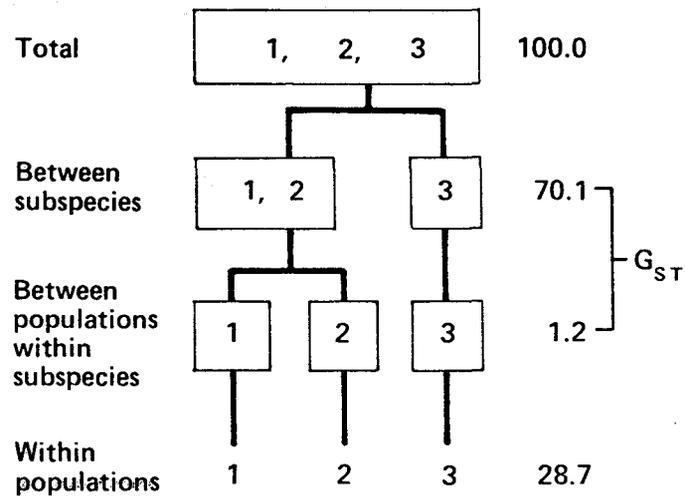


Figure 6A. Hierarchy of gene diversity analysis for data of Figure 5 and Table 1.

## GENE DIVERSITY

Relative

Absolute

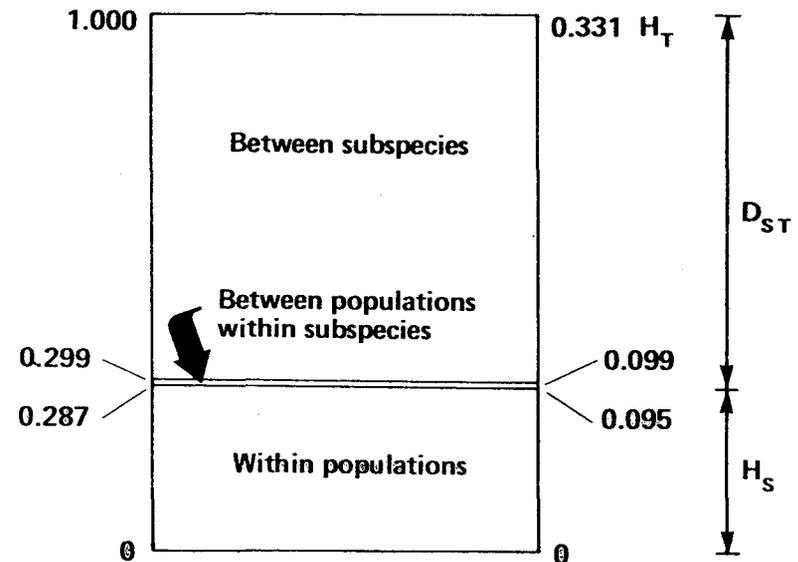


Figure 6B. Histogram comparing relative and absolute measures of gene diversity.

## APPENDIX

(For calculations from data of Table 1)

The reader is referred to Ferguson (1980) and Hartl (1980) for details of additional calculations involving Mendelian data collected by electrophoresis for protein-coding loci.

### Proportion of loci polymorphic

The proportion of polymorphic loci is the fraction of loci that are polymorphic of the loci examined. It is customary to specify a particular level of polymorphism (e.g., 0.95, 0.99). For the individual populations the proportion is the same, i.e.,

$$1/3 = 0.333.$$

For locus 2, populations 1 and 2 and population 3 are fixed for different alleles. Consequently, the species is considered polymorphic for this locus as well (although no heterozygous individuals were observed) and the proportion of polymorphic loci is

$$2/3 = 0.667.$$

### Average number of alleles

The average number of alleles per locus is the number of different detected alleles divided by the number of examined loci. For each population, this number is

$$(2 + 1 + 1)/3 = 1.3.$$

For combined samples, this number is

$$(3 + 2 + 1)/3 = 2.0$$

### Hardy-Weinberg proportions

For locus 1 and population 1 the expected number of AA genotypes in the sample of 50 individuals is

$$p(A)^2 \times 50 = (0.95)^2 \times 50 = 45.1.$$

Similarly, the expected numbers of AA' and A'A' genotypes are respectively

$$2p(A)p(A') \times 50 = 2(0.95)(0.05) \times 50 = 4.8$$

and

$$p(A')^2 \times 50 = (0.05)^2 \times 50 = 0.1.$$

If each of the samples of 50 individuals were drawn from the same panmictic population, the expected numbers of the total genotypes should approximate the numbers predicted from the binomial expansion of the mean values of the allele frequencies, i.e.,

$$p(A) = (0.95 + 0.75 + 0)/3 = 0.567,$$

etc. The expected numbers of the six genotypes for locus 1 are

$$AA = p(A)^2 \times 150 = (0.567)^2 \times 150 = 48.2$$

$$AA' = 2p(A)p(A') \times 150 = 2(0.567)(0.350) \times 150 = 59.5$$

$$A'A' = p(A')^2 \times 150 = (0.350)^2 \times 150 = 18.4$$

$$AA'' = 2p(A)p(A'') \times 150 = 2(0.567)(0.083) \times 150 = 14.1$$

$$A'A'' = 2p(A')p(A'') \times 150 = 2(0.350)(0.083) \times 150 = 8.7$$

$$A''A'' = p(A'')^2 \times 150 = (0.083)^2 \times 150 = 1.0.$$

It is clear that Hardy-Weinberg proportions of genotypes are not approximated from comparing these values with the observed totals of Table 1. Less obvious deviations may be tested statistically by chi-square or G-statistic (see contingency test, below). Appropriate degrees of freedom for tests of Hardy-Weinberg proportions are the number of phenotypes minus 1, minus the number of alleles minus 1; with three alleles the degrees of freedom would be

$$(6 - 1) - (3 - 1) = 5 - 2 = 3.$$

Heterozygosity at individual loci is the proportion of observed or expected heterozygotes relative to the total genotypes, e.g.,

$$5/50 = 0.10 \text{ (observed heterozygosity)}$$

$$4.8/50 = 0.09 \text{ (expected heterozygosity)}$$

for locus 1 and population 1.

Average heterozygosity of a population is the sum of the heterozygotes at individual loci divided by the number of loci, e.g., for the observed heterozygotes of locus 1 and population 1

$$0.10/3 = 0.033.$$

The average heterozygosity within populations is the sum of the heterozygosities for each population divided by the number of populations, e.g.,

$$(0.033 + 0.127 + 0.127)/3 = 0.096$$

for the observed average heterozygosity within populations.

Total gene diversity is the expected heterozygosity based on the mean values of allele frequencies averaged over all loci, i.e.,

$$[2(0.567)(0.350) + 2(0.567)(0.083) + 2(0.350)(0.083) + 2(0.667)(0.333)]/3 = 0.331$$

#### Contingency tests

A contingency table for the observed and expected (in parentheses) numbers of A and A' alleles in populations 1 and 2 is

	Populations			Total
		1	2	
Alleles	A	95(85)	75(85)	170
	B	5(15)	25(15)	30
	Total	100	100	200

where the expected values are the averages of the two populations for alleles A and A'.

A chi-square test for these data with

$$(\text{number of alleles} - 1)(\text{number of populations} - 1) = 1$$

degrees of freedom is the sum of

$$(O - E)^2/E$$

where O is the observed and E the expected value, i.e.,

$$(10)^2/85 + (10)^2/85 + (10)^2/15 + (10)^2/15 = 15.68.$$

A G-test for the same data set and degrees of freedom is

$$2[(f \ln f \text{ for the allelic frequencies}) - (f \ln f \text{ for the row and column totals}) + (n \ln n)]$$

where  $f$  is the number of alleles observed in each cell and  $n$  is the total number of alleles, i.e.,

$$2[(95 \ln 95 + 5 \ln 5 + 75 \ln 75 + 25 \ln 25) - (100 \ln 100 + 100 \ln 100 + 170 \ln 170 + 30 \ln 30) + (200 \ln 200)] = 16.9.$$

The distribution of the G-statistic approximates that of chi square and significance levels of both tests can be found in tables of chi square.

#### Genetic distance

Genetic distance ( $D$ ) as given in the text is

where

$$I = \frac{-\ln(I)}{\sum x_i y_i \sqrt{\sum x_i^2 \sum y_i^2}}$$

and  $x_i$  and  $y_i$  are frequencies of specific alleles in populations  $x$  and  $y$ .

Calculation of  $D$  for populations 1 and 2 is as follows:  
In locus 1,

$$I = \frac{(0.95)(0.75) + (0.05)(0.25)}{[(0.95)^2 + (0.05)^2][(0.75)^2 + (0.25)^2]} = 0.964.$$

For both loci 2 and 3

$$I = \frac{(1)(1) + (0)(0)}{[(1)^2 + (0)^2][(1)^2 + (0)^2]} = 1$$

The combined value of  $I$  for the 3 loci is

$$(1 + 1 + 0.964)/3 = 0.988,$$

and

$$D = -\ln(0.988) = 0.012.$$

Calculation of D for populations 1 and 3 is as follows:

In locus 1

$$I = (0.50)(0.75) / [(95)^2 + (0.05)^2][(0.75)^2 + (0.25)^2]^{0.5} = 0.050$$

In locus 2, I=0 because of no common alleles and a numerator of 0.

In locus 3, I=1 in the same manner as populations 1 and 2, loci 2 and 3.

The mean value for I over all three loci is

$$1.05/3 = 0.350,$$

and

$$D = -\ln 0.350 = 1.05$$

In a similar manner, the I values for populations 2 and 3 are

locus 1 - 0.249  
locus 2 - 0  
locus 3 - 1  
average - 0.416

and

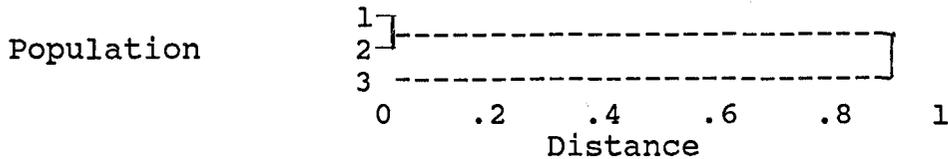
$$D = 0.876.$$

#### Cluster analysis

The unweighted pair-group method (UPGM) of clustering for constructing a dendrogram starts with a matrix of paired distance (or similarity) values. The first two groups to be clustered are those with the lowest distance (or highest similarity) value. A new matrix is then formed - reduced by one row and column - containing these paired groups as a single member. The recalculated values involving this new member are the means of the values of its two component groups with each of the other members from the original matrix. The process is repeated (in large matrices) through the ultimate averaging of values from two remaining groups. In the present case, populations 1 and 2 join at a D value of 0.012. The new member (comprising populations 1 and 2) joins population 3 at a D value of

$$(1.050 + 0.876)/2 = 0.963.$$

The cluster then, appears as



### Gene diversity analysis

The initial calculations for gene diversity analysis have already been outlined under different calculations of expected heterozygosity in the section presenting Hardy-Weinberg calculations. An alternate formula for expected heterozygosity at a locus is

$$(1 - \text{the sum of squared allelic frequencies}).$$

The mean expected heterozygosity of Table 1 is  $H(S)$  as outlined in the text; likewise, the total diversity is  $H(T)$ .

The only additional value that requires calculation in the present hierarchy is the increase in expected heterozygosity from  $H(S)$  at the level of populations within subspecies. This value is obtained by pooling the allelic frequencies of the populations within each subspecies (in this instance, populations 1 and 2) and recalculating the mean heterozygosity from  $H(S)$  at the level of populations within subspecies. This value is obtained by pooling the allelic frequencies of the populations within each subspecies (in this instance, populations 1 and 2) and recalculating the mean heterozygosity as if the subspecies themselves were the individual populations. This calculation can be weighted assuming the sampling is representative of the actual number of population units existing in the species (Chakraborty 1980); i.e., for locus 1

$$[2(2)(0.85)(0.15) + 2(0.75)(0.25)]/3 = 0.295.$$

Alternatively, the calculation can be unweighted, assuming the sampling is independent of the actual number of units (D. Campton, personal communication; see also Chakraborty and Leimar, 1987); i.e.,

$$[2(0.85)(0.15) + 2(0.75)(0.25)]/2 = 0.315$$

The difference between either of these figures and  $H(S)$  is that portion of the structuring that is due to population heterogeneity within subspecies depending on which assumption is most appropriate. Under the first assumption,

$$.295 - .282 = .013.$$

The remainder of the structuring within the species, i.e.,

$$0.268 - 0.013 = 0.255$$

is that portion due to differences between subspecies.

For locus 2, all diversity is due to differences between groups because of fixation of alternate alleles in different subspecies. The absolute gene diversity calculated on the weighted allele frequencies is

$$2(0.667)(0.333) = 0.444.$$

The average absolute gene diversity between subspecies is

$$(0.255 + 0.444)/3 = 0.232.$$

Similarly, the average gene diversity between populations is

$$(0.013)/3 = 0.004.$$

Calculations of coefficients of gene diversity are based on that proportion of H(T) that is due to population heterogeneity at a particular level of the hierarchy: i.e., for the within population component

$$0.095/0.331 = 0.287;$$

the combined diversity from subdivision at both hierarchical levels (G(ST)) is

$$(0.331 - 0.095)/0.331 = 0.713;$$

the coefficient of gene diversity due to subspecies is

$$(0.232)/(0.331) = 0.701;$$

the coefficient of gene diversity due to populations within subspecies is

$$(0.004)/(0.331) = 0.012.$$

# IDENTIFICATION AND INTERPRETATION OF MITOCHONDRIAL DNA STOCKS IN MARINE SPECIES

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## INTRODUCTION

My assigned task is to evaluate the use of mitochondrial DNA (mtDNA) in genetic stock identification of marine fishes, but in some respects the timing for this review is less than ideal. First, relatively few published data are yet available on mtDNA variability in organisms (fishes or otherwise) inhabiting the marine realm. Second, the far more abundant information on mtDNA in terrestrial and freshwater species has recently been summarized from diverse perspectives. Thus, Brown (1981, 1983, 1985) emphasizes molecular mechanisms and rates of evolution of animal mtDNA; Avise and Lansman (1983), Avise (1986) Avise et al. (1987a), and Wilson et al. (1985a) review evolutionary genetic aspects of mtDNA polymorphism in natural populations; Lansman et al. (1981) summarize laboratory techniques as well as potential applications of mtDNA approaches in population analysis; and Ferris and Berg (1987) and Quinn and White (1987) evaluate mtDNA approaches as they have been applied to fishes and birds, respectively. All of these reviews should be consulted by those interested in a deeper appreciation of many issues that will also be dealt with here.

However, this symposium is well-timed if the intent is to evaluate prospects and to influence direction of mtDNA research with marine organisms. Previous surveys of mtDNA in terrestrial and freshwater species have provided a solid foundation of information about the molecular and evolutionary dynamics of mtDNA polymorphism, and have shown that mtDNA genotypes often readily distinguish geographically separated, conspecific populations. While the potential for marine stock identification thus appears great, preliminary data suggest that some marine species may show little or no mtDNA differentiation over large geographic areas. It seems likely that interplays between life history-mediated dispersal potential and the physical environment (past and present) exert major influence on magnitude and pattern of mtDNA population divergence.

Here I will reiterate general features of mtDNA that make it a powerful genetic marker for microevolutionary study. I will specifically summarize available mtDNA information for marine species, and will conclude with some thoughts about meaningful

research directions with marine organisms. This review is geared for a general audience.

## BACKGROUND

In this section major features of mtDNA variability in higher animals will be listed. For those interested in detailed justification, one or a few primary references of particular importance will be cited for each topic, and together with the reviews mentioned above, can be utilized as entrances into the literature.

### Molecular Properties and Transmission Genetics

i) Molecular characteristics. MtDNA is a closed-circular, duplex molecule usually about 16,000 base pairs long in higher animals. It encodes about a dozen proteins involved in electron transport and oxidative phosphorylation, as well as transfer and ribosomal RNAs that participate in protein translation on mitochondrial ribosomes (Bibb et al. 1981; Anderson et al. 1981). The great majority (>90 percent) of the molecule is transcribed to functional RNA, the major exception being a "D-loop region" of about 1000 base pairs which contains an origin of mtDNA replication. There are no introns within transcribed genes, essentially no spacer sequences between genes, and no classes of repetitive DNA.

ii) Molecular basis of variation. MtDNA gene content and arrangement are conserved over long spans of evolutionary time. For example, man, mouse and cow (representing three mammalian orders) have identical mtDNA gene order, which also appears similar if not identical to that of the clawed frog Xenopus (Brown 1983). Nonetheless, mtDNA evolves rapidly at the nucleotide sequence level (see beyond). Most mutational changes (perhaps >90 percent) are single-base substitutions, primarily transitions (Greenberg et al. 1983; Aquadro and Greenberg 1983). Particularly in non-coding regions, small-size (one or a few base pair) addition/deletions are also common (Cann and Wilson 1983), but in routine population assays these are not distinguishable from base substitutions. Large-size (e.g., tens or hundreds of base pair) length changes within species have been documented in some lower vertebrates (Densmore et al. 1985; Bermingham et al. 1986) and insects (Harrison et al. 1985).

iii) Intraindividual homoplasmy. Various somatic and germ cells of an individual animal usually appear homoplasmic--that is, exhibit a single detectable mtDNA nucleotide sequence. Since

most animal cells contain many (often thousands) of mtDNA molecules, lack of significant within-individual sequence heterogeneity was unexpected, and remains somewhat a mystery. One possible explanation involves sorting of mtDNA molecules into homogeneous populations by random genetic drift in intermediate germ cells, where mtDNA numbers might be small (Upholt and Dawid 1977; Birky et al. 1982; Chapman et al. 1982; Solignac et al. 1984). Notwithstanding recent reports of occasional intraindividual heteroplasmy (Bermingham et al. 1986; Densmore et al. 1985; Harrison et al. 1985), and the likelihood that additional heteroplasmy remains undetected by conventional assays, the clear pragmatic result is that heteroplasmy is not a serious complication in natural population analysis.

iv) Maternal inheritance. Mitochondria are housed in the cell cytoplasm. In higher animals, since zygote cytoplasm derives overwhelmingly from the egg, mtDNA is predominantly if not exclusively maternally inherited (Lansman et al. 1983a).

v) Effective lack of recombination. It is uncertain whether a potential for mtDNA recombination in cells of higher animal is realized, as it is in some yeasts (Fonty et al. 1978) and in artificial cell hybrids (Belliard et al. 1979; Dawid et al. 1974). However, even if physical recombination does occur, its effectiveness in generating novel genotypes should be minimal because of the uniparental transmission and usual homoplasmy of mtDNA. In effect, in contrast to the recombining nuclear genome, mtDNA genotypes have a linear evolutionary history of asexual (matriarchal) transmission.

#### Data acquisition and analysis

Much of our understanding of the detailed molecular characteristics of mtDNA has come from labor intensive techniques of nucleotide sequencing. For purposes of population-level analysis and stock identification, mtDNA markers are far more readily revealed as restriction fragment or site polymorphisms, by the procedures outlined below.

i) mtDNA isolation. MtDNA may be isolated either in highly purified or in crude form (contaminated with nuclear DNA), depending upon how subsequent assays are to be conducted. In its covalently-closed-circular form, mtDNA exhibits a unique density (in CsCl gradients) which provides the basis for its separation from nuclear DNA, RNA, and other contaminants. Purification begins with a series of low speed centrifugations of homogenate derived from fresh liver, brain, kidney, ovary, or other soft tissue. (Freezing of tissue decreases yield dramatically by introducing single-strand breaks in mtDNA and altering its migration in gradients.) Pelleted mitochondria are then lysed, and mtDNA purified by high speed CsCl density gradient

centrifugation (Lansman et al. 1981). Rate-limiting steps are the CsCl centrifugations (36 hours), and subsequent dialyses (48 hours) which remove ethidium bromide and excess ethylene diamine tetraacetic acid.

An alternative procedure which eliminates these rate-limiting steps produces a crude mtDNA preparation that can be used with "Southern-blotting" gel assays. Fresh or frozen tissue is homogenized, proteins are removed by phenol/chloroform extraction, and nucleic acids precipitated with ethanol (Maniatis et al. 1982). In this approach, mtDNA "purification" can be thought of as being effectively achieved at the gel electrophoretic stage, where mtDNA genotypes are specifically revealed by hybridization to purified mtDNA "probes" on Southern blots (see below).

Numerous minor variations on these mtDNA isolation themes exist, and it is to be expected that methodological advances will streamline the process (Chapman and Powers 1984). In some cases, nature aids in mtDNA isolation: mature oocytes have a ratio of mtDNA to nuclear DNA of up to 100:1, whereas this ratio is often 1:100 in somatic cells (Dawid 1972).

ii) Restriction digestion and electrophoretic separation. Restriction endonucleases cleave duplex DNA at specific recognition sites, either 4, 5, or 6 base pairs long. For example, the restriction enzyme EcoRI recognizes GAATTC. If three such recognition sites are present in a given mtDNA, digestion by EcoRI will produce three linear mtDNA fragments whose sizes are determined by the positions of these sites on the mtDNA molecule. Such fragments can be separated by molecular weight on agarose or acrylamide gels, resulting in mtDNA digestion profiles which constitute the raw data in mtDNA population surveys (Figure 1).

Many restriction enzymes can be purchased commercially, with digestion conditions specified by the manufacturer. The method of visualization of digestion products on gels is dictated by the purity and amount of mtDNA available. For purified mtDNA, fragments can be readily identified by staining with ethidium bromide, or by "end-labeling" with radioactive nucleotides followed by autoradiography (Brown 1980). End-labeling offers the advantage of greater sensitivity in detecting small amounts of DNA. Thus, beginning with 2-5 grams of liver tissue, we routinely isolate enough mtDNA for at least 20 end-labeling digestions, while only 5-10 digestions might be scored using ethidium bromide.

For crude mtDNA preparation, digestions and electrophoretic separations are carried out as usual (although larger amounts of restriction enzyme are required), and the fragments are

transferred (blotted) to a filter such as nitrocellulose. The filter is then incubated under DNA/DNA hybridization conditions with radioactivity labeled "probe". The probe is mtDNA from the same or a related species, highly purified by an extension of approaches outlined above, or by molecular cloning in an appropriate vector. Autoradiography reveals the positions of successfully-hybridized probe, and hence the positions of mtDNA digestion fragments in the original gel.

Several considerations influence choice of techniques. End-labeling or ethidium bromide procedures require purified mtDNA from each sample, and hence normally involve the expense and time-constraints of an ultracentrifuge. However, since gradient centrifugations require little "hands-on" effort, they can readily be integrated with other laboratory work. Major advantages of Southern-blotting methods are decreased sample preparation time, and the possibility of utilizing frozen or limited tissue. These advantages are offset somewhat by the additional expense of restriction enzymes, the need for purified or cloned mtDNA from appropriate source, and the usual difficulty of detecting smaller fragments (<500 base pairs) on gels due to inefficient transfer during blotting.

iii) Data analysis. Base substitutions and very small addition/deletions account for most sequence evolution in mtDNA. These genetic changes are often reflected in interpretable changes of mtDNA digestion profiles on gels. For example, Figure 1 shows several distinct EcoRI mtDNA profiles in a sample of 18 American and European eels, Anguilla. Individuals in lanes 1, 2, and 4-12 (from the left) exhibit 4 mtDNA fragments, of sizes 7.9, 4.5, 3.1, and 1.0 kilobases (kb). Individuals in lanes 16-18 show 5 fragments, of sizes 5.0, 4.5, 3.1, 2.9, and 1.0 kb. Evolutionarily, these two genotypes (arbitrarily called EcoRI patterns B and C) are interrelated by a single EcoRI restriction site gain or loss, interconverting the 7.9 kb fragment in B with the 5.0 and 2.9 kb fragments in C. This restriction site difference is most likely due to a single base substitution (or small addition/deletion) creating or destroying an EcoRI site (GAATTC), depending on whether site presence or absence was the ancestral condition. Similarly, inspection of fragment sizes reveals that the individual in lane 3 has an EcoRI mtDNA genotype (labeled A) which differs from B by a single site loss; and individuals in lanes 14 and 15 have a profile D differing from C by a single site gain. A useful check on band scoring is provided by total mtDNA genome size, which in Anguilla is about 16.5 kb. Thus, in profile E (Figure 1), two mtDNA fragments must have the same molecular weight, 3.1 kb. Altogether the five EcoRI mtDNA genotypes in the figure can be connected into a single most-parsimonious evolutionary network, which is shown in the figure.

A reasonable population survey might involve 5-20 restriction endonucleases, each with a different recognition site. Data from such a survey can be summarized in a composite letter code, and the genotypes interconnected into a parsimony network by an extension of the above reasoning (Avice et al. 1979a). For larger or more complex data sets, a matrix of restriction fragment (or site) presence versus absence in each individual may serve as a qualitative data base for evolutionary reconstruction by any of numerous computer algorithms.

With further effort (involving "double" and "partial" digestion procedures -- Maniatis et al. 1982), restriction sites can be mapped on the mtDNA molecule (Figure 2). Site maps provide additional information about molecular aspects of mtDNA variation, as well as clarify interpretations of fragment pattern interconversions. For example, suppose the EcoRI patterns B and C (Figure 1) had not been observed in the survey of Anguilla. From fragment comparisons alone, by hard criteria it would be unclear whether patterns A and E differed by a series of independent site changes, or whether genome rearrangements (e.g., inversions or translocations) might be responsible. Site mapping would help provide the answer.

For either restriction fragment or site data, nucleotide sequence divergence ( $p$ ) between samples can be estimated from formulae derived under reasonable assumptions about mode of mtDNA evolution (e.g., Nei and Li 1979). Matrices of  $p$  values between samples provide useful quantitative summaries of data (and can also be used in estimation of phylogeny). A complete analysis of an mtDNA data set includes both qualitative and quantitative approaches.

### Evolutionary Properties

i) Rate of evolution. MtDNA appears to evolve at a rate 5-10 times greater than that of average, single-copy nuclear DNA (Brown et al. 1979). From the initial slope of a curve relating mtDNA sequence divergence to time since speciation for 19 pairs of mammalian species, Brown et al. (1979) calculated a rate of sequence divergence of about two percent (one percent per lineage) per million years. Reasons for this rapid evolution are unclear, but may include faulty mtDNA replication and/or inefficient repair mechanisms (Brown 1983). Relaxation of selection due to genome redundancy (presence of many mtDNA copies per cell) is presumably not an adequate explanation because most individuals are homoplasmic. Furthermore, most observed nucleotide substitutions are in genomic positions where they would be expected to be tolerated by natural selection -- e.g., the D-loop region, and silent positions in coding sequences (Brown et al. 1982).

ii) Intraspecific polymorphism. In nearly all species surveyed to date, mtDNA polymorphism is extensive (Awise and Lansman 1983). Estimates of mean nucleotide sequence divergence ( $p$ ) between conspecific individuals commonly range from 0.3 to 4 percent, with maximum  $p$  values sometimes approaching 10 percent. Major published studies have included terrestrial mammals (Brown and Simpson 1981; Lansman et al. 1983b), reptiles (Wright et al. 1983), amphibians (Spolsky and Uzzell 1984), and freshwater fishes (Awise et al. 1984a; Bermingham and Awise 1986). Data for marine species will be detailed later.

iii) Geographic structuring of genotypes. Of particular relevance to the current report is the observation (for terrestrial and freshwater species) that mtDNA genotypes are usually strongly patterned geographically (Awise et al. 1987a). Thus, for many species the rate of geographic spread of mtDNA variants by dispersal and gene flow has not been sufficient to override historical patterns of population subdivision supposedly revealed in mtDNA phylogeny reconstructions. Furthermore, an early comparison between mtDNA and allozyme data sets for one species, the pocket gopher (Geomys pinetis) led us optimistically to suggest "that restriction analysis of mtDNA is probably unequalled by other techniques currently available for determining phylogenetic relationships among conspecific organisms" (Awise et al. 1979a). With some qualifications, this suggestion still appears valid today. The following exemplify studies from our laboratory in which results of mtDNA and conventional multi-locus allozyme surveys were compared.

a) Geomys pinetis, pocket gopher. --An mtDNA and allozyme survey of 87 gophers collected from across the species' range in the southeastern United States revealed two major genetic forms (eastern and western "races") that had remained unrecognized by morphologic criteria (Awise et al. 1979a). The two forms were distinguished by mtDNA restriction site differences for 5 of 6 endonucleases ( $p=0.034$ ), but by only a single fixed electromorph difference at 25 allozyme loci (Nei's (1972)  $D=0.065$ ). Allozyme variation in G. pinetis was low and inadequate to provide much further population resolution. However, 23 mtDNA genotypes were observed in the study, all of which were geographically localized. Furthermore, a parsimony network demonstrated that geographically contiguous samples were usually closely related genetically.

b) Peromyscus maniculatus, deer mouse. --Eight endonucleases were used to generate restriction site maps for mtDNAs of 135 animals collected across North America (Lansman et al. 1983b). A total of 61 mtDNA genotypes was observed. Each genotype was confined to a small portion of the species' total range, and genetically related genotypes were usually proximate

genotypes, in eastern and western North America, respectively, were highly distinct in mtDNA composition ( $\bar{p} \approx 0.05$ ). The conspecific population structure evidenced by the mtDNA genome was largely invisible to allozyme analysis (Awise et al. 1979b). Thus in a conventional protein-electrophoretic survey, at 16 monomorphic or mildly polymorphic loci the same electromorph predominated in all samples; and at 6 highly polymorphic loci the same alleles usually recurred in high, intermediate, and low frequencies throughout the continent.

c) Lepomis macrochirus, bluegill sunfish. --In the southeastern United States, two bluegill subspecies originally described by morphologic criteria proved to exhibit essentially fixed allelic differences at two of 15 surveyed allozyme loci (Awise and Smith 1974). MtDNA restriction site maps, generated from 12 endonucleases (Figure 2), distinguished the subspecies by 20 restriction site changes (Awise et al. 1984a).

Thus, in each of the above studies, restriction enzyme analysis of mtDNA provided more genetic markers and proved to be a more sensitive indicator of intraspecific differentiation than did conventional allozyme analysis of a moderate number of nuclear gene products. The enhanced resolution offered by this mtDNA approach is presumably attributable to one or both of the following: (1) a more rapid pace of evolution in the mtDNA genome; (2) ability of the restriction endonuclease assay to detect all classes of nucleotide substitution, including those in non-coding regions and in silent positions of protein-coding genes.

#### RESULTS--mtDNA DIFFERENTIATION IN MARINE SPECIES

These background considerations raise the hope that mtDNA analysis may also be an especially powerful approach for identifying genetic stocks in marine species. This section summarizes the extent to which this hope has thus far been realized.

i) Limulus polyphemus, American horseshoe crab. --L. polyphemus is an estuarine species with geographic range from New Hampshire to the Yucatan Peninsula. Adults are benthic and relatively sedentary. Eggs laid in the intertidal zone hatch into trilobite larvae that presumably remain near shore and quickly settle on intertidal flats.

Saunders et al. (1986) employed 12 informative restriction endonucleases to survey mtDNAs purified from 99 horseshoe crabs collected from New Hampshire to the panhandle of Florida. MtDNA fragment profiles produced by selected enzymes are shown in Figure 3, and their geographic distributions in Figure 4. For

example, two BstEII fragment patterns differing by a single restriction site were observed: pattern C in all Limulus collected north of Brunswick, Georgia; pattern B in crabs south of Cape Canaveral, Florida and in the Gulf of Mexico. Geographic distributions of mtDNA genotypes virtually identical to this were elucidated by HindIII, HincII, and BclI (Figures 3 and 4), BamHI, NdeI, and SacI (not shown). This dramatic genetic "break" ( $p \approx 0.02$ ) between northern and southern samples was a surprise, because Limulus is continuously distributed along the coastline and there are no obvious barriers to gene flow in the transition region. Potential explanations will be considered in the Discussion. Whatever the reason, numerous genetic markers provided by mtDNA clearly distinguish northern from southern "stocks" of the horseshoe crab.

Additional but apparently minor mtDNA population substructure in Limulus was revealed with some enzymes. For example, a variant HincII pattern (A) was observed only in the western panhandle of Florida; and the variants XbaI "B" and BclI "Y" appeared confined to the region from Panacea, Florida to the Florida Keys (Figures 3 and 4). One mtDNA variant (XbaI "D") was observed in several widely separated geographic locales, and in mtDNA genotypes that were otherwise very different from one another. XbaI "D" differs from the common pattern XbaI "C" by a restriction site loss which could be due to a substitution in any of the six bases in its recognition site, TCTAGA. Thus, it is likely that XbaI "D" has arisen from XbaI "C" more than once in evolution. Similar evidence for occasional convergent site evolution has been presented in other mtDNA surveys (Lansman et al. 1983b).

A total of ten mtDNA genotypes was observed in the Limulus study --three in the northern populations and seven in the southern. Figure 5 shows a parsimony network interconnecting the composite genotypes.

Overall, the results for Limulus document that at least some continuously distributed marine organisms can exhibit dramatic mtDNA differentiation geographically. On the other hand, most of the mtDNA divergence was associated with the major genetic break in the north Florida region. Particularly in collections from Georgia to New Hampshire, observed mtDNA genotypic diversity was very low.

An earlier allozyme survey gave some indication that northern and southern Limulus might be genetically divergent. Selander et al. (1970) assayed protein products of 24 nuclear loci in 64 individuals from four localities: (1) Massachusetts, (2) Virginia, (3) Panacea, Florida, and (4) Panama City, Florida. At seven of nine polymorphic loci, there were consistent (though relatively minor) allele frequency differences between the

Atlantic and Gulf Coast populations. These findings appear to parallel the results presented earlier for Geomys and Lepomis -- general concordance between allozyme and mtDNA data, but greater distinguishing power and resolution afforded by mtDNA.

ii) Anguilla rostrata and A. anguilla, American and European eels. --North Atlantic eels have a catadromous life history. Spawning takes place in the western tropical mid-Atlantic Ocean (the Sargasso Sea), and larvae disperse by ocean currents to coastal regions. Young inhabit estuarine and fresh water until sexual maturation when they return to the Sargasso Sea to spawn. Important questions raised by this unusual life history pattern include the following: (1) is spawning within either A. rostrata or A. anguilla essentially panmictic? (2) is larval dispersal largely passive, such that recruits at any geographic locale represent random draws from perhaps a single gene pool? and (3) might the two nominal species actually belong to a single randomly breeding population? From a consideration of morphological, life history, and protein electrophoretic data, Williams and Koehn (1984) propose that answers to the first two questions are "yes", and to the third "no".

Awise et al. (1986) used 18 restriction endonucleases to assay the mtDNA of 138 eels from seven North American and two European locales. In the American eel, A. rostrata, four results argued against significant divergence among samples collected from Maine to Louisiana: (1) one composite mtDNA genotype (composed of 80 restriction sites) was most common in all collections; (2) variant genotypes present in two or more individuals were observed in widely separated geographic locales; (3) a strong polymorphism (provided by HincII) was not significantly different in frequency among collections; and (4) no genotype differed by more than two assayed mutation steps from the common composite pattern. The apparent lack of significant mtDNA divergence of A. rostrata samples along this 4,000 kilometer stretch of shoreline contrasts sharply with the geographic structuring of mtDNA genotypes normally observed in terrestrial and freshwater vertebrates sampled over large geographic areas. These results are certainly consistent with the conventional postulate of a single spawning population and random dispersal of larvae for A. rostrata. Nonetheless, it remains possible that separate spawning populations do exist, but that they have been separated for too short an evolutionary time for accumulation of significant genetic differences.

A. rostrata was, however, easily distinguishable from A. anguilla in mtDNA composition. Eleven of 18 endonucleases produced distinct mtDNA digestion profiles and the overall proportion of shared digestion fragments was only  $F = 0.62$  (corresponding to a sequence divergence estimate of  $p = 0.035$ ). KDThe EcoRI digests in Figure 1 faithfully reflect the general

mtDNA patterns observed with many enzymes, i.e., identity of the common genotype in American eels from all locales, occasional variants usually differing from the common genotype by one restriction site change, and clear distinction of American from European eels.

A. rostrata and A. anguilla have previously proved difficult to distinguish by criteria other than continent inhabited by juveniles. A single morphological trait (number of vertebrae, genetic basis unknown) separates most specimens, as does one known allozyme locus (Mdh-2; Comparini and Rodino 1980). MtDNA provides the first convincing evidence that the two nominal species differ in a large number of genetic characters.

iii) Arius felis, hardhead catfish. --Life history patterns as extreme as that in Anguilla rostrata apparently are not necessary for the observation of relative geographic uniformity of mtDNA genotypes. A survey of 13 informative restriction enzymes on 60 hardhead catfish from 10 locales between North Carolina and Louisiana revealed 11 distinct mtDNA genotypes (Awise et al. 1987b), but as was the case in American eels, there was little evidence for geographic structuring of those genotypes. Thus the most common genotype greatly predominated all collections, and rarer genotypes were also usually observed in widely separated locales. A similar picture also emerged within the gafftopsail catfish (Bagre marinus), which was however sampled much less extensively (Awise et al. 1987b). Marine catfish are mouthbrooders of eggs and fry, so gene flow must be mediated by adult movement. These catfish are certainly strong and active swimmers, and the mtDNA data suggest that populations along the Atlantic and Gulf Coasts have had extensive historical interconnectedness.

iv) Opsanus tau, oyster toadfish. --Toadfish are bottom-dwelling, sluggish fish that lay demersal eggs and lack pelagic larvae. A survey of 43 specimens of O. tau, collected from across most of its range from Massachusetts to Georgia, revealed two distinct mtDNA phylogenetic groupings that were also geographically partitioned (Awise et al. 1987b). The approximate dividing line between the two groups is the Cape Hatteras region of North Carolina, which is also a well-known boundary between zoogeographic provinces. The northern versus southern forms of O. tau were clearly distinguishable by restriction site differences at HindIII, MspI, and StuI, (out of 13 endonucleases utilized), and the estimate of mean nucleotide divergence was  $\bar{p} \approx 0.01$ . Opsanus beta, a related species in the Gulf of Mexico, was also included in the study, and proved to exhibit some minor geographic differentiation. However, O. tau and O. beta were highly distinct from one another in mtDNA composition, exhibiting a nucleotide divergence value of  $\bar{p} \approx 0.10$ , and apparently fixed restriction site differences for all 13 of the endonucleases

utilized. Thus both by the criteria of species' ranges, and geographic pattern of intraspecific mtDNA phylogeny, populations of marine toadfish in the western Atlantic have had lower historical interconnectedness than have the marine catfish.

v) Katsuwonus pelamis, skipjack tuna. --Skipjack tuna lack discrete spawning areas and their larvae are found circumtropically in pelagic waters. Adults are known to move great distances, often thousands of kilometers. Potential thus exists for extensive gene flow, including between Atlantic and Pacific Ocean basins via the Cape of Good Hope (Graves et al. 1984).

Graves et al. (1984) used nine restriction endonucleases to survey mtDNA of 16 K. pelamis from Hawaii, Brazil, and Puerto Rico. Four of the nine enzymes revealed polymorphic mtDNA restriction sites, but none of the variant patterns was confined to either the Atlantic or Pacific collections. Since monomorphic mtDNA sites were also shared by all samples, it was concluded that there were no significant genetic differences between tuna from the two oceans. This conclusion, although provisional because of the small number of restriction sites scored, was consistent with previous morphometric and protein-electrophoretic results.

vi) Salmo salar, Atlantic salmon. --Individual Atlantic salmon exhibit either an anadromous or nonanadromous life history, and sometimes the two strategies occur in fish in the same drainage. From morphological and protein-electrophoretic data, it is uncertain whether any genetic differences exist between the forms (Ryman 1983).

Birt et al. (1986) produced 27 mtDNA fragments by digestion with 11 endonucleases. With the exception of an XbaI variant in one individual, all eight nonanadromous and seven anadromous salmon surveyed shared a single composite mtDNA genotype. The authors suggest that the inland non-anadromous population examined has been separated from ancestral anadromous stock for too short an evolutionary time to have accumulated significant differences in mtDNA.

vii) Salmo gairdneri, rainbow and steelhead trout. --A similar pattern of anadromous versus nonanadromous life histories is exhibited by Salmo gairdneri. Rainbow trout (S. g. irideus of some authors) remain in freshwater throughout their lives; steelhead (S. g. gairdneri) are anatomically almost identical, but spend most of their lives in salt water before returning to streams to spawn.

Wilson et al. (1985b) observed high levels of mtDNA genotypic diversity in rainbow and steelhead trout in the U.S. northwest. Particularly within rainbow trout, there was also

considerable mtDNA variation geographically. In one instance, rainbows and steelheads from the same river could be distinguished readily; but in another case, "subspecies" from different locales shared the same mtDNA genotype. At least three factors probably complicate evolutionary reconstructions in S. gairdneri: (1) occasional hybridization between life history forms; (2) artificial transplantations of various stocks; and (3) the potential for multiple independent origins of anadromous forms from nonanadromous ancestors (Willers 1981). Nonetheless, from analysis of the mtDNA and other evidence, Wilson et al. (1985b) suggest "that natural populations of rainbow trout and steelhead are genetically distinct subspecies, potentially capable of hybridization; but normally reproducing within their own subspecies."

viii) Clupea harengus harengus, Atlantic herring. --This non-anadromous clupeid utilizes discrete spawning regions in the western North Atlantic (Kornfield and Bogdanowicz 1987). An important question is whether genetically distinct groups home to specific spawning sites. Genetic heterogeneity among groups has not been found in allozyme surveys, despite the availability of many polymorphic markers.

Kornfield and Bogdanowicz (1987) examined mtDNA variation in spawning herring from the Gulfs of Maine and St. Lawrence. From seven endonucleases yielding informative digestion patterns, 11 mtDNA genotypes were observed in a sample of 20 fish. The three most common genotypes were shared between locales, while eight genotypes were observed in single individuals. The small size and high mtDNA genotypic diversity conspired to inhibit firm conclusions about possible genetic divergence among putative stocks. Nonetheless, from joint inspection of a parsimony network and collection sites for mtDNA genotypes, the authors suggested that the spawning stocks were probably not distinct.

Kornfield and Bogdanowicz (1987) also assayed a sample of Pacific herring (Clupea harengus pallasii) from Puget Sound. The Pacific and Atlantic subspecies showed differences in restriction sites for five endonucleases, and estimated sequence divergence was  $p=0.026$ .

Most other completed studies of mtDNA in marine organisms have focused primarily on molecular characterization (Komm et al. 1982) or on between-species phylogeny (Berg and Ferris 1984). Additional studies of intraspecific mtDNA differentiation and stock identification are no doubt underway and quite possibly will have appeared before this review reaches print.

## DISCUSSION

In fishery biology, the term "stock" has no precise genetic meaning but often loosely refers to a population or set of populations under harvest (Koehn 1984). Here, "stock" will be used strictly to describe genetically differentiated populations within species. Even with this restricted definition, however, ambiguities arise because magnitude of genetic differentiation may range along a continuum from small to great, and the evolutionary significance of (or reasons for) differentiation may often be unclear. Furthermore, mitochondria are maternally inherited, so genetic stocks identified by mtDNA may sometimes be different from those registered by the biparentally inherited nuclear genome. In fact, there are several examples in the literature (not involving marine species) of an apparent lack of concordance in species' relationships as reflected by mitochondrial versus nuclear genotypes (see reviews by Avise 1986 and Wilson et al. 1985a). The reasons for such discrepancies are currently under debate, and include the possibilities of past hybridizations, patterns of stochastic maternal lineage sorting across speciation events, and selection-driven responses to epistatic interactions between nuclear and cytoplasmic genomes. In the context of discussions about intraspecific genetic stocks, it is thus also worthwhile and necessary to consider further the "meaning" of genetic divergence of mtDNA.

### MtDNA population dynamics

Figure 6 presents a simplified evolutionary tree of mtDNA lineages within a species. Each node denotes a female individual, and branches summarize matriarchal genealogy. The tree is non-anastomosing; it reflects the asexual inheritance of mtDNA within what is otherwise a sexually reproducing species. If mtDNA differentiation is time-dependent, the genetic distance between extant individuals is proportional to the "depth" of their separation in the tree; that is, to the time elapsed since they last shared a female ancestor.

Avise et al. (1984b) modeled stochastic survivorship and extinction of female lineages under a variety of demographic scenarios. The models are analogous to those used to study "male surname evolution" in human societies. In general, stochastic lineage sorting can be very rapid under biologically plausible demographic conditions. Suppose, for example, that in a stable-sized population adult females produce daughters according to a Poisson distribution with mean 1.0. In any generation, the probability that a given potential mother will produce zero female offspring (which also equals the proportion of female lineages lost from the population) is 0.37. Extinctions

accumulate, such that within even 100 generations, a population initiated with 100 females would likely retain descendants of only about 2 foundresses. For nonexpanding populations founded by  $N$  females and/or regulated about a carrying capacity  $K=N$ , at any point in time all extant individuals will very likely trace ancestries to a single foundress less than  $4N$  generations earlier (Awise et al. 1984b). Evolutionary trees in stable-size populations are thus continually "pruned", such that frequency distributions of mtDNA distance will normally be truncated at relatively low levels.

Any process which inhibits lineage extinction will allow larger mtDNA distances to accumulate within a species. One such process (which can operate only over short evolutionary time) is population expansion, where mothers have a high probability of leaving daughters. Another process, which is almost certainly of great significance in nature, is density regulation in subdivided populations. Suppose a species is structured into a number of populations between which gene flow is severely limited. As long as each population is buffered against extinction through density-dependent population growth, it will necessarily retain at least one mtDNA lineage since time of divergence from other populations (Figure 6). I suspect that such density regulation in subdivided species is the major reason for extensive intraspecific mtDNA divergence, and for the mtDNA genetic "breaks" commonly observed to distinguish geographic populations. If this is true, the major mtDNA "stocks" identified in natural population surveys will largely coincide with population units that would be of potential significance to the resource manager.

On the other hand, it is also true that because of the non-recombining history of mtDNA molecules, grossly different mtDNA genotypes can be present within a single random-mating population, either as long-retained ancestral polymorphisms, or as more recent admixtures (in hybrid zones) of allopatrically evolved mtDNA differences. The major point is that information from mtDNA should be interpreted cautiously, integrated where possible with data on nuclear genes, and evaluated carefully against known or suspected life histories and evolutions of the species involved. To dramatize this important point, I will provide three empirical examples from work in our laboratory.

#### Interpretation of mtDNA differences

i) MtDNA differences within a random-mating population. -- Two grossly different mtDNA genotypes (Figure 2) are observed in bluegill sunfish (*Lepomis macrochirus*) in the southeastern United States. In one well-studied pond (Lake Oglethorpe) in north Georgia, both mtDNA genotypes are present in roughly 50 percent frequency, although any individual bluegill exhibits either "A"

or "B" type mtDNA (Awise et al. 1984a). Suppose this were the only information available. One might be tempted to speculate that two separate genetic populations, perhaps even sibling species, were present in the lake. A broader data base yielded a very different conclusion.

As already mentioned, the "A" and "B" mtDNA genotypes are distributed geographically in accord with the ranges of two bluegill subspecies as originally described by morphologic and fixed allozyme differences. By these same criteria, L. m. macrochirus and L. m. purpurescens (which probably differentiated in allopatry) now engage in extensive hybridization in Georgia and South Carolina. In Lake Oglethorpe, allozyme genotype frequencies are in agreement with Hardy-Weinberg expectations for a random-mating population, and mtDNA genotypes are associated essentially at random with various nuclear genotypes (Awise et al. 1984b). From a population biology perspective, it would thus be incorrect to distinguish two bluegill "stocks" in Lake Oglethorpe, except in the sense that two distinctive forms originally contributed genotypes which are now thoroughly mixed through hybridization. Because mtDNA genotypes are asexually transmitted, they cannot alone provide definitive conclusions about mating relationships, but they can be highly informative when used in conjunction with data from nuclear genes.

ii) MtDNA differences and life history information. -- Could an analogous situation to that for bluegill sunfish in Lake Oglethorpe also apply to the pattern of mtDNA divergence described for American and European eels, Anguilla (Awise et al. 1986)? Recall (from the RESULTS section) that A. rostrata and A. anguilla are easily distinguished by mtDNA genotype. In this case, however, the catadromous life history pattern renders it very unlikely that the mtDNA genotypes characteristic of A. rostrata and A. anguilla coexist within a single random-mating population. If these mtDNA genotypes were present in a single panmictic assemblage, they would almost certainly be in gametic phase equilibrium with various nuclear genotypes. Any postulated dispersal behavior taking larval A. rostrata to North America and A. anguilla to Europe would then have to be influenced cytoplasmically, presumably by mtDNA itself! It seems far more likely that American and European eels simply represent largely separate breeding populations.

iii) MtDNA differences in a continuously distributed species. --Theoretical models suggest that major mtDNA "breaks" may also appear in continuously distributed species with greatly limited dispersal and gene flow (Neigel and Awise 1986). Consider again Figure 6, and suppose that the 18 extant lineages in the lefthand population are aligned along a continuous linear habitat, such as a coastline. As the figure is drawn, there have been no lateral "leapfrogging" of lineages (equivalent to an

assumption that female-mediated gene flow is small compared to the total length of habitat occupied). At the position indicated by the arrow, there is a major mtDNA "break", due to the chance survival of lineages belonging to two deeply divided branches of the phylogenetic tree. Geographically oriented mtDNA genetic breaks can thus arise in continuously distributed species through stochastic lineage survival and extinction (if gene flow were extensive, those mtDNA differences would not be geographically oriented, but would be reflected as a widely distributed polymorphism).

Such an explanation might conceivably account for the major mtDNA "break" observed in Limulus horseshoe crabs in north Florida (see RESULTS section, and Fig. 4). If true, there would be no particular evolutionary or management significance to the differentiation between these northern and southern mtDNA "stocks" (stochastically-generated mtDNA breaks are distributed haphazardly in computer simulations -- Neigel and Avise 1986). Alternatively, the north-south mtDNA break in Limulus could be due to some particular deterministic evolutionary force(s) reducing or eliminating effective gene flow specifically in the north Florida inshore area. Deterministic possibilities might include physical "barriers" to dispersal, such as ocean current patterns, or selection differentials such as salinity or temperature gradients associated with water mass differences. As noted by Saunders et al. (1986), the north Florida inshore area is a long-recognized transition zone between warm-temperate and tropical marine faunal assemblages, suggesting that the mtDNA break in Limulus might be related to the same ecological factors that influence distributional limits of other species. If some deterministic force has indeed provided a barrier to Limulus gene flow in north Florida but nowhere else, differentiation should also be observable with suitable assays of the nuclear genome. The two major "stocks" already identified by mtDNA would then clearly be of significance to any management decisions for this species.

#### CONCLUSIONS AND RESEARCH DIRECTIONS

The interplay between life-history-mediated dispersal capability, and environmental limitations to gene flow (past and present), should be an important influence on the magnitude and pattern of geographic population structure reflected in mtDNA or other genetic assays. Thus, if all else were equal, freshwater species confined to distinct drainages for at least moderate periods of evolutionary time might be expected to exhibit greater overall population structure than their counterparts inhabiting the potentially more continuous marine realm. Furthermore, many marine fishes and invertebrates have tremendous dispersal capabilities at one or more stages of their life cycle (Burton

1983; Scheltema 1971; Strathmann 1974; Thresher 1984). For example, the great majority of coral reef fishes have pelagic larvae which remain in the water column for a few days to several months (Brothers and McFarland 1981; Thresher 1984).

On the other hand, considering the great diversity of life-history patterns (with respect to dispersal) exhibited by marine and other species, it is probably unwise to overgeneralize about expected patterns of population subdivision. Indeed, one of the most exciting challenges in the realm of stock identification is to determine whether predictable relationships exist between life history and population structure (Awise et al., 1987b). For empirical tests, it will be important to continue to survey a number of closely related and otherwise similar species that differ sharply in dispersal capacity.

It is still an open empirical question whether conspecific populations in marine species will normally exhibit as much geographic differentiation in mtDNA genotype as has typically been found for terrestrial and freshwater vertebrates. Preliminary results, taken at face value, suggest that they may not. Restriction site surveys of mtDNA in American eels from across the North American coastline,, skipjack tuna from two ocean basins, horseshoe crabs from Georgia to New Hampshire, marine catfish along the Atlantic and Gulf coasts, Atlantic herring from separate (but nearby) spawning regions, and Atlantic salmon with contrasting spawning behaviors, have all failed to detect significant mtDNA differences. These were tough test-cases for the mtDNA approach, however, because they usually represented instances in which earlier attempts to differentiate the populations (such as by protein electrophoresis, morphological comparison, etc.) had also "failed". Given that an assay is capable of detecting even small genetic differences which do exist, lack of significant divergence is of course a positive biological finding. Thus, for example, all assayed American eels probably indeed belong to a single spawning population; and interoceanic gene flow in skipjack tuna may indeed have been sufficient to prevent population differentiation.

There is abundant empirical support for the sensitivity of the mtDNA approach to distinguish conspecific populations. Although most of the data derive from studies of freshwater and terrestrial species, indications are that restriction site variability in mtDNA may also be the most powerful available population genetic technique for distinguishing closely related marine stocks. For example, American and European eels are very readily distinguishable in mtDNA genotype, despite near lack of morphological and allozyme differences; the gulf and oyster toadfish are grossly different in mtDNA genotype, and some populations within each species can also be distinguished; and the northern versus southern forms of horseshoe crabs, readily

identifiable by mtDNA, had remained unrecognized by other criteria. As a very rough "rule-of-thumb" (one to which exceptions are bound to be found), if two populations show any evidence of allozyme divergence (e.g., large allele frequency shifts at even one or a few polymorphic loci), they seem likely to exhibit fixed and cumulative differences at many mtDNA restriction sites.

For purposes of meaningful stock identification, increasing power of an assay to detect differences is not the only issue. Suppose, for example, that it became technically feasible to determine the nucleotide sequence of the entire 16 kilobase mtDNA genome in a large number of individuals. Very likely, nearly every individual would have a different mtDNA genotype, but some would be more closely related than others. Of particular interest and potential significance to the resource manager would be any discontinuities in levels of divergence -- situations where the ratio of genetic distances between versus within arrays of individuals was large. Such appears to be the case for the north-south mtDNA "break" in Limulus polyphemus, or the American-European mtDNA "break" in Anguilla. Major genetic breaks of potential relevance to stock identification may well be generally detectable with the level of sensitivity already provided by multi-endonuclease mtDNA surveys.

As noted by Koehn (1984), "The existence of a pattern of population differentiation does not in and of itself provide information as to why it exists, even when the genetic pattern of spatial variation is statistically correlated with environmental patterns." Koehn was specifically referring to the usual difficulty of identifying natural selection and adaptation as direct agents influencing the distributions of genetic traits such as allozyme frequencies. I believe there is one important sense in which studies of mtDNA genotypes may provide enhanced understanding of the reasons for geographic structuring of populations. Because of the asexual and non-reticulate history of mtDNA lineages within species, and because of the cumulative nature of mtDNA mutational steps observable with restriction-site assays, it is usually possible to demonstrate a phylogenetic component to population structure (Avice et al. 1987a). In other words, geographically separated populations usually occupy different branches of an intraspecific evolutionary tree. This pushes the issue of the adaptive significance of observed geographic differences (in mtDNA) to a problem relating to the reasons for the survival and extinction of various female lineages. Furthermore, a joint comparison of mtDNA and allozyme (for example) genotype distributions may reveal situations in which an allozyme frequency shift would unlikely be attributable to phylogenetic separation.

These considerations reopen the general question of the meaning and significance of mtDNA stocks. Observable mtDNA genotypes in any species represent the current termini of branches in a matriarchal evolutionary tree (as in Figure 6). The histories reflected in such genealogies will have been influenced by myriad evolutionary forces (both stochastic and deterministic) impinging on demography and female lineage survival. From a strictly pragmatic management perspective, it may sometimes be sufficient to discover mtDNA markers which distinguish subsets within a species. These subsets will in principle be hierarchically arranged, reflecting different depths of twigs and branches in an evolutionary tree; even the finest twigs on a branch might be differentiable with a sufficiently sensitive genetic assay. But in most instances, we will also be interested in larger branches within a tree, and in the evolutionary processes responsible for these divisions. As in several examples discussed in this paper, it may be best to interpret mtDNA data sets on a case-by-case basis, with due consideration given not only to the ramifications of the uniparental mode of mtDNA transmission but also to the zoogeography, biology and life history of the particular species involved.

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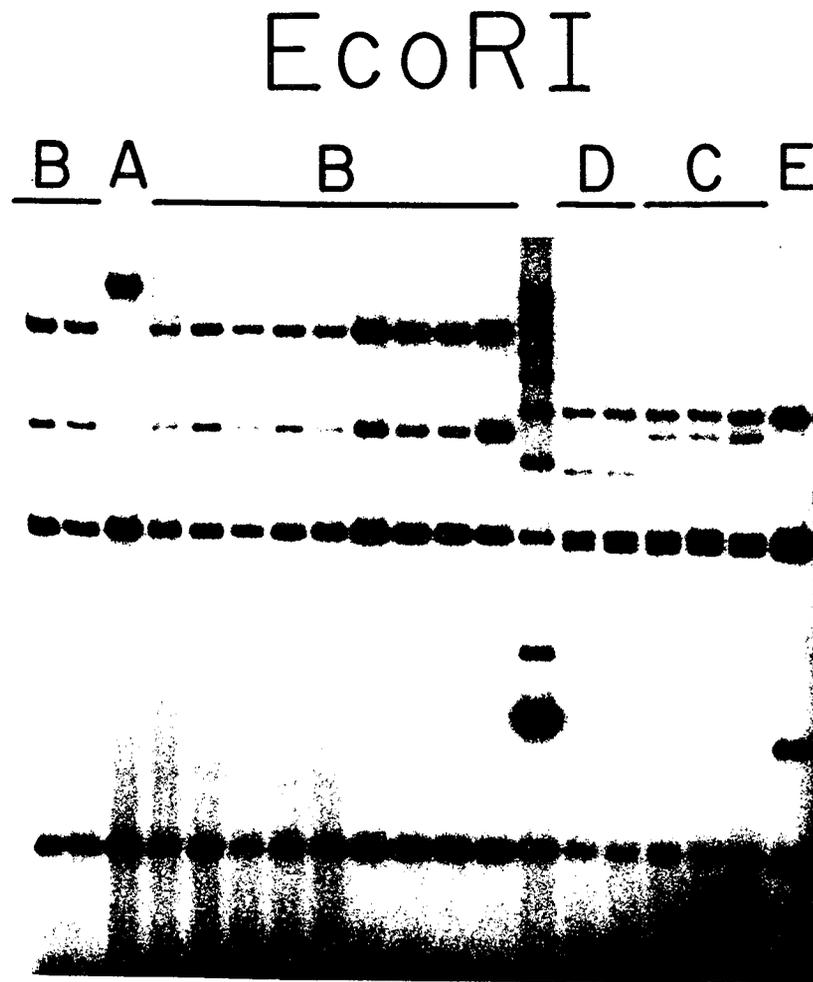
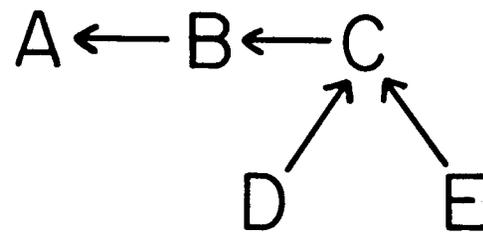


Figure 1. Autoradiograph showing examples of EcoRI digests of mtDNA from Anguilla rostrata (leftmost 12 lanes) and A. anguilla (rightmost 6 lanes). Lane 13 is a molecular weight marker, in which the darkest band is 1.6 kilobases (kb), successive bands above it are approximately 2,3,4,5,6,7,8..kb, and the band below it is 1.0 kb. A parsimony evolutionary network connecting the mtDNA genotypes is shown below the gel. Arrows indicate direction of restriction site loss, and not necessarily direction of evolution. Pattern E apparently has a "doublet" (two bands of indistinguishable molecular weight) at 3.1 kb.



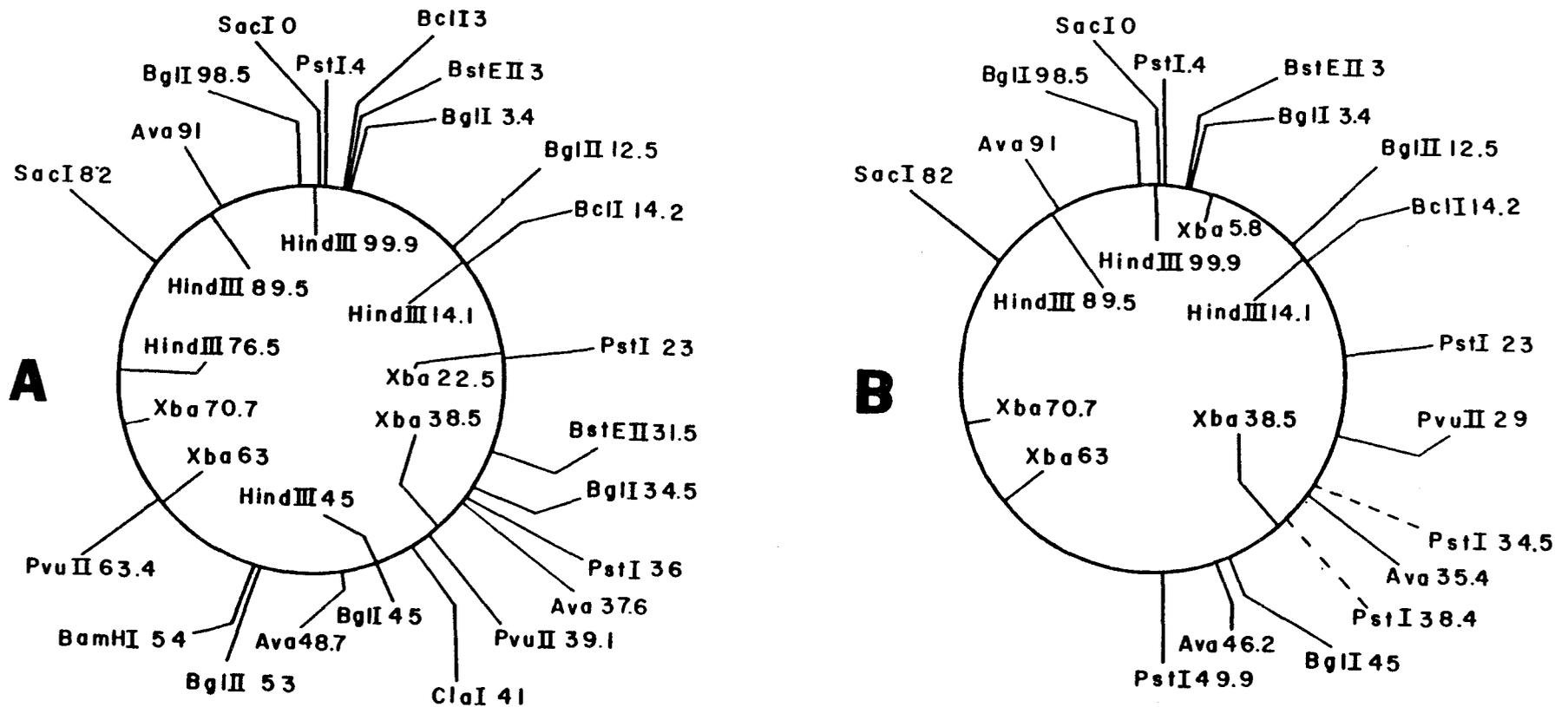


Figure 2. Restriction site maps of the two common mtDNA genotypes in bluegill sunfish (*Lepomis macrochirus*) (from Avise et al. 1984a).

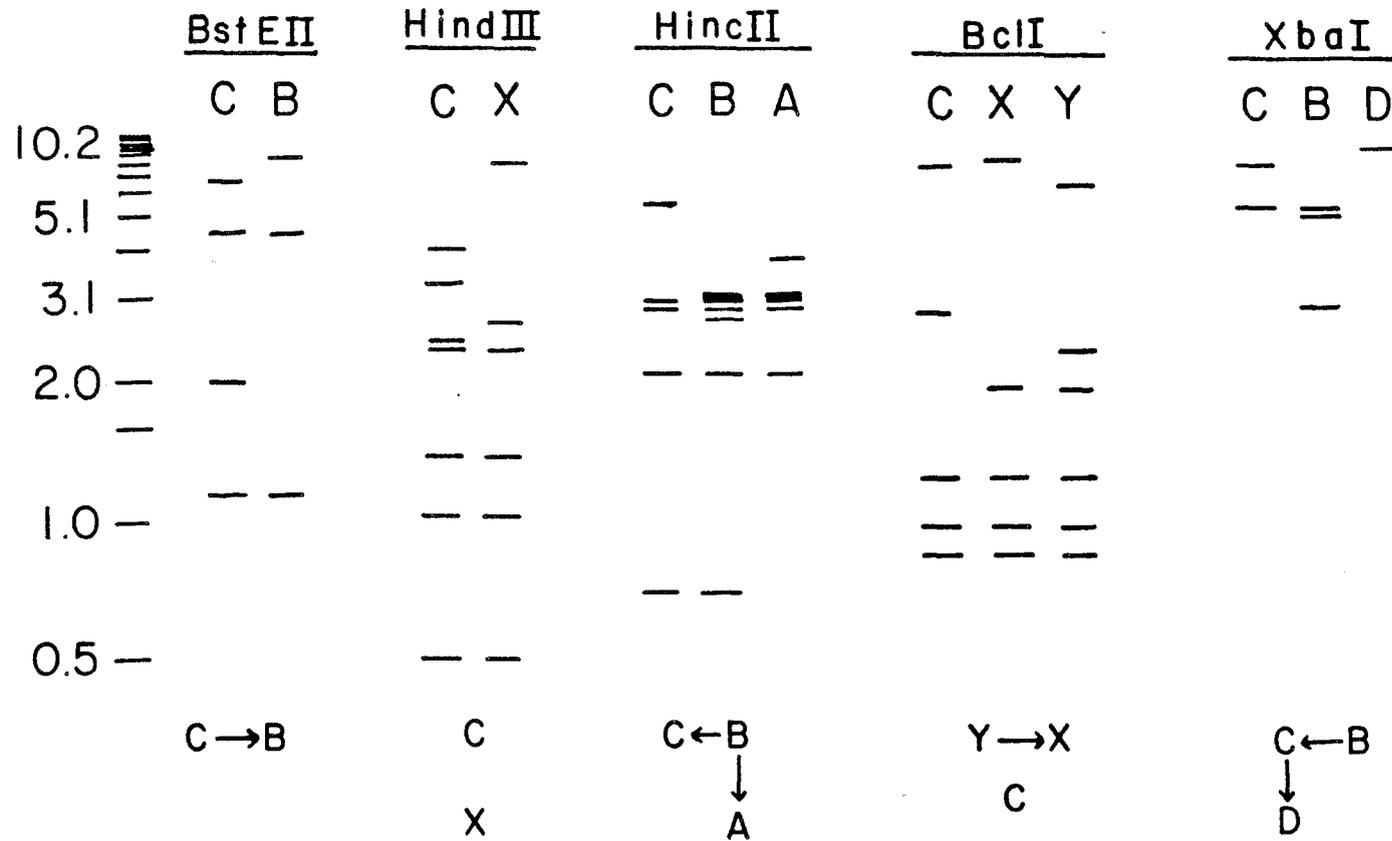


Figure 3. Diagrammatic representation of selected mtDNA digestion profiles in the horseshoe crab (*Limulus polyphemus*) (Saunders et al. 1986). Evolutionary relationships among the patterns are also shown, where arrows indicate direction of restriction site loss, and lettered patterns not connected by arrows differ by two or more restriction site changes. Sizes (in kb) of particular molecular weight markers are indicated to the left.

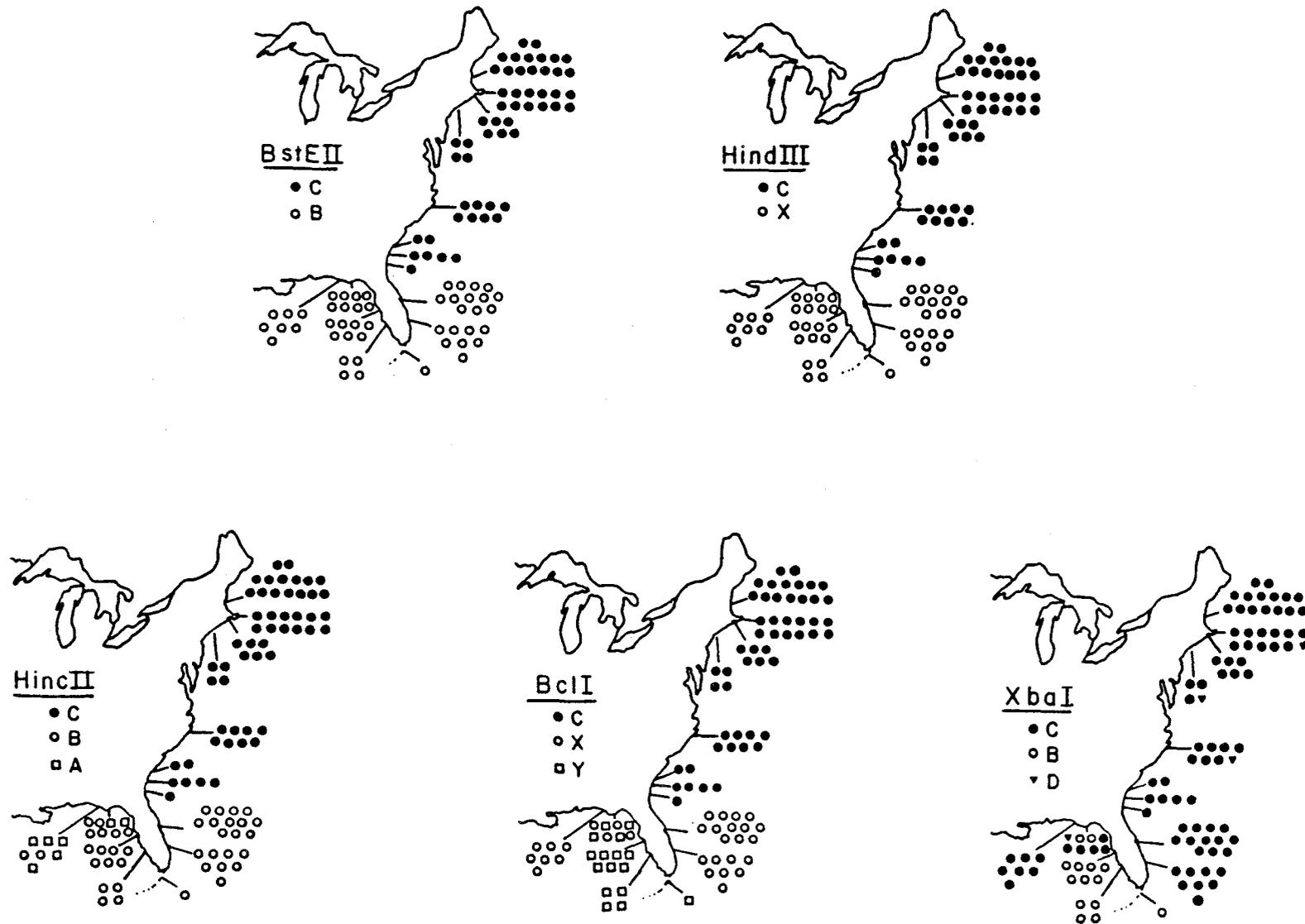


Figure 4. Geographic distributions of selected mtDNA genotypes in the horseshoe crab (*Limulus polyphemus*) (Saunders et al. 1986). Genotypes are labeled as in Figure 3.

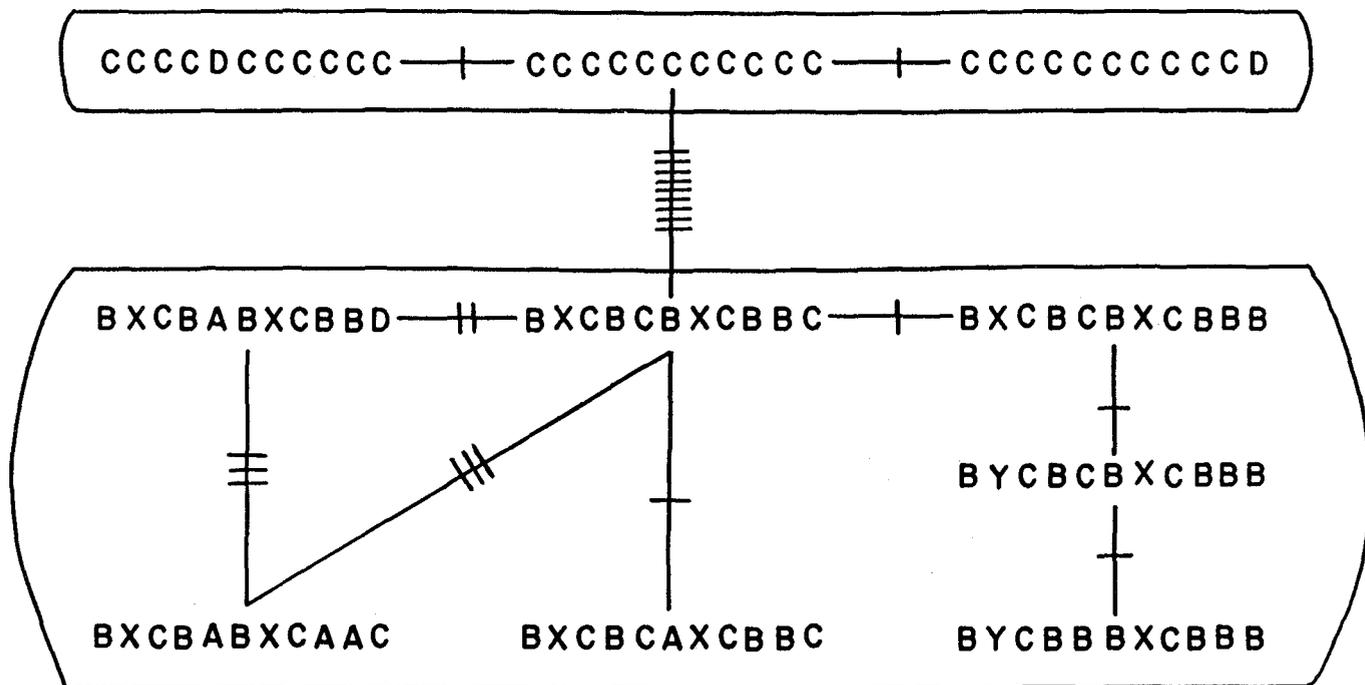


Figure 5. Parsimony network interconnecting the composite mtDNA genotypes in the horseshoe crab (*Limulus polyphemus*) (Saunders et al. 1986). Larger ovals encompass the northern and southern genotypic assemblages. Slashes crossing branches of the network indicate numbers of restriction site changes along a path.

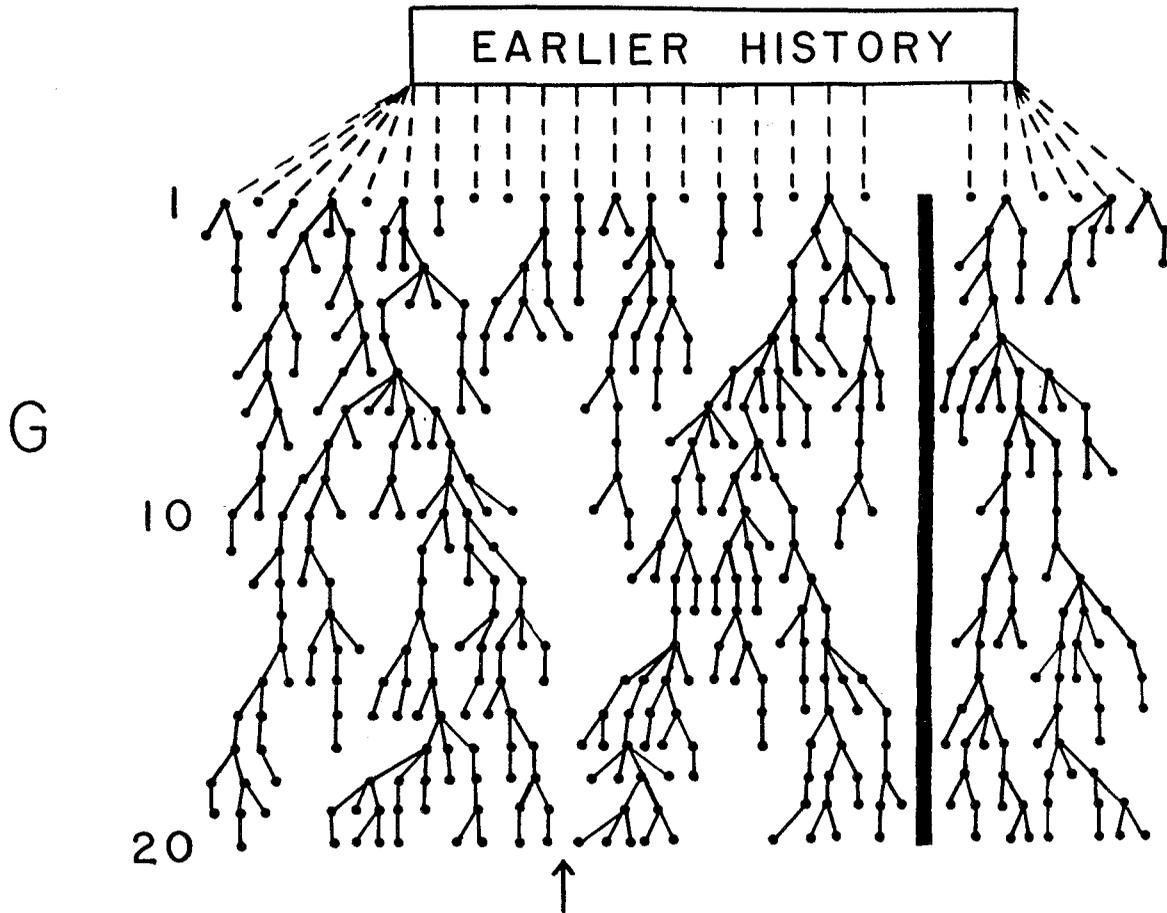


Figure 6. Schematic representation of an intraspecific evolutionary tree (genealogy) for mtDNA through 20 generations. Each node represents a female individual, and branches lead to daughters. The probability distribution of progeny numbers is approximately Poisson with mean 1.0 per female. As drawn, there are no lateral leapfrogging of lineages, as might approximate the case for a species with very limited gene flow. With higher gene flow, extant lineages in each generation would be geographically mixed to a greater degree. The arrow indicates the position of a large mtDNA break between adjacent individuals, due to their chance membership in deeply separated branches of the tree. The vertical bar indicates an extrinsic (environmental) barrier to gene flow.

THE POTENTIAL USE OF MONOCLONAL ANTIBODIES FOR IDENTIFICATION  
OF FISH STOCKS

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Immunochemical methods for the analysis of fish species and fish stocks are currently more of historical than practical significance. Serological techniques have been applied to anthropological studies of a wide range of organisms (Sarich and Wilson 1966; Wallace et al. 1973; Prager and Wilson 1976). Serological investigations of inter- and intraspecific variation among fishes have also had a history of use (Deligny 1969; Cushing 1956; Suzuki et al. 1981; Ridgway 1963; Krauel and Ridgway 1963; Ridgway et al. 1962). Despite the great promise initially held for the use of immunological methods, the promise has largely gone unfulfilled. Technical problems eventually resulted in the virtual abandonment of immunological methods as a serious tool for studying fish stocks when electrophoretic methods became popular in the 1960s (Ihssen et al. 1981). Electrophoresis eventually became the dominant technique, since it was possible to collect a large amount of genetic based data with only moderate effort (Utter et al. 1974). An analysis of the "technical problems" associated with the immunological (serological) methods reveals two major problem areas: the stability of the antigens, and the specificity and reproducibility of the antisera preparations.

Conventional antisera are produced according to the following generalized scheme: The process begins by administering the antigen to an animal, typically a rabbit. The animal's immune system recognizes the antigen as foreign and initiates a complex chain of events culminating in the production of a large array of antibodies directed against the various antigenic determinants associated with the antigen. The antibodies, which are present in the blood, may be recovered by the researcher by bleeding the animal, allowing the blood to clot, and retaining the serum fraction which will contain the antibodies reactive with the antigen of interest. Antibodies unrelated to the antigen under study may also be present from prior exposure of the animal to bacteria, virus, etc. A good antiserum will contain antibodies which perform the desired function (cell lysis, cytotoxicity, precipitate soluble antigens, etc.) and will have a high degree of specificity toward the antigen of interest. The papers by Ridgway (1963) and Ridgway et al. (1962) serve to illustrate some of the key problems encountered in the production and use of

antisera for immunogenetics. There is considerable variability in the immunological response to the same antigen. Ridgway et al. (1962) could not raise suitable antisera in goats or chickens but found that rabbits gave suitable antisera. However, not all bleedings from the same rabbit or from different rabbits were equally useful in detecting stock differences. To obtain enough antisera, it was necessary to combine several bleedings. Many applications require purification of the antisera to remove non-specific or cross-reacting antibodies. This purification step can often be quite extensive with multiple adsorptions required to remove the unwanted contaminants. Whether additional purification is needed or not, each antiserum is unique and available in finite amounts.

This classical type of antisera production was the norm up until the year 1975 when the landmark paper by Kohler and Milstein (1975) virtually revolutionized the field of immunology. Their work on the genetics of immunoglobulin production led directly to the technology of making monoclonal antibodies. Monoclonal antibodies differ from conventional antisera in that the monoclonals are monospecific, they react with single antigenic determinants, while antisera contains a mixture of different antibodies (polyclonal antibodies). Monoclonal antibodies can be produced in virtually unlimited quantities, all of it exactly the same. They can be selected for particular effector functions and specificities and can be combined to produce a predefined, reproducible "antiserum".

Although there are numerous variations in techniques now available for producing monoclonal antibodies, the following describes the process in general. The test animal is usually a BALB/c mouse. The mouse is immunized with the antigen in much the same way as were the rabbits in antisera production. A series of antigen injections with or without adjuvant is given over a period of weeks or months. The precise immunization scheme is variable depending on a multitude of factors, but a proper immunization culminates with the mouse's production of specific antibodies. Several booster immunizations may be given to increase the proportion of immune cells secreting antibody against the desired antigen. Whereas in serology, it is at this point that the animal would be bled to recover the antibody, the technique used for monoclonal antibody production takes the process a step further. A final injection is given and 3-4 days later the mouse's spleen is removed. Thus, antisera production recovers the antibodies from the animal, while the monoclonal antibody technique recovers the cells responsible for antibody production. The spleen is gently massaged and teased apart to release a suspension of antigen stimulated B-lymphocytes (or B-cells) from the spleen. Each B-cell produces one particular antibody. The B-cells are then mixed with myeloma cells (a cancerous plasma cell, or plasmacytoma). The B-cell and myeloma

are fused together by briefly exposing the cells to polyethylene glycol. This fusion treatment causes adjacent B-cells and myelomas to fuse together into a hybrid cell known as a hybridoma. The hybridoma cell has properties derived from each of the parental cells. It has the B-cell parent's ability to produce one specific antibody and the myeloma cell parent's ability to grow continuously in vitro. From the fusion mixture, viable hybridoma cells are isolated in a selective media containing hypoxanthine, aminopterin, and thymidine (HAT media), which allows only the hybridoma cells to grow. By plating the surviving hybridoma cells at a very low density into 96-well tissue-culture plates, it is possible to grow individual colonies derived from single hybridoma cells (limiting dilution cloning). The hybridomas can be grown continuously in tissue culture where the monoclonal antibody can be recovered from the spent media. The hybridomas may also be propagated in vivo as ascitic tumors. The ascites fluid contains very large amounts of the monoclonal antibody.

A current monoclonal antibody project at the National Marine Fisheries Service Gloucester Laboratory in Gloucester, MA involves the creation of monoclonals capable of identifying species in seafood products (Lundstrom 1985). It is largely our experience with species identification which leads us to speculate on the potential use of monoclonal antibodies for stock identification. Monoclonal antibodies for identifying species have been created by immunizing mice with crude protein extracts from different seafood species. After immunization, the mouse's B-cells are fused with myeloma cells as previously described. The monoclonal antibodies from individual clones are then screened for reaction with the immunizing species, and for any cross-reaction with a panel of about 100 different seafood species. Although it would seem logical to select for species-specific monoclonal antibodies, we have found that approach to be very inefficient (you would need to derive 200 different monoclonal antibodies to identify 200 different species). Instead, we select monoclonal antibodies that recognize particular antigenic determinants which are present in some species but absent in others. By constructing a panel of monoclonal antibodies which detect a variety of antigenic determinants, it is possible to identify a species by noting the pattern of positive and negative reactions relative to the antibody panel. The data shown in Table 1 illustrates this concept. The 10 monoclonal antibodies listed at the top of the figure were derived by immunizing BALB/C mice with an extract of heat stable (100 degrees C) muscle tissue antigens from cod (Gadus morhua). The monoclonal antibodies were tested for specificity using a simple ELISA assay in which each monoclonal antibody was tested for reaction with heat stable muscle tissue extracts of the species shown in Table 1.

The ELISA assay was conducted using the following procedure. The antigens (heat stable muscle tissue extracts) were allowed to bind to the wells of a 96-well vinyl assay plate by incubating the plates overnight at 4 degrees C. The next morning, any unbound antigen was removed by repeated washing of the plates with phosphate buffered saline containing 0.01% Tween-20 (PBST). Monoclonal antibody (undiluted spent tissue culture media from stationary phase hybridoma cultures) was added to each well and allowed to incubate for one hour at room temperature. If a monoclonal antibody recognized its corresponding antigen (or a closely related antigen), the antibody bound to the antigen. Unbound monoclonal antibody was then washed out of the wells with PBST. Next, to each well was added an enzyme labeled "second" antibody reactive with the mouse monoclonal antibody (i.e., goat anti-mouse IgG - horseradish peroxidase conjugate). Wells containing mouse monoclonal antibody (bound to the antigen which was in turn bound to the plastic of the assay plate) bound the second antibody, thus tagging a positive well with the enzyme, horseradish peroxidase. Unbound second antibody was again washed out of the wells with PBST. Wells containing the bound sequence of antigen-monoclonal antibody-enzyme labeled second antibody were detected by adding a chromogenic peroxidase substrate (o-phenylenediamine) to each well. Positive wells turn a yellow color as the normally colorless reduced o-phenylenediamine is oxidized by the peroxidase. Positive reactions were assumed to be any well in which the absorbance was five times the absorbance of a control well containing a nonrelevant antigen (bovine serum albumin).

Using a panel of ten monoclonal antibodies, the reaction patterns for ten species are shown in Table 1. It is evident that the first two species shown, Atlantic and Pacific cod, have the same pattern of reaction against this antibody panel and additional monoclonal antibodies with differing specificities would be needed to resolve these two species. The other eight species shown, however, have unique patterns of reaction against this antibody panel. This pattern of positive and negative reactions, in effect, defines an "immunophenotype" for a species. The data shown here represent only a small portion of the work done to date in this area.

We have derived 24 different monoclonal antibodies and have tested them for reaction against over one hundred different species in both the raw and cooked states. Many instances of different species reacting in the same way to our antibody panel have been found, but we are continuing to produce new monoclonal antibodies in an effort to find a combination which reliably differentiates all species of commercial interest.

In the course of our research on the development of the monoclonal antibody species identification panel, we have noted

the occasional occurrence of monoclonal antibodies which react with some individuals within a species, but do not react with other individuals. Of course, this is undesirable for species identification purposes, and we have routinely discarded hybridoma clones which produce monoclonal antibodies reactive with polymorphic antigenic determinants. This observation suggests that it may be possible to use monoclonal antibodies for fish stock identification.

Perhaps the simplest use to envision for monoclonal antibodies in stock identification is its application as a direct substitute for the currently popular electrophoretic methods. Electrophoresis is used to separate and identify various alleles at polymorphic loci. Individual stocks are characterized on the basis of allelic and genotypic frequencies. Data from a large number of individuals and from many loci are needed to make meaningful comparisons. The effort expended in running the electrophoresis experiments and in interpreting the results for even a modest survey is considerable. The survey samples, which typically include eye fluid, muscle, liver, and heart samples, must be analyzed immediately or frozen at low temperatures to avoid loss of enzyme activity, since the specific enzyme staining techniques depend on the enzyme being active.

Monoclonal antibodies offer a way to detect enzyme alleles directly, without prior electrophoretic separation, and in an assay system which can be completed in less than two hours. The use of currently available biotechnology instrumentation would allow complete automation of the procedure from sample preparation to collection of data. Monoclonal antibodies could be created to recognize enzyme alleles previously shown by electrophoretic methods to be important in differentiating stocks. A marker, a fixed allele present in one specific stock, would be an ideal candidate for creation of a monoclonal antibody. In any case, the isozyme would be isolated using conventional electrophoretic and chromatographic techniques to immunize one or more mice (nanogram to microgram amounts are usually sufficient). After cell fusion, the resulting hybridomas would be screened for positive reaction with the particular allele of interest, for lack of cross-reaction with other alleles, and for lack of cross-reaction with other components which might cause false positive reactions. In theory, it should be possible to create monoclonal antibodies against all the relevant polymorphic loci now commonly detected by electrophoresis, although this would be a major undertaking. The benefits necessary to justify such an undertaking must be considerable. Probably the best justification would be the gain in efficiency.

Although the monoclonal antibody development stage is complex and time consuming, once the antibody producing hybridoma

has been created, the monoclonal antibody can be generated in large amounts very inexpensively. The assay for the enzyme allele could be based on the popular ELISA technique. The test sample would be distributed into the wells of a plastic 96-well immunoassay plate where the antigens will bind to the plastic after a suitable incubation period. Monoclonal antibodies against various alleles are then used as probes; when a monoclonal antibody recognizes its corresponding antigen attached to the plastic, it binds to the antigen. Unbound monoclonal antibody is then washed away. The antigen-antibody complex bound to the plastic is detected by adding a "2nd antibody." The 2nd antibody is typically an anti-mouse immunoglobulin conjugated with an enzyme label (i.e., goat anti-mouse IgM-horseradish peroxidase). The 2nd antibody binds to any mouse monoclonal antibody present in the wells. Unbound 2nd antibody is then washed away leaving a complex of bound antigen-monoclonal antibody-labeled 2nd antibody in samples originally containing the antigen. This complex is detected by adding a chromogenic substrate mixture which changes color when exposed to the enzyme labeled 2nd antibody. Instrumentation is currently available which can automatically carry out the necessary functions including sample preparation, pipetting of antigen, monoclonal antibody, 2nd antibody, and substrate solutions into the assay plate, washing out unbound substances between reagent additions, and reading, recording, and analyzing the results. Additional advantages may lie in the fact that ELISA assays detect quite small amounts of antigens (nanogram amounts or less) and use small amounts of reagents. The detection of the enzyme depends only on the monoclonal antibody recognizing the antigenic determinant. The determinant need not be on the enzyme's active site which may obviate the need to have an active functioning enzyme for the assay. It may, in fact, be preferable to generate monoclonal antibodies which recognize sites on a denatured enzyme since this might allow storage of samples under less severe conditions.

A technique related to the ELISA technique described above, known as "dot-blotting," uses a nitrocellulose matrix to immobilize the antigen. The dried blotting paper can be stored for long periods at room temperature while still allowing monoclonal antibodies to bind with the antigen. An additional advantage is that the antibodies can be removed from the blotting paper to allow probing with other monoclonal antibodies.

The previous stock identification approach is limited in that it only deals with a restricted subset of alleles, those previously detected by electrophoresis. Monoclonal antibodies may also detect null alleles, those with charge differences too small to allow separation by electrophoresis, but a greater promise lies in detecting genetic differences not yet investigated by electrophoresis. We have taken a similar approach in

our species identification work, since we do not know to what the monoclonal antibodies react, but define them by their function. For stock identification purposes, the screening stage would include provision for selection of antibodies which detect epitopes present in some individuals and absent in other individuals. If the frequency of occurrence of these epitopes differed in reproductively isolated groups, the monoclonal antibody would be useful for stock identification. The genetic basis of these epitopes could be ascertained in the same way they were for the electrophoretic methods. Monoclonal antibodies also expand on the number of polymorphic determinants available for examination, since bound antigens such as red blood cell surface antigens can easily be probed. The use of fixed cells bound to the plastic wells as the antigen obviates many of the problems associated with collection, handling and storage of labile samples like red blood cells.

As mentioned previously, the ideal situation for a monoclonal antibody stock identification technique would occur when a stock has a fixed allele at a minimum of one loci that genetically marks it relative to another stock. In this situation, individual fish can be detected in mixed populations as having originated from a particular stock. These genetic differences may be naturally occurring or they may be created by artificial breeding to alter allele frequency intentionally at a particular locus (Reisenbichler and McIntyre 1977; Allendorf and Utter 1979).

Another type of "tagging" technique could be based on an immunological basis where a fish is immunized against an antigen not normally found in its environment (e.g., bovine serum albumin, a killed virus, a synthetic polypeptide, etc.). This fish will produce antibodies against the foreign substance, and if the antigen is properly chosen and administered, the resulting immunity could be quite long-lived, although this would need to be determined. Although not designed as a tagging method, Stolen et al. (1983) have used an immunological technique which detects antibodies to human enteric bacteria in several fish species from past contact with these potentially pathogenic microorganisms. Teleost fishes have well developed immune systems as demonstrated in other studies as well (Snieszko 1970; Anderson and Klontz 1970; Bowers and Alexander 1981). Assuming the fish could be immunized en-masse and maintain the antibody for a sufficiently long time, these fish could be identified at a later date using one of the several possible immunological techniques.

A simple highly sensitive test based on the ELISA technique might work as follows: Samples or serum from individual fish would be pipetted into the wells of a plastic microtiter plate previously coated with the immunizing antigen. If the specific antibody against this antigen is present in the serum of a

particular fish, the antibody will bind to the antigen. Any unbound fish antibody would then be washed out of the wells. The antigen-antibody complex would then be detected by an enzyme labeled second antibody reactive with the fish antibody. The second antibody would bind in wells containing the antigen-fish antibody complex. After washing out any unbound enzyme labeled second antibody, substrate is added to each well and a color change is noted in samples containing the antibody tag. Different antigens could be used to immunize different stocks, thus producing a number of different possible tags.

These potential uses of monoclonal antibodies for fish stock identification are at present purely speculative. The use of monoclonal antibodies in human genetics stands on firmer ground having been under study for over ten years. Slaughter, et al. (1980) have produced monoclonal antibodies to human alkaline phosphatases. In humans, this multilocus enzyme system has two loci with fixed alleles giving six common phenotypes in most human populations and an extensive series of rare alleles found in heterozygous combination with one of the more common alleles. They used their monoclonal antibody panel to make a determinant-by-determinant comparison of the different alleles and also to make comparisons with various organ-specific alkaline phosphatases in nonhuman animals. Other studies have also demonstrated the ability to create isozyme specific monoclonal antibodies for cytochrome P-450 in human tissues (Fujino et al. 1982), for phenotyping leukemic T-cell lines (Martin et al. 1982), for human phosphofructokinase on blood cells and in cultured cell lines (Vora 1981), and for creatine kinase (Roberts and Parker 1981).

In summary, the monoclonal antibody technique is a powerful method which obviates many of the problems associated with conventional serological techniques. The ability to create monoclonal antibodies which recognize different isozymes has been demonstrated to be useful in human genetics. The successful application of monoclonal antibodies to the identification of bacterial species and strains (Conway de Macario and Macario 1983) and the promising preliminary results of the application of the technique to fish species identification indicates a good potential for the study of inter- and intraspecific variation in fishes using monoclonal antibody technology.

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Table 1. ELISA species cross-reaction assay.

MONOCLONAL ANTIBODY NUMBER SPECIES	4A-B2	4A-F11	4B-G2	4B-G11	23B-D10	23C-A2	23E-F8	23G-A6	23G-C4	26C-B2
Atlantic Cod ( <u>Gadus morhua</u> )	+	+	+	+	+	-	+	+	-	+
Pacific Cod ( <u>Gadus macrocephalus</u> )	+	+	+	+	+	-	+	+	-	+
Wolffish ( <u>Anarhichus lupus</u> )	+	+	+	+	-	-	+	-	-	+
Red Snapper ( <u>Lutjanus campechanus</u> )	+	+	+	+	-	-	+	+	-	+
Pacific Halibut ( <u>Hippoglossus stenolepis</u> )	+	-	+	-	-	+	+	+	-	+
Bluefish ( <u>Pomatomus saltatrix</u> )	+	-	+	-	-	-	-	-	-	-
Tilefish ( <u>Lopholatilus chamaeleonticeps</u> )	+	-	+	+	-	-	+	+	+	+
Cusk ( <u>Brosme brosme</u> )	+	+	+	+	-	-	+	-	-	+
Bluefin tuna ( <u>Thunnus thynnus</u> )	+	-	-	-	-	+	-	+	+	-
Lobster ( <u>Homarus americanus</u> )	-	-	-	-	-	-	-	-	-	-

"+" = Positive reaction between antigens of species and monoclonal antibody.

"-" = Negative reaction between antigens of species and monoclonal antibody.

A BRIEF REVIEW AND GUIDE TO SOME MULTIVARIATE METHODS  
FOR STOCK IDENTIFICATION

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INTRODUCTION

The realistic application of many population dynamics models implies that a unit stock, which has consistently recognizable characteristics, has been identified. The ability to identify unit stocks successfully is useful and important to some fishery management decisions, especially those of a long-term nature. However, stock identification is not always easy to apply in practice, and some questionable logic and methodologies may have been utilized in the past. A review of various stock discrimination procedures in marine fishes has been made by Templeman (1983), and the Canadian Stock Concept International Symposium (1981) provides considerable recent background concerning stock identification to the interested reader.

In general terms, a unit stock can be thought of as a population of organisms which should be treated independently, because each stock may respond uniquely to exploitation or to environmental perturbations. The Stock Concept International Symposium perspectives and policy recommendations (Spangler et al. 1981) indicate that it is not necessary to agree on a single definition of a "stock" to communicate effectively the concept for fishery management purposes. The major aspects of the stock concept appear to include temporal and spatial discreteness as well as reproductive isolation. It is generally recognized that for management purposes, a unit stock does not necessarily correspond to a genetically distinct group, because phenotypic characteristics are often used for stock identification.

Early work in stock identification should be critically and cautiously examined before accepting some of its findings as valid. This statement is in keeping with Kutkuhn's (1981) warning that many attempts at stock identification have failed due to faulty logic, faulty sampling procedures and questionable statistical and analytical procedures. It is the purpose of this review to point out a few of the more obvious pitfalls in classification and to suggest some potentially useful methodologies, especially with reference to multivariate techniques suitable for stock identification.

For most problems in stock identification, identification with certainty is not possible, either because too many characters are variable within a stock, or because all components of character states are subject to some measurement error. Therefore, it is usually necessary to utilize probabilistic identification methods. The relevant question then becomes: which stock is most likely to have produced the specimen requiring identification? This question is different from that answered by the genetic

identification method based on electrophoretically detected genetic variation as reviewed by Mackie (1980). In this latter method, genotype frequencies from two data sets are used to obtain maximum likelihood estimates of the proportional contributions of various stocks to a mixed fishery. The sample size requirements of the genetic identification method are relatively high and accuracy and precision are related to sample size and grouping of data. The genetic identification technique is not considered further in this review.

The general problem of stock identification involves pattern recognition, which is a broad and rapidly evolving discipline. More specifically, stock identification is analogous to classification, the ordering of organisms into groups on the basis of their relationships. These relationships may be genetic and evolutionary (phylogenetic) or may simply refer to similarities in phenotype (phenetic relationships). However, the temporal variation in phenotypic characters should be carefully considered.

There are two major approaches to multivariate classification: discrimination and clustering. Discrimination techniques begin with either a priori conceptual distinctions or with data divided into a priori groups. Clustering techniques use a priori selection of a measure of similarity (a criterion) and a class description to find inherent structure in data (i.e., clusters). Discrimination uses externally supplied labels associated with each member of a set of objects to aid in establishing rules for sorting objects into groups. In clustering (objects or variables), we seek to find data-derived groups based on internal similarity between objects.

Discrimination is called "supervised learning", whereas clustering techniques are termed "unsupervised learning" procedures. In a practical application, clustering might be utilized in an effort to obtain parsimonious groupings of species based on geographic variables (Murawski et al. 1983), whereas discrimination is applied to groups which have been previously identified (Van Winkle and Kumar 1982).

Scientists in many disciplines have taken advantage of rapid data acquisition procedures and the general availability of computers to develop various classification procedures. These procedures have been translated into algorithms and to computer programs. Unfortunately, a particular computer program does not assure the user of success--unless the user has a good definition and understanding of the stock identification problem, the limitations of the data, and the assumptions implicit in the application of a given methodology.

#### A FEW PROBLEMS

Part of the motivation for the development of many multivariate classification techniques has risen from the desire to reduce the summarization that can mask similarities in multivariate data.

A very simple example of one possible problem area is provided in Figure 1. In this figure three dissimilar data sets yield identical means and covariance matrices. From Figure 1 it is evident that the mean and the covariance matrix do not tell everything about these data, unless they are

normally distributed. Indeed, classification techniques that use only values of a mean vector and a covariance matrix, and which do not use original values of the data again after the mean and covariance matrix have been calculated, cannot distinguish among the three data sets illustrated in Figure 1. It should be pointed out, however, that there are available operational classification techniques (such as Sneath 1967) which distinguish these differences.

A second possible problem area involves clustering techniques, and it concerns what is meant by a natural cluster or grouping. Consider Figure 2 to examine how scaling of individual variables can affect the objects to be placed into a cluster. In Figure 2a it seems reasonable, on inspection, to consider clusters 1-4 as distinct. By rescaling the x-axis (Figure 2b) it now appears that clusters 1 and 2 form a larger cluster, as do clusters 3 and 4. Note, however, that rescaling the y-axis also produces two larger clusters, but this time they consist of clusters 1 and 3 and clusters 2 and 4.

It should also be recognized that seldom, if ever, are truly random samples available for stock identification, because most fishing gear tends to be selective for length and/or girth. It, therefore, is desirable to test for a correlation of any variable with length (age) in order to minimize difficulties caused by gear selectivity. If no significant correlation of the meristic variables of interest with length is found, then the samples can be construed as reasonably representative and usable for stock identification. Morphometric characters can also be standardized statistically to remove size effects.

Another problem pointed out by Royce (1953) is that statistically significant differences may be found between closely related groups by increasing sample size, using a larger number of variables, using more critical testing procedures or some combination of the above. The only advice concerning this problem is that one should clearly establish the probable existence of separate stocks and obtain samples when the stocks are presumed or known to be spatially isolated before attempting statistical separation procedures.

#### A FEW QUESTIONS

The user of multivariate techniques for sorting fish (or other aquatic organisms) into unit stocks (groups) must be able to answer certain fundamental questions in order to be routinely successful. Some of these questions include the following:

- 1) What assumptions are made about the underlying structure of the available data?
- 2) How are the variables selected, scaled and counted or measured?
- 3) Can the fishery scientist assume that knowledge of class membership is sufficient to guide the grouping procedure?
- 4) What techniques are available and appropriate for the problem at hand?

## A FEW ANSWERS

- 1) Structure of data--Assumptions regarding the structure of data should affect the choices of classification technique. The assumptions can be characterized in terms of two dimensions. The first dimension considers the amount of overlap assumed to exist between groups. The second dimension considers the variety of form and shapes of the data in a group. A simplistic representation of these two dimensions is shown in Figure 3. In Figure 3a there are two groups of identical shapes widely separated from each other. In Figure 3i there are two groups which differ considerably in shape but the means are identical.

In general, the importance of the structure of the data relates to assumptions made in order to elicit information from the data. For example, in Figure 3c, it can be assumed that the number of groups is two and that they have equal covariance matrices. With these assumptions it may be possible to obtain satisfactory estimates of the characteristics of both groups, thus, permitting identification of unknown specimens. However, if it is known that the assumptions are incorrect, the analysis (identification) may actually impose a structure on the data rather than finding the true structure in the data. In the context of stock identification, we might mistakenly infer that there are two stocks when in fact only one stock exists or vice versa.

- 2) Variable selection--Clearly variables must be chosen before applying a particular classification technique. For example, in discrimination, one wishes to find variables that enhance differences between groups while retaining some amount of within-group similarities. The actual number of variables to utilize will be discussed later. After variables have been chosen, they can be scaled, transformed and coded in a variety of ways. It has already been shown that scaling affects particular statistical techniques such as clustering. It is less evident how each variable should be weighted, or whether natural or transformed variables are more appropriate for the purposes at hand. There is no easy answer to this question. However, if an a priori grouping of data tends to be relatively invariant to transformation changes, then this provides some indication that the empirically derived groupings are not artifacts.
- 3) Class membership--It is considered highly desirable in stock identification to be able to identify tentatively well-defined groups (stocks) which form the basis of discrimination schemes. This can be done by obtaining training samples from known breeding areas during the breeding season. The problem of trying to find underlying structure in a relatively undifferentiated set of organisms is difficult, and assigning unknown specimens to statistically-derived groups is even worse. In our experience, classification techniques involving discrimination seem to perform better than those which rely on obtaining internal structure from

ungrouped data sets (i.e., clusters). However, this comparison has not been made critically.

#### SOME METHODOLOGIES

Some major methods of multivariate statistical analysis include cluster analysis, factor analysis, principal components analysis, linear and non-linear discriminant analysis and canonical correlation analysis. Discriminant functions, especially the linear discriminant function, have been a powerful tool which has been successfully applied to stock identification in many cases. Their use is based on the assumption that one knows that the variables needed to characterize an organism are highly relevant to the identification problem, and that all samples (organisms) belong to one of the initially-specified classes, in addition to well-defined statistical assumptions. Clearly, these assumptions are not always fulfilled completely in fish stock identification. Recently, in stock identification it has become increasingly easy to obtain a multitude of measurements on a single organism (sample), using such techniques as high pressure chromatography, electrophoresis, spectrographic techniques, x-ray diffraction, and digitizing pads. In some cases, sample sizes for linear or non-linear discriminant analyses may be inadequate due to the large number of variables which have been measured. We will, therefore, consider briefly another form of discrimination, which is less dependent on sample size. Specifically, we will briefly describe linear discriminant analysis and some of its possible limitations and then describe a newer technique of discrimination with acronyms ranging from Statistical Isolinear Multiple Component Analysis to Soft Independent Modeling by Class Analogy (SIMCA). It is believed that the example provided herein is the first application of this methodology to fish stock identification.

#### Linear Discriminant Analysis

Linear discriminant analysis was originally developed by Sir Ronald Fisher and was first applied to a problem dealing with the characterization of skulls into archaeological time periods by Barnard (1935). From a representative sample of each skull, the average value of each measurement was computed for each period. A new skull could then be classified by comparing differences in the observed value with the expected value of each of seven measurements. Rather than sum these differences with equal weight, Fisher devised a technique for computing a coefficient for each difference. The desired set of coefficients would be the one that gave greater weight to those measurements which provided greater discrimination between time periods. This set of coefficients yields a linear discriminant function. The optimum set of coefficients is found by maximizing the ratio of the among-category-sum-of-squares of this function to its pooled within-categories-sum-of-squares. Rao (1952) gives the necessary theoretical discussion and proofs for discriminant analysis to the reader interested in formal details.

An attempt will be made herein to provide only a very simple geometric interpretation of discriminant analysis. This is shown in Figure 4. In this figure the two sets of concentric ellipses represent the bivariate swarms for two groups in idealized form. The two variates, X and Y, appear to be moderately correlated. Each ellipse is the locus of points of equal density

(or frequency) for a group. For example, the ellipse for group A might define the region in which 95 percent of group A lies, and similarly for B. These ellipses are sometimes called centours (centile contours). The two points at which the centours intersect define a straight line, labeled II in this case. If the second line (labeled I) is constructed perpendicular to line II and if the points in two-dimensional space are projected onto I, the overlap between the two groups will be smaller than for any other possible line. The discriminant function transforms individual attributes (variables) to a single discriminant value, and that value is the sample's location along line I. The point b where II intersects I will divide the discriminant space into two regions, one indicating probable membership in group A and the other in group B. Notice that this diagram depends on the equality of the two group dispersions. If either the variances of X and Y or the X,Y covariance were different for the two groups, then the centile contours for the two groups would not have the same shape and orientation, and the boundary value (line II) would not be a straight line. The sizes of the two groups do not have to be the same--only the dispersions need be the same for application of the linear discriminant function.

When the objective is to distinguish between k populations (instead of two), the two-dimensioned space used in Figure 4 may be partitioned in k parts, one for each of the swarms of points around the different population means. The statistical methodology resembles principal components analysis. At first, a single discriminant function is computed to separate between the k populations by means of a set of (k-1) parallel planes which are perpendicular to a straight line in p-dimensional space on which the k population means would fall.

Multiple discriminant analysis has been applied to striped bass morphometrics and to a combination of electrophoretic and morphometric data by Fabrizio (this workshop). Indeed, the use of discriminant functions in fish stock identification has a relatively long and successful history. See, for example, the citations given in Appendix 1. Multivariate alternatives to this method have seldom been considered in fish stock identification.

Some might question whether alternative multivariate stock identification techniques are required in view of the apparent success of the discriminant function. There are some reasons for considering alternatives, which include:

- 1) The use of the discriminant function requires that the number of samples (n) be much larger than p, the number of variables. A rule of thumb is that n should be at least three times p. Automated and semi-automated techniques make it increasingly easy to obtain a large number of measurements on a single sample. Therefore, there will be an increasing frequency of times when n is not larger than p, and alternative methodologies need to be considered which do not have this sample size requirement. Stepwise discriminant analysis may relieve the problem of sample size in some cases.
- 2) In linear and non-linear discriminant analysis, each individual is assigned to one of a pre-determined number of groups. There is no provision for assigning an individual to an unknown group, nor is it

easy to determine which observations are outliers except by changing probability levels in the analysis program. Sometimes, detecting outliers or unknown categories is valuable in stock identification.

- 3) It is sometimes desirable to make use of several iterative procedures for stock identification for comparative purposes.
- 4) Missing data should be accommodated in the analysis. The discriminant function does not permit this.
- 5) The statistical assumptions of multivariate normality and equal variance-covariance matrices may limit application of the linear discriminant function in some instances. However, use of the quadratic discrimination procedure relaxes the latter assumption.

#### SIMCA

The SIMCA method makes less demands on sample size ( $n$ ) relative to the number of variables ( $p$ ) than discriminant analysis. In fact, the sample size ( $n$ ) considered minimal for application to the method is about 5 per group and ( $p$ ), the number of variables, may be substantially higher than  $n$ . The SIMCA procedure permits assigning individuals to "unknown" classes and provides for easy detection of outliers. It is also an iterative procedure which permits considerable flexibility.

SIMCA is a classification (discrimination) method based on latent factors. It has been developed by a chemist and applied to chemical identification problems primarily. Wold (1976), Wold and Sjostrom (1977), Wold et al. (1981), Dunn et al. (1984), and Smith et al. (1985), have provided some background, theory and applications of the SIMCA method. Software is commercially available from Principal Data Components, 2505 Shepard Blvd., Columbia, MO 65202.

The basis of the SIMCA method is to approximate the multivariate data of each group (which has been established a priori) by means of a principal components model. A multi-dimensional space (sometimes called M-space) is constructed by letting each of the variables measured in a group define one coordinate axis. This space may be multi-dimensional, but it is of course possible to visualize only 3-dimensional space. However, higher multi-dimensional spaces have the same mathematical properties.

In  $p$ -dimensional space all the data defining one object (say, a fish) are represented by a single point. See Figure 5 for a 3-dimensional space with one object. An attempt will be made to describe briefly the principal components model geometrically as well as by data arrays. Figure 6 illustrates  $n$  objects (fish) represented as a swarm of points in  $p$ -space. These fish are also represented in the figure as  $n$  rows in the data table labeled  $X$ . The data swarm of Figure 6 can be represented by its mid-point. The coordinates of this point are the averages of the variables  $x_{jk}$ , which form the row vector  $\bar{X}$ , as is demonstrated in Figure 7. Next, subtract these averages from the data to get residuals  $e_{jk}$ , elements in a matrix  $E$ . This corresponds to moving the coordinate system to be centered at  $\bar{X}$ . If we now rename the residuals  $E$  as  $X$ ,

then we have scaled and centered the data matrix, X. We next fit a straight line to the n points in p-space so that the deviations from this line are as small as possible in the least squares sense, as is shown in Figure 8. The direction coefficients of the line are the so-called loadings, one for each variable k. These are denoted by  $p_{1k}$  forming the row vector  $p_1$ . When each point is projected on the line we get the score  $t_{i1}$ . These are the coordinates of point i along the axis  $p_1$  as shown in Figure 8. The residuals,  $e_{ik}$  are obtained by subtraction of  $t_i p_k$  from  $x_{ik}$ . Again, rename the coordinate E as X, and this will correspond to removing the direction  $p_1$  from the data. When the row X is used to fit a straight line to the points (n), this corresponds to a second line through x which is orthogonal to the first line, as shown in Figure 9. Figure 10 shows how the first and second principal components can be used to define a plane in the p-dimensional space. This plane can be visualized as a 2-dimensional window in p-space.

Figure 11 illustrates that the distance between a point and a class model is proportional to the residual standard deviation. The one-dimensional principal components model shown in Figure 12 illustrates asymmetric data, and confidence bands are indicated around the model.

In general, SIMCA involves analyses of multivariate data with the purpose of finding similarities in groups of objects (samples). The patterns of similarities as group principal components models are then used to assign unknown individuals to given groups. This is done according to the degree of fit to the group models. In its simplest form, the steps in this analysis are as follows:

- 1) Define the groups of interest in terms of variables that are associated with individuals in these groups. At least five or more individuals are needed for each group, but the number of variables may be substantially higher.
- 2) Normalize all data so each variable has a zero mean and unit variance over all groups. This is done to give each measured property equal weight in the classification. If there is prior knowledge about the relative importance of variables, they can be weighted according to their importance.
- 3) Define the data structure for each group by fitting a principal components model of the following form to each group:

$$x_{ki} = \bar{x}_i + \sum_{a=1}^A t_{ka} b_{ai} + e_{ki} \quad (1)$$

Where:

$x_{ki}$  = elements of the data matrix,

$\bar{x}_i$  = group mean for variable i,

A = number of component terms in the model,

$t_{ka}$  = component score for each principal component term,

$b_{ai}$  = loading for each principal component term,

$e_{ki}$  = residuals representing the contribution of measurement error and model error.

- 4) Use the data structure of step 3 above to classify unknown objects. This is done by fitting the data from each unassigned object to each group model, with the  $\bar{X}_i$ , and  $b_{ai}$  values fixed as those determined in step 3. Each such fit corresponds to a multiple linear regression, and provides among other things, a residual standard deviation  $s_k^q$  (for object k in group q) by means of the following equation:

$$s = \left[ \sum_K e_k^2 / (n-p) \right]^{1/2} \quad (2)$$

where p is the number of parameters.

A non-assigned object is classified as belonging to the group for which this standard deviation  $s_k^q$  is smallest, provided that it is of the same magnitude as the typical residual standard deviation for that class,  $s_0$ .

#### SIMCA Application Using Striped Bass

A subset of the total sample of striped bass used by Fabrizio (this workshop) was utilized for the work. This subset contained 15 striped bass of known Hudson origin, 15 striped bass of Chesapeake origin and 31 unknown samples consisting of a mixture of Hudson and Chesapeake fish. The origin of the 31 unknown samples was actually known, but not made available for this study until after the classification had been applied. For this example, a set of ten optical density peaks electrophoretically identified from eye lens proteins of each fish were utilized as the variables. In our example,  $n = 61$  of which there were two training sets of 15 each and 31 unknowns.  $M$  was 10, and defined the number of reasonably consistent peaks found. Optical density peak numbers 5, 8, 9, 14, 15, 23, 24, 25, 28, and 30 were used from Fabrizio's data.

The first difficulty we faced in running the SIMCA analysis was that the data appeared to be extremely noisy. Biological data are usually noisy, so this finding was not unexpected, but it did pose a problem. The SIMCA procedure requires one to decide how many principal components (PCs) are to be retained in the analysis. The suggested procedure is to limit the number of PCs to those which pass a SIMCA "significance" test. Unfortunately, none of the striped bass PCs were "significant", according to the SIMCA suggested test. There are possible objections to the test, however, so we decided to proceed with the analysis and attempt to develop our own decision criteria for the number of PCs to be retained.

The ultimate goal of the SIMCA procedure is to classify fish of unknown stock origin into either of two stocks, Hudson River stock or Chesapeake stock. Two classification schemes were used. The first, the Range Test, is conceptually simple. To be included in the class, the unknown fish must fit the training set model at least as well as the worst fitting training set fish. In other words, the upper limit on the fit-to-the-model is set by the upper limit of the range. This test gives a simple yes/no answer to the question "does this unknown fish belong in the training set class?" The Range Test is fast, but suffers from the disadvantage that statistical significance levels have not been developed for it.

The second test, the F Test, is the test suggested by the author of the SIMCA procedure. In this test, the fit-to-the-model of the unknown fish is compared to an average error (deviation) in fit obtained from the training set. This comparison in fit is distributed as the F-distribution, so F-tables are used to make the decision as to whether or not the unknown fish belongs to the training set class. The test is somewhat more difficult to use than the Range Test, but the F Test allows one to assign a statistical significance level to the result. In this study, we used a 95 percent significance level.

The actual SIMCA procedure was applied to two data sets. The first consisted of 30 striped bass, 15 from the Hudson and 15 from the Chesapeake. Ten fish from each stock were used for each of the training sets. Thus, the Hudson model was based on the ten Hudson fish training set and the Chesapeake model was based on the ten Chesapeake fish training set. The classification procedure was applied to the remaining 10 fish.

For the second data set, the 30 fish of the first set were combined with an additional 31 fish, 6 Hudson fish and 25 Chesapeake fish. The training sets consisted of 15 Hudson fish and 14 Chesapeake fish. In this case, 46 fish were considered "unknowns" for the classification using the Hudson model, while the Chesapeake model was used to classify 45 "unknowns" (one Chesapeake fish was considered to be an outlier and was dropped from the analysis). The second group of 61 total fish included several striped bass collected from Chesapeake tributaries; these fish may potentially belong to groups which can be distinguished within the Chesapeake.

Since all fish used in this analysis were of known origin, it was possible to compare SIMCA classification results with the actual classes. The results of the analysis are given in Tables 1 and 2. These results vary with the number of principal components retained for the analysis. The best results were:

	Percent correctly classified	
	Range Test	F Test
30 fish data set		
Hudson model	95%	75%
Chesapeake model	84%	79%
61 fish data set		
Hudson model	78%	74%
Chesapeake model	74%	74%

From the above, it is clear that the SIMCA procedure has promise in stock identification. In most cases, almost three-quarters of the fish were classified correctly, in spite of noisy data and small sample sizes.

The validity of the classification results depends to some extent on the number of PCs one should use. Our preliminary results suggest in a nutshell, that one should use only one PC if the Range Test is to be employed. For the F Test, one should probably use as many PCs as are required to account for approximately 50 percent of the variation in the training set. These preliminary suggestions are based on the trends displayed in Tables 1 and 2.

Generally, the validity of classification for the Range Test displayed no consistent trend with the number of PCs used in the analysis. There is some suggestion, however, that the ability of the Range Test to detect members of its own class decreases as the number of PCs increases.

In contrast, the validity of the F Test appears to increase as the number of PCs used in the analysis increases. In this case, one should use more PCs to increase the chance of correctly classifying a fish. We believe that PCs should be limited to the number that accounts for approximately 50 percent of the deviation in the training set. This suggestion is based on the desire to not "over-specify" the class: as the number of PCs retained for the analysis increases, the unexplained deviation goes down, but the stability of prediction is generally thought to also decrease. For the striped bass, allowance for about half of the deviation seemed to provide a balance between over-specification of the class and prediction validity in this case at least.

For the striped bass example presented here, our future research should concentrate on the following areas:

- a) Examination of the classification results for the presence of sub-populations.
- b) Inclusion of more proteins in the analysis.

One of the advantages of the SIMCA method is that one may have fewer fish than variables in the training set. We have not studied the stability of classification as a function of number of fish included in the training set. This also would be a suitable topic for further study.

Based on these preliminary results, it would appear that the SIMCA method shows promise in the classification of fish to different stocks. In this limited sample three-quarters of the "unknown" fish were correctly described. Clearly, the SIMCA procedure should receive the attention of fisheries biologists interested in stock identification.

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#### APPENDIX

##### Applications of Discriminant Functions of Stock Identification

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Table 1. Percent of fish correctly classified--30 fish data set.

No. PCs	Unexplained deviation	Hudson Model					
		Range Test			F Test (95% level)		
		H	C	A	H	C	A
4	0.53	40	100	85	80	73	75
3	0.59	100	33	50	100	60	70
2	0.76	100	47	60	100	13	35
1	0.86	100	93	95	100	7	30
		Chesapeake Model					
4	0.60	93	25	79	73	100	79
3	0.68	93	50	84	60	100	68
2	0.84	80	50	74	20	100	37
1	1.00	73	75	74	7	100	26

H = Hudson fish  
 C = Chesapeake fish  
 A = All fish

Example: For the Hudson model using four principal components and the Range Test, 40% of the Hudson fish and 100% of the Chesapeake fish in the "unknown" set were correctly classified.

Table 2. Percent of fish correctly classified--61 fish data set.

No. PCs	Unexplained deviation	Hudson Model					
		Range Test			F Test (95% level)		
		H	C	A	H	C	A
5	0.53	50	83	78	83	73	74
4	0.61	17	80	72	83	60	63
3	0.72	50	68	65	100	18	28
2	0.82	67	60	61	100	8	20
1	0.90	33	70	65	100	3	15
		Chesapeake Model					
4	0.52	95	24	57	67	72	70
3	0.60	81	40	59	57	88	74
2	0.71	81	28	52	14	92	56
1	0.89	67	80	74	0	96	52

H = Hudson fish  
 C = Chesapeake fish  
 A = All fish

Example: For the Hudson model using five principal components and the Range Test, 50% of the Hudson fish and 83% of the Chesapeake fish in the "unknown" set were correctly classified.

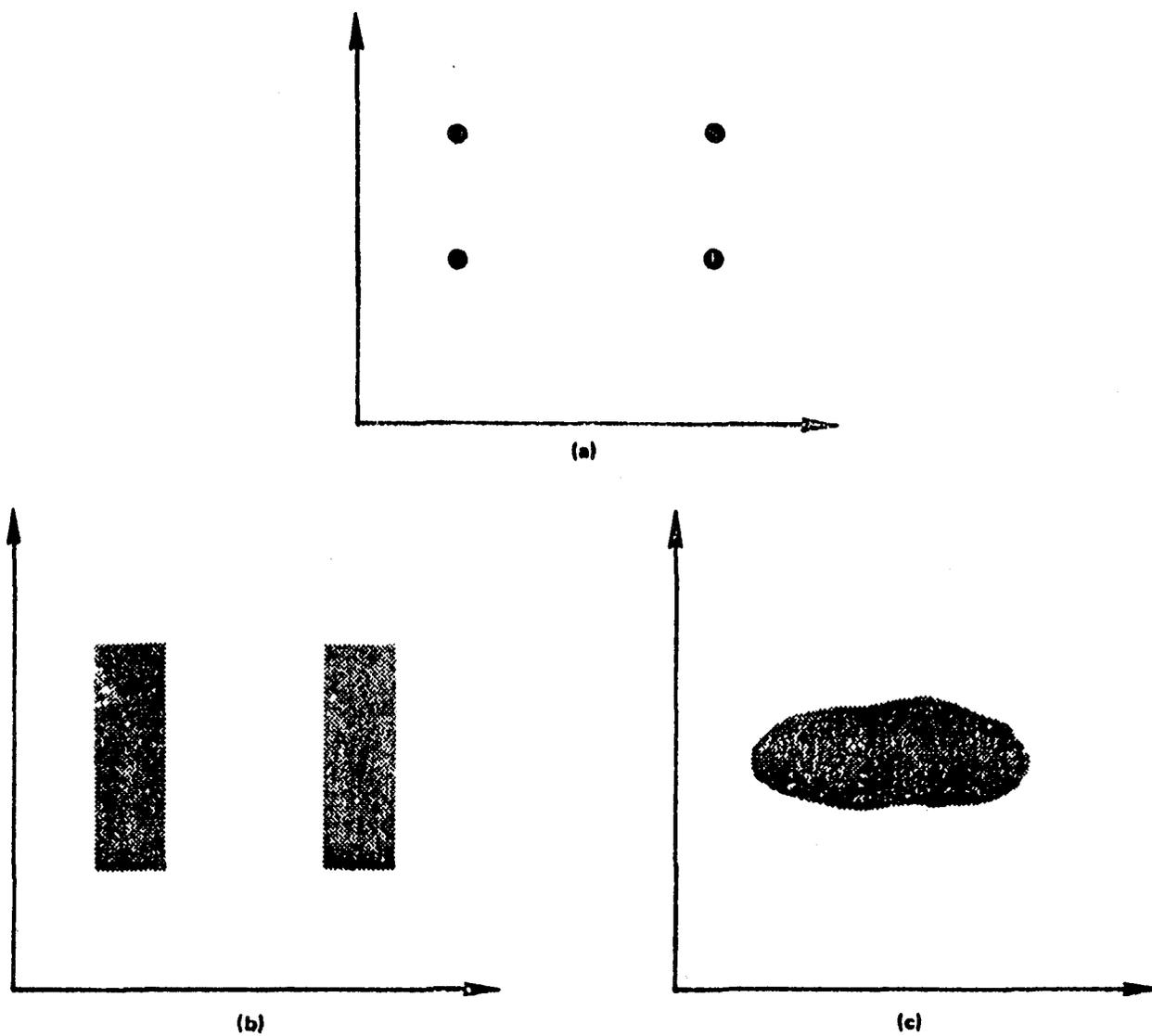


Figure 1. Three dissimilar data sets, each with the same mean and the same covariance matrix.

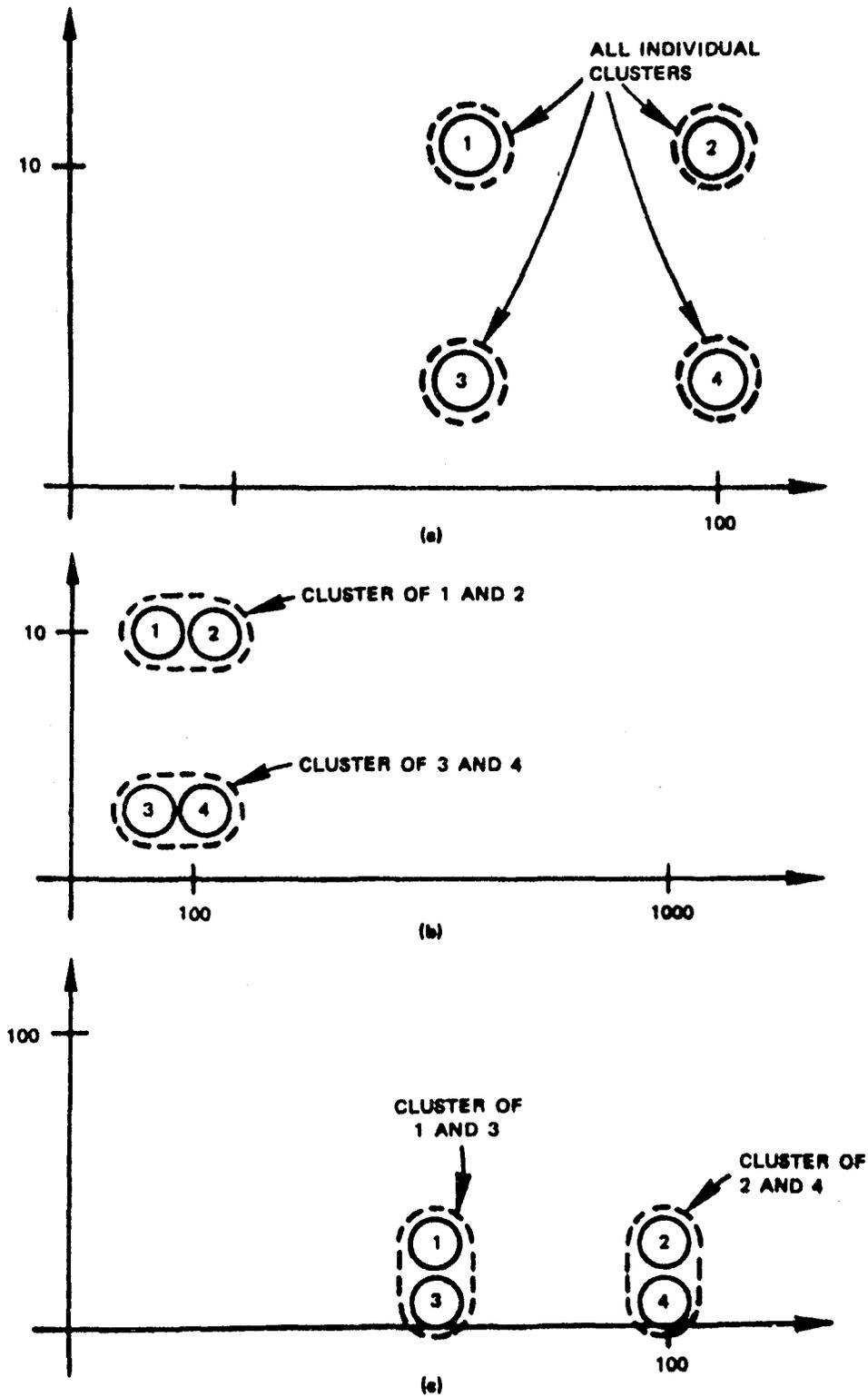


Figure 2. The effect of scaling on which clusters look similar.

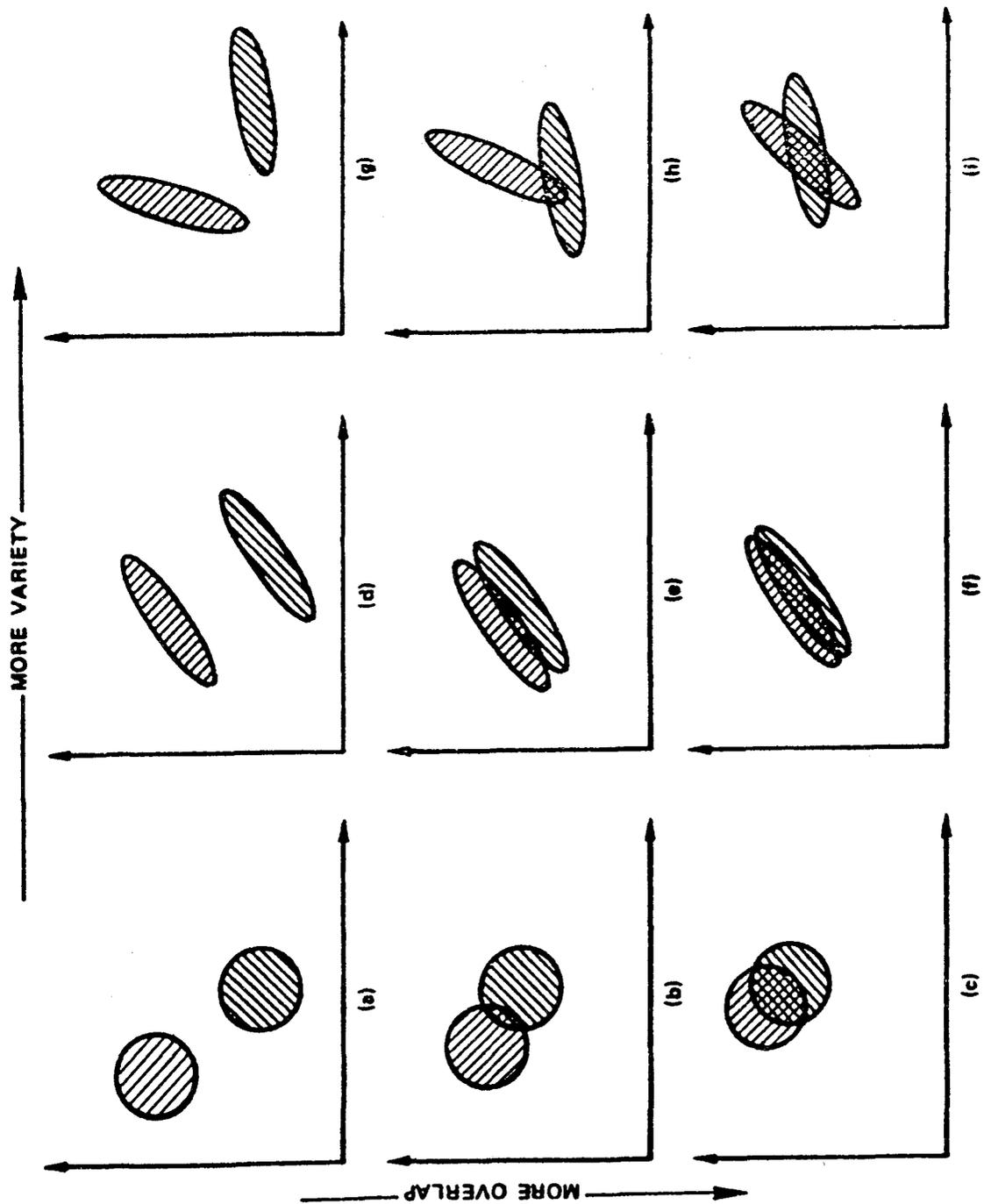


Figure 3. Illustration of the variable structure of data.

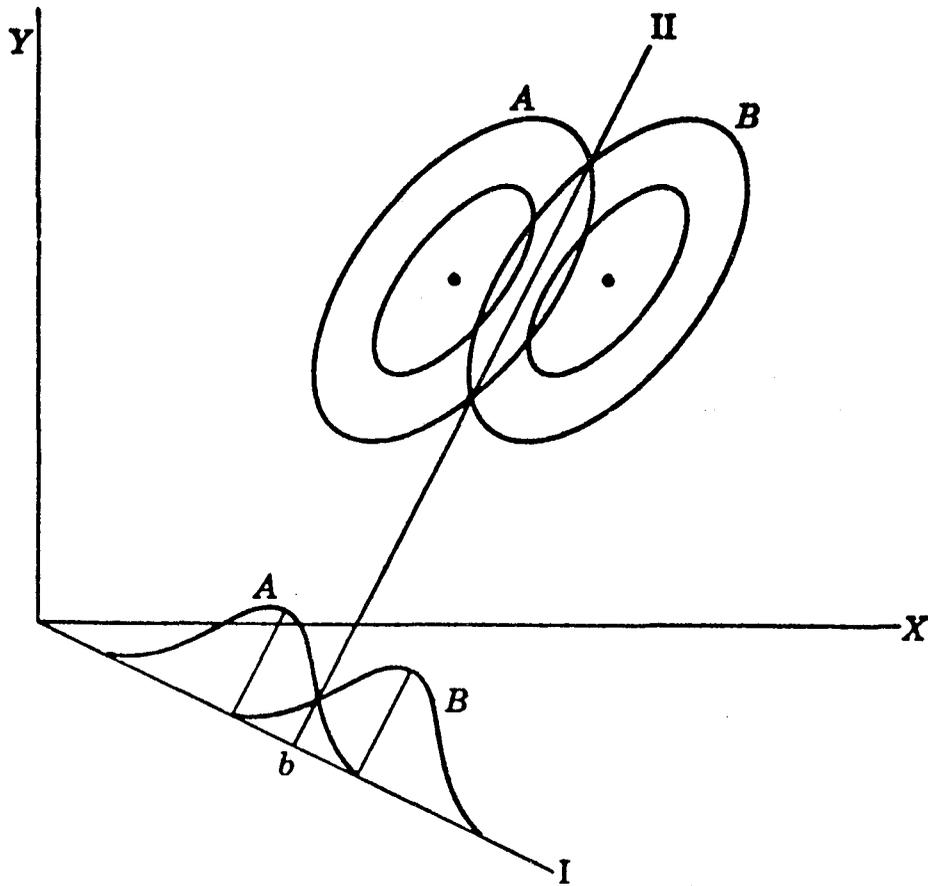


Figure 4. Geometric interpretation of the linear discriminant function for the two sample case.

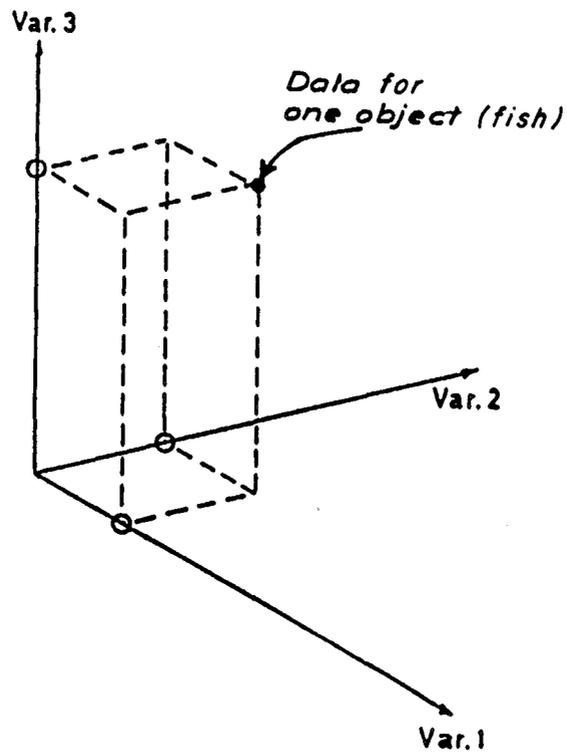


Figure 5. Three-dimensional space representation of one object (fish).

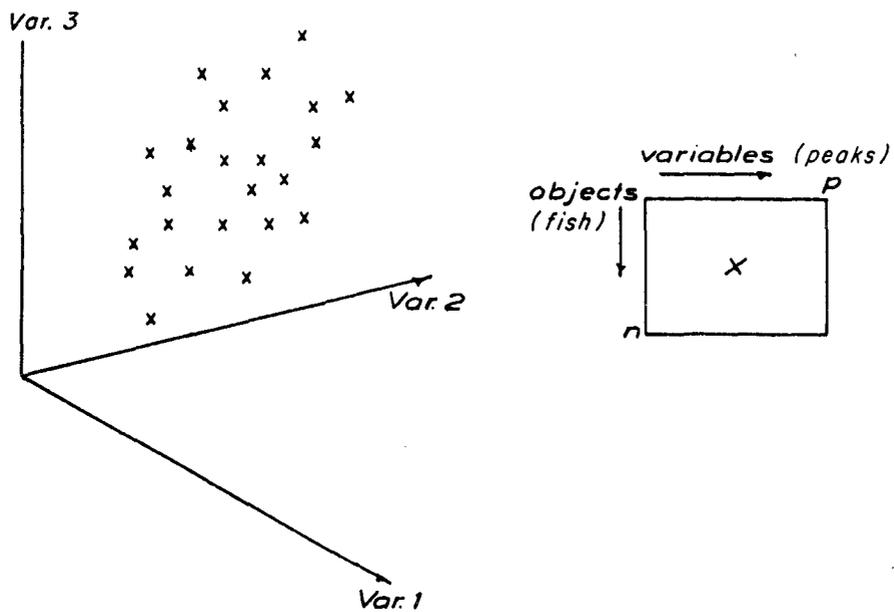


Figure 6. The  $n$  objects (fish) in the data set constitute a swarm in  $p$ -dimensional space.

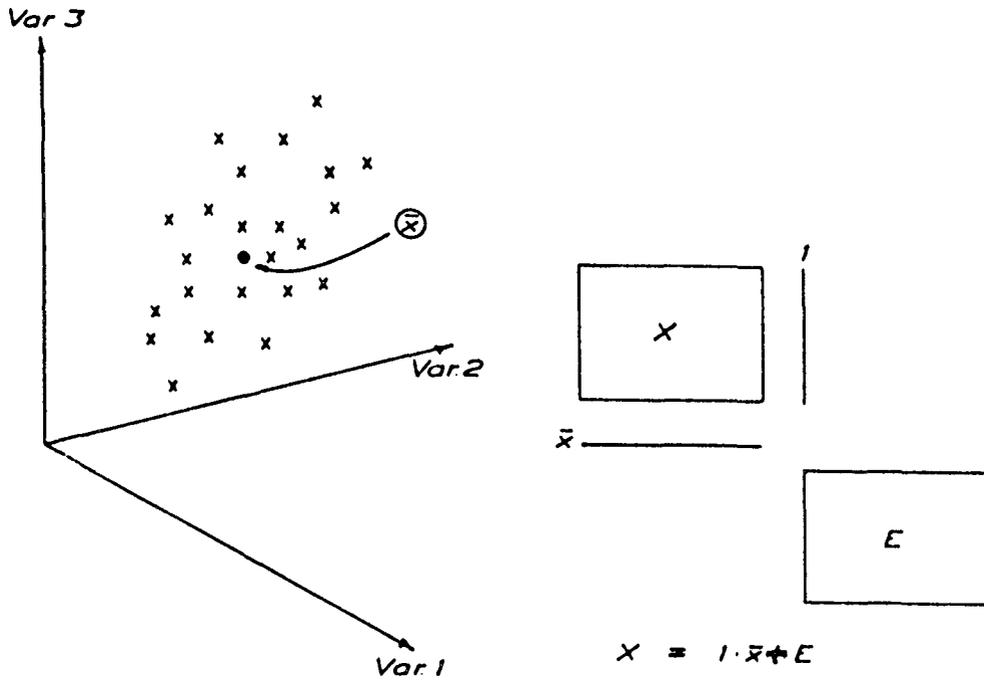


Figure 7. Data set modeled by its mean  $\bar{x}$ . This has the coordinates defined by the average of the variables. The matrix  $E$  contains the residuals  $e_{ik}$ .

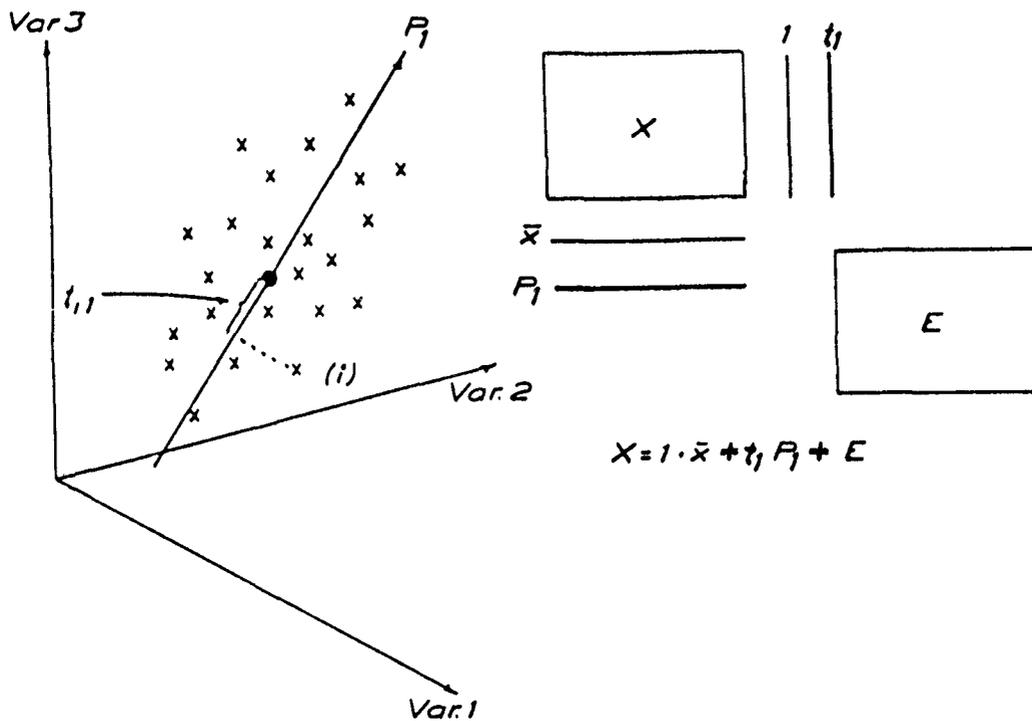


Figure 8. Data modeled by a least-squares line through  $x$ . This line is the first principal component and its equation is defined by the loading vector  $p_1$ .

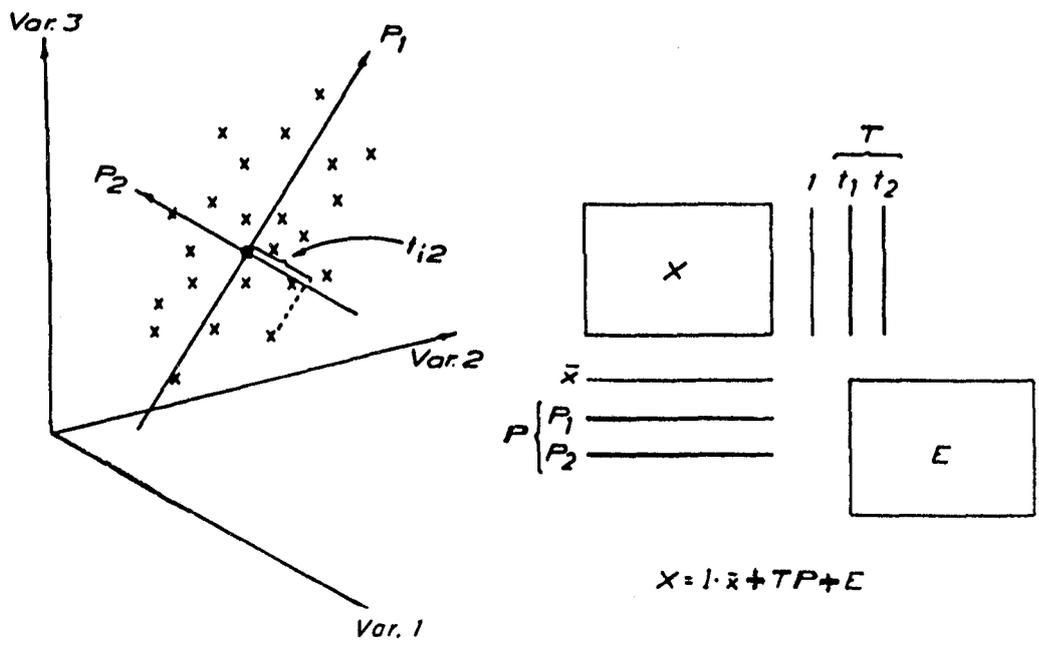


Figure 9. The second principal component line through  $x$ , perpendicular to  $P_1$ .

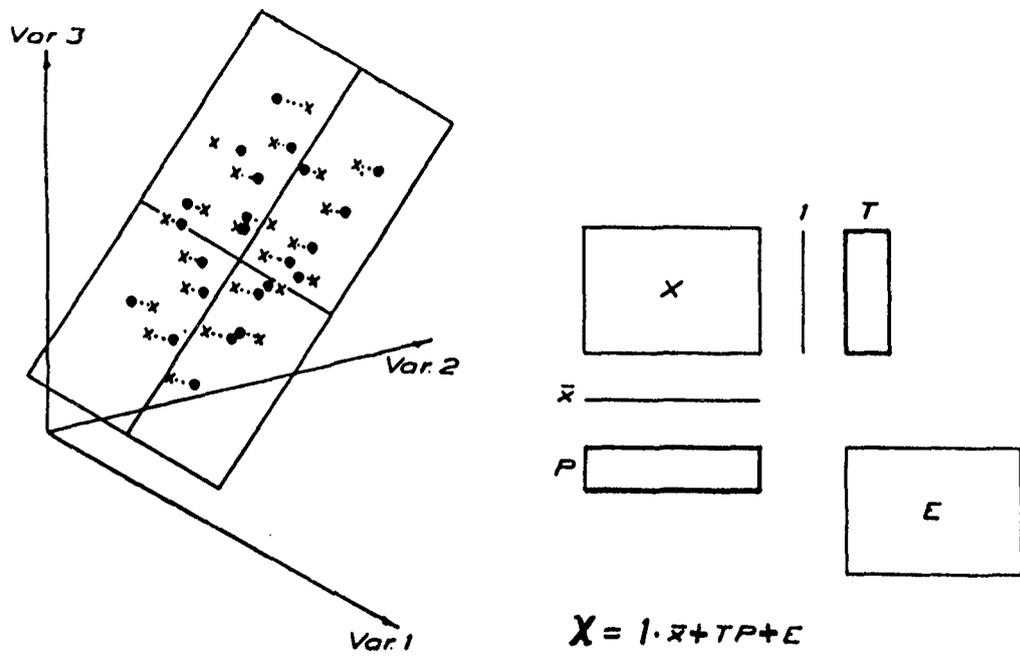


Figure 10. Principal component lines 1 and 2 define a plane. This is a 2-dimensional window into  $p$ -space.

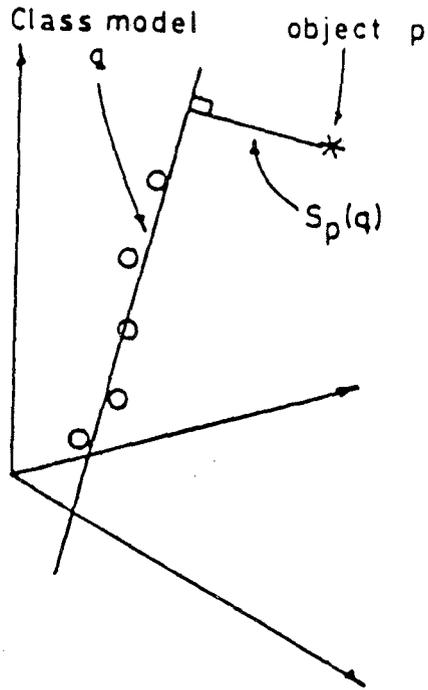


Figure 11. The distance between a point and a class model is proportional to the residual standard deviation.

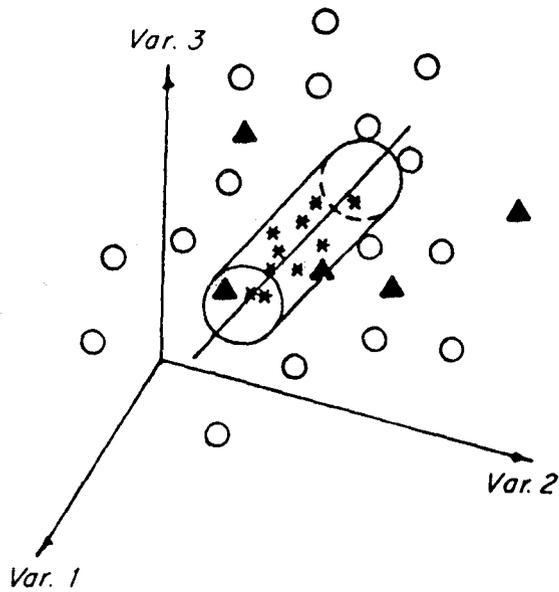


Figure 12. One dimensional principal component model and asymmetric data. Confidence bands are shown around the model.



ON THE USE OF MITOCHONDRIAL DNA AS A TOOL FOR STOCK ASSESSMENT

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The introduction of mitochondrial DNA analyses into the study of natural populations has presented population biologists with a powerful tool. As the number of studies employing this technique have expanded, it has become clear that: 1) most individuals possess a single mtDNA molecule (i.e., are homoplasmic); 2) individuals often possess mtDNAs that can be distinguished by changes in nucleotide sequence or by increases in the size of the molecule; 3) individuals can often be related by the number of mutation events since common ancestry; and 4) mtDNA is for the most part maternally inherited. Exceptions to these generalities do exist, but they have held for a wide variety of organisms.

Given the above observations, it would seem that mtDNA analyses would rapidly be incorporated into the repertoire of laboratories studying population dynamics. However, a major limitation to the extensive use of mtDNA has been the tedious methods used to isolate the molecule and expensive radionucleotides used to identify restriction fragments. It is not unusual to spend a week isolating a few samples of highly purified mtDNA and several days thereafter autoradiographing restriction fragments. This paper focuses upon a rapid isolation protocol and its application to problems in population dynamics. The procedure has several advantages over existing methodologies; 1) simultaneous preparation of 24 or more samples in under six hours; 2) low cost per sample; 3) use of standard laboratory equipment; 4) inexpensive methods to identify restriction fragments; and 5) results that are identical to the highly purified mtDNA used in other laboratories.

Intensive studies of two commercially important species, Morone saxatilis and M. americana, have revealed some unusual patterns of mtDNA variation. In both species, mtDNA variation is characterized more by size polymorphisms than by restriction site changes which are typical of mammals and some fishes. These species differ in the manner by which size variants are packaged. M. saxatilis variation occurs between individuals, while M. americana individuals may carry as many as 10 distinct mtDNA molecules. Intraindividual heteroplasmy is known to occur in some species but M. americana heteroplasmy is virtually 100% and far exceeds that of any known species. The molecular and/or cellular basis for differences in M. americana and M. saxatilis mtDNA expression is not known. Geographic distributions of mtDNA genotypes in the Chesapeake Bay suggest the presence of three distinct populations of M. saxatilis and at least four distinct populations of M. americana.

Laboratory studies on mtDNA transmission genetics in M. saxatilis show that the molecule may on occasion be transmitted by males which contrasts sharply with the strict maternal transmission that has been assumed to date. In summary, analyses of mtDNA variation in the genus Morone urges caution in interpreting patterns of variation for management purposes.

ANALYSIS OF KING MACKEREL STOCKS USING HIGH  
PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

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Polymorphisms in nuclear eye lens proteins have been used to distinguish populations within species. The proteins of the eye lens core or nucleus possess qualities which make them a desirable source for biochemical genetic information: 1) in vertebrates the lens nucleus has a higher concentration of protein than any other tissue; 2) as the nucleus is easily isolated, there is a minimum of contamination from other tissue proteins; 3) a resistance to denaturation ameliorates tissue handling procedures; and 4) as the nucleus is a nonmetabolizing tissue, there is a minimum of turnover.

Herein we report a new technique having applications in biochemical studies of fish populations: high pressure liquid chromatography (HPLC) of eye lens nuclear proteins. Research to date has shown this procedure to be a fast, reliable, and reproducible method of analyzing eye lens proteins for intraspecific polymorphisms. After initial start-up expense (which will vary according to the quality of the system), the cost of HPLC operation is minimal.

HPLC has two essential elements: a mobile phase and a stationary phase. The mobile phase is a solvent or combination of solvents which carries a small aliquot of sample through the system and through the stationary phase. The stationary phase is a separatory column which acts as a sieve to retain preferentially fractions of the sample for varying time spans. These retention times are dependent upon characteristics of the sample fractions such as molecular weight, net charge, chemical affinities, etc. As the fractions elute from the column they pass to an absorbance detector which monitors eluent concentration. The detector generates a series of electrical signals which are translated into an elution chromatogram by an integrator or other data handling device. The

chromatogram is a record of detector response (proportional to eluent concentration) plotted against time. The retention times of the major peaks and their relative heights are the two types of data which can be used in comparisons of eye lens proteins between populations.

The objectives of this report are to describe HPLC of eye lens nuclear proteins as a new research technique for biochemical analyses of fish populations, and to present data on HPLC investigations of king mackerel (Scomberomorus cavalla) populations.

King mackerel were collected from January 1985 to July 1985 from nine areas in the western north Atlantic Ocean and Gulf of Mexico. Numbers of fish samples were North Carolina = 10, South Carolina = 24, Florida Keys = 20, Florida Panhandle = 24, Louisiana = 22, Texas = 6, Veracruz, Mexico = 20, Yucatan, Mexico = 17, and Cuba = 20. Eye lenses or whole eyes were excised from fresh specimens, placed in individually labelled plastic bags, and immediately iced. Dry ice was used when available. All lenses were stored at  $-5^{\circ}$  C until time of analysis.

Prior to homogenization, the capsule and cortical layers of the lenses were removed. The lens nucleus was then pulverized in a hand-held, glass tissue homogenizer with two ml of an extraction medium. The medium dissolved not only the water-soluble (crystalline) fraction of the nucleus, but also the water-insoluble (albuminoid) fraction. An additional one ml of extractant was added post-homogenization. Particulate material was sedimented by centrifugation for three minutes at 12,800 x g in an Eppendorf microcentrifuge. Fifteen microliters of the supernatant fraction was injected for each analysis.

A dual pump HPLC system (Waters Assoc., Milford, MA 01757) was used for the analysis of king mackerel eye lens proteins. Reverse phase HPLC was accomplished using the following mobile phase: solvent A 0.02% trifluoroacetic acid (TFA) in a 50:50 solution of acetonitrile ( $\text{CH}_3\text{CN}$ ) and water, and solvent B 0.02% TFA in water. The mobile phase was run using various solvent gradients.

All analyses were performed at room temperature. Elution chromatograms were graphically depicted as absorbances at a detector wavelength of 220 nm.

Preliminary results indicate that eye lens protein HPLC patterns are consistent within most areas sampled: the Carolinas, Louisiana, Texas, Florida Keys, Veracruz, Yucatan, and Cuba. In one area, northwest Florida, two distinct patterns were noted. One similar to the Key West group and one similar to the Louisiana group. When patterns were compared visually certain similarities among regions were noted and were grouped as:

- a. North Carolina, South Carolina, Florida Keys, northwest Florida (in part),
- b. Texas and Yucatan,

- c. Cuba and Veracruz,
- d. Louisiana and northwest Florida (in part). In previous runs Louisiana, Texas, and Veracruz shared common characteristics.

Information demonstrating stock structure for the king mackerel off the east coast of the United States, in the Gulf of Mexico and the Caribbean is urgently needed as a management tool. HPLC appears promising as a technique to identify different mackerel groups. King mackerel eye lens proteins from within areas sampled (except northwestern Florida) exhibited a monomorphic chromatogram. In northwestern Florida two chromatographic forms were found, one similar to Louisiana mackerels and one similar to mackerels from southern Florida.

We are of the opinion that these forms are indicative of migratory groups of the Gulf of Mexico and the Atlantic. However, we feel that many more samples must be run in order to establish significant chromatograms of the results. We are also attempting to quantify the results in a meaningful statistical manner in order to compare regions accurately. During the next 12 months we propose to gather eye lens data synoptically from regions in the Gulf, from various areas of the Caribbean, and off the east coast. We will strive to collect and analyze groups of eye lenses from 25 different king mackerel during each season for each area. An objective will be to gather all samples seasonally within a week or two of each other in order to decrease the chance that migrations and mixing will affect the results. If sampling is done with sufficient frequency we anticipate learning of different groups appearing seasonally off different areas.

PATTERNS OF GENETIC VARIATION AND SUBPOPULATION STRUCTURE  
IN NARROW-BARRED SPANISH MACKEREL

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The narrow-barred Spanish mackerel (*Scomberomorus commerson*) is a neritic species distributed throughout much of the Indo-west Pacific from South Africa to Fiji. In northern Australia, the Torres Strait, and Papua New Guinea this mackerel supports significant commercial, recreational, and/or subsistence fisheries. An investigation of genetic aspects of stock structure in this species was initiated to provide information necessary for the future management of these fisheries.

Over 2,000 Spanish mackerel were collected from 19 different localities in the southwestern Pacific and Indian Oceans. To date, a total of approximately 1,000 mackerel (from four Australian localities, three localities in Papua New Guinea, and from New Caledonia, and Fiji)

have been analyzed for genetic variation using horizontal starch gel electrophoresis. Thirty-five loci were surveyed in all fish. Twenty-seven loci exhibited scorable genetic variation; ten of these were polymorphic at the P(0.95) level. The distributions of presumed genotypes in each collection were in close agreement with Hardy-Weinberg expectations in all cases. Statistical analyses indicated the existence of three major genetic groups of mackerel (one in Australia, a second in Papua New Guinea, and a third in Fiji). Substantial genetic differentiation (involving large allele frequency differences at many loci) was observed among these three groups. Additionally, evidence of considerable regional differentiation within Papua New Guinea and of limited heterogeneity within Australia was obtained.

These data reveal an absence of panmixia among Spanish mackerel in the Australian region of the Indo-west Pacific and suggest the existence of several more-or-less discrete stocks. The observed genetic differentiation between mackerel in Australia and those in Papua New Guinea suggests that fish in the Torres Strait, a politically sensitive area, may belong to one or both of these major stocks (or even a third stock).

Preliminary electrophoretic analyses of Spanish mackerel from South Africa reveal that these fish are very distinct from those in the Australian region, emphasizing the high degree of geographic differentiation within the species.

The genetic relationships established in this study should provide an initial basis for management of the Spanish mackerel fisheries in the Australian region on a stock basis.

This study was conducted at the CSIRO Laboratory at Cleveland, Queensland and was funded by the Australian Department of Primary Industry and the Fishing Industry Research Trust Account.

STOCK ASSESSMENT USING ELECTROPHORETIC AND BODY COLOR INDICES:  
COMPLEX PATTERNS PRESENT A UNIQUE STOCK IDENTIFICATION  
PROBLEM IN STONE CRABS (Menippe mercenaria)

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Electrophoretic and body color patterns used to assess geographic variation in the commercially valuable stone crab (Menippe mercenaria) throughout the southeastern United States reveal that the species is actually a taxonomic supergroup composed of two taxa, one fitting the description of M. mercenaria and the other an undescribed subspecies, here called the Western Gulf Form (WGF). These two taxa meet and hybridize extensively in northwest Florida, where a significant

commercial stone crab fishery is located. Color patterns suggest that the population in the hybrid zone is well-mixed, but allele frequencies at three diagnostic loci show a significant deficit of hybrid forms. Indices created for allele frequencies (genotype index) and color patterns (phenotype index) enable a detailed analysis of the hybrid zone population through simultaneous evaluation of both data sets. Combined index scores show that the population is composed principally of animals more closely resembling M. mercenaria, but with a significant proportion of WGF-like forms and "pure" forms of both taxa. To examine more closely phenotype-genotype relationships, phenotype index scores were grouped into five categories, ranging from one pure form to the other (mercenaria, mercenaria-like, intermediate, WGF-like, and WGF). Genotype patterns within and among these groups show: 1) an overall introgression of the mercenaria genotype into the WGF phenotype; 2) that the intermediate phenotype group is not significantly different in allele frequencies from the two mercenaria phenotype groups; and 3) sex-specific selection against the female WGF phenotype-genotype combination is apparently occurring.

The Gulf of Mexico stone crab fishery is regulated in federal waters by the Gulf of Mexico Fishery Management Council stone crab fishery management plan, and, in Florida waters, by the State. Regulations governing the fishery at both levels recognize only a single stock ranging throughout the Gulf of Mexico. The combined techniques of electrophoresis and color morphology clearly demonstrate that two separate stocks exist, and that these stocks hybridize extensively in an area where the stone crab fishery is of regional economic importance. Other studies have suggested that the two taxa are also physiologically, ecologically, behaviorally, and reproductively differentiated to varying degrees. All of these factors probably create seasonal and areal differences in the genetic composition of the commercial catch within the hybrid zone. Management strategy for the stone crab population in the hybrid zone is a complex issue; a successful strategy will require precise knowledge of spatial and temporal variation in the proportions of pure forms and hybrids comprising the commercial harvest.

ANALYSIS OF GENETIC VARIANCE OF SEA TURTLES COMMON IN THE SOUTHEAST U.S.  
AND THE CARIBBEAN, USING PAG-IEF COUPLED WITH SPECIFIC ENZYME STAINING

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A pilot study to analyze the degree of genetic variance in each of five species of sea turtles was conducted in order to determine if full scale sampling and analyses should be performed. Loggerhead (Caretta caretta) turtles were sampled from North Carolina and Florida; green

(Chelonia mydas) turtles, from Florida, the Caribbean and Hawaiian Islands; olive ridley (Lepidochelys olivacea) and hawksbill (Eretmochelys imbricata) turtles, from Florida and the Caribbean Islands; and Kemp's ridley (Lepidochelys kemp) turtles from Mexico. Blood samples were collected from the five species and were analyzed for genetic variance utilizing twelve specific enzyme stains following polyacrylamide gel isoelectric focusing. Frequency of alleles and number of polymorphic loci were tabulated for four enzyme systems: lactate dehydrogenase, acid phosphatase, peptidase (alanine-leucine), and an esterase combination; all other enzyme stains were monomorphic. Only two species, Caretta caretta and Chelonia mydas, showed significant polymorphism. Insufficient data were obtained on the remaining species.

#### BLUEBACK HERRING AND THE STOCK CONCEPT

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A total of 664 blueback herring (Alosa aestivalis) was analyzed for variation at 28 loci using horizontal starch gel electrophoresis. The fish represented 8 samples from 6 locations across 4 rivers in Georgia and South Carolina. The species was polymorphic at 22 of the loci but averaged only 36% on the loci polymorphic at the 0.01 level within each sample. The average heterozygosity per population was 5%, which is slightly less than the average value expected for fish. Significant differences in allele frequency among locations were detected for 9 loci. In addition, differences in allele frequency were also detected between locations in the same drainage. Year-to-year variation in allele frequency was also noted for 3 loci at one location. Considerable interpopulational differentiation exists in this species and different genetic populations may be collected at the same location in successive years. A land-locked population along the Savannah River in the Par Pond reservoir was the most different genetically from all of the other samples. The other samples contained migratory forms and had lower interpopulational genetic differences than comparisons involving the Par Pond population. Significant temporal and spatial heterogeneity in gene frequency was observed at every hierarchical level within the statistical analysis. Identification of major stocks in this species must await additional data because of the high degree of spatial and temporal heterogeneity observed in this study. Future studies need to examine fish over a broader geographical range to test for existence of major stocks.

OPTICAL PATTERN RECOGNITION  
FOR STOCK IDENTIFICATION: PAST, PRESENT, AND FUTURE

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Growth patterns exhibited on salmon scales are known to differ among stocks. These differences are influenced by genetic and environmental factors. The complex mechanisms affecting scale growth are not well understood; however, the extent of these differences is often sufficient to provide stock composition estimates for fisheries research and management. The earliest studies relied on manual measurement to obtain circulus spacing data for discrimination. This process is tedious, time consuming, and expensive. As a result the number of studies was restricted. The use of digitizing tablets partially relieved these constraints and as a result there are many different stock identification studies in progress. The availability of image processing systems has further relieved these logistic constraints and has provided a quantum leap in the amount of information available for analysis. To examine the potential of this new technology a successive approximation converter (low end video digitizer) was used to examine the circuli spacing patterns on 14 races of sockeye salmon from the Fraser River. Statistical methods were adapted to utilize these data for real-time interactive discriminant analysis and mixing proportion estimation.

A microcomputer-based system with a television camera interface was utilized to make luminance measurements in a 256 by 256 array over the surface of the scale. From this array a subsample of 200 luminance values along the longest axis of the scale for a distance of 1.0 mm was extracted to identify and measure circuli. This distance insured inclusion of the lacustrine growth zone and a variable portion of early marine growth. By filtering this time series of luminance measurements, the circuli could be identified as local minima below the mean of the series. Additional variables were constructed from these data to identify regions where circuli were closely spaced, widely spaced, or changing rapidly in spacing. Fish length was recorded and incorporated into the variable set for each specimen.

Interactive discriminant analyses were conducted to examine the systems potential for in-season management. Generally the system was sufficiently accurate to make determinations for about half of the stocks within those management scenarios examined. Classification accuracies ranged from 100 percent to 35 percent for the samples from 1981. For other years on the Fraser River, the differences are much more apparent and the system should be much more effective. Regardless, additional measurement variables are needed to improve classification accuracy before the system can be used for comprehensive stock identification.

For in-season use on the Fraser River, variables are needed that exhibit an effect of race much greater than an effect of year (within cycles).

To accomplish this a more extensive search of the video image for variables is warranted. An imaging system with a higher resolution should be used so that a larger portion of the scale may be perused. The system is currently limited by the stock specific differences that have been discovered in the scales. Only a small portion of the scale has been examined for this study. It is likely that additional information from the video image could be well utilized for stock identification purposes.

The amount of data contained in a high resolution video image is overwhelming. Problem knowledge is required to make sense out of this huge volume of information. Undoubtedly, there are many stock specific differences that may be extracted from video images. For applications where the differences have been identified, the use of video is straightforward; those features need only be quantified. For other situations the human element is very important in directing the feature extraction process. Based upon his knowledge of the problem to be studied, the investigator must conceive of the possibilities and explore them.

STOCK ANALYSIS OF BLUEBACK HERRING (*Alosa aestivalis*)  
FROM SANTEE AND COOPER RIVERS, SOUTH CAROLINA

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Adult blueback herring collected from two adjacent rivers were examined for meristic and electrophoretic variations. Herring were sampled over four separate periods during the 1985 spawning season. Univariate and multivariate analysis found significant differences ( $P < 0.05$ ) in meristic characters of populations between and within rivers. Differences were more apparent in females than male herring. Adjusted Mahalanobis distances indicate a high degree of overlap in anatomical characters. Horizontal starch-gel electrophoresis revealed that 19 of 35 isozyme systems were polymorphic. Allelic frequency analysis also showed significant variations ( $P < 0.05$ ) between and within rivers. Genetic variability measures are being calculated to evaluate further the electromorphic data. Impacts of the redirection of 80% of Cooper River's

water to the Santee River will be discussed in relation to these findings and future management implications.

RESTRICTION ENDONUCLEASE CLEAVAGE PATTERNS IN MITOCHONDRIAL DNA:  
WHAT DO THEY SAY ABOUT RELATIONS BETWEEN AND AMONG POPULATIONS OF  
SPINNER DOLPHINS?

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The National Marine Fisheries Service regulates the number of spinner dolphins (Stenella longirostris) which can be killed by U.S. vessels incidentally to purse seining operations for yellowfin tuna in the eastern tropical Pacific ocean. Mortalities are enumerated for four populations--Costa Rican, eastern, northern whitebelly, and southern whitebelly--so designated based on morphology and distribution. However, the relationships between these populations remain puzzling (direction and extent of interchange) and analysis of genetic material was undertaken to provide additional information.

Since previous work has shown a very low degree of even interspecific genetic variability, analysis of the rapidly evolving mtDNA molecule was selected, rather than protein electrophoresis, as a promising tool to assist in clarifying these relationships. Restriction endonuclease fragment patterns were analyzed using total liver cell DNA isolated from over 100 samples obtained from eastern and whitebelly animals killed during purse seine operations. Fragment patterns (morphs) were observed with 7 enzymes (Ava I, Ava II, Bam HI, Hae III, Hinc II, Hinf I and Hpa II) using Southern transfer and hybridization with <sup>32</sup>P-labeled mtDNA cloned from Commerson's dolphin (Cephalorhynchus commersonii). Each individual was assigned a composite pattern (mtDNA type) based on the 7 morphs.

These mtDNA types were compared with modal school population type (population designation for quota purposes), morphology of the individual animal, geographic origin, and herd membership.

GROWTH INVARIANT DISCRIMINATION AND CLASSIFICATION OF STRIPED BASS  
(*Morone saxatilis*) STOCKS IN NEW YORK, CONNECTICUT, AND RHODE ISLAND  
COASTAL WATERS BY MORPHOMETRIC AND ELECTROPHORETIC METHODS

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Four morphometric variables and six eye lens protein abundance variables measured from striped bass from the Hudson River, Chesapeake Bay, and Roanoke River were studied for consistent differences among stocks. Differences in the size frequency distributions of the spawning stock samples precluded discrimination of stocks without adjustments for these sampling differences. A growth invariant discriminant function analysis was utilized since it is capable of eliminating size effects from the discrimination procedure. Growth invariant discrimination of the three stocks indicated that the Hudson River stock is clearly distinguishable from the Chesapeake Bay and Roanoke River stocks. Misclassification rates for the Chesapeake Bay and Roanoke River stocks were high (22.2 to 28.0 %, respectively). An attempt to discriminate better between groups was made by pooling the two most similar stocks. The growth invariant discriminant function calculated for the Hudson River stock and pooled Chesapeake Bay - Roanoke River group correctly classified 92.7% and 90.6% of the samples, respectively. The high fidelity of the growth invariant function permitted reliable classification of striped bass taken from New York, Connecticut, Rhode Island and Massachusetts coastal waters.

Results of the classification of the coastal samples gave estimates of the relative composition of the exploitable coastal populations for the spring and fall seasons. In the spring of 1984 a significantly greater overall proportion of Hudson River striped bass was apparent in the Connecticut sample (67.6%); Rhode Island's striped bass population had the lowest overall contribution from the Hudson River stock (22.9%). However, the contributions of the three stocks changed during the course of a sampling season. This is evident from the Rhode Island spring 1984 data; in the early spring (before May 7) 9.1% of the migratory population classified as Hudson River striped bass. In the late spring (after May 22) 41.5% of the population classified as Hudson River striped bass. Size related differences occur in conjunction with changes in the relative composition of this sample as well. The bass which migrate along the Rhode Island coast in the early spring are mostly smaller fish; the larger individuals, hence older year classes, appear later in the season. The proportion of Hudson River bass in the fall of 1984 within the coastal waters of New York, Connecticut, and Rhode Island was consistently low, 7.3% to 11.0%. The composition of the migratory population in the fall was significantly different from that in the spring for each coastal area. Mixed stock fisheries in New York, Connecticut, and Rhode Island are subject to stock specific changes in

the contribution of year classes. Furthermore, the migratory movements of year classes appear to be time dependent. Estimates of the composition of the coastal populations are sample dependent. The size composition of the sample and the segment of the migratory population sampled (early, middle, or late migrating bass) will contribute to observed changes in the overall relative composition of the population.

DELINEATION OF KING MACKEREL (Scomberomorus cavalla) STOCKS ALONG  
THE U.S. EAST COAST AND IN THE GULF OF MEXICO

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King mackerel are widely distributed along the U.S. south Atlantic coast and in the Gulf of Mexico where they support both recreational and mixed-gear commercial fisheries. Because catches are landed within the boundaries of eight states, two regional fishery management jurisdictions and Mexico, management of the fisheries is a problem of both regional and international concern. Regional Fishery Management Councils (FMC) currently recognize two migratory groups for management purposes, one along the southeast U.S. coast and one in the Gulf of Mexico, with overlapping ranges in southeast Florida. To manage these fisheries most effectively it is important to know the identity of any component stocks, and how fishing mortality is distributed among them.

Biochemical (starch-gel electrophoresis) and mark-recapture techniques are being used to evaluate the stock structure of king mackerel. Preliminary results suggest that at least two breeding groups may exist; a western Gulf of Mexico group, and a second group in the eastern Gulf of Mexico and along the Atlantic coast.

Biochemical genetic data provide the strongest evidence. Analysis of allelic frequencies of peptidase using glycl-L-leucine as substrate in king mackerel from ten locations from the Gulf of Mexico and Atlantic coast indicates that fish sampled in Texas and Campeche, Mexico are significantly different from other sampling locations. These data do not confirm or refute the existence of a separate Atlantic migratory group. May's (1983) results for an additional seven sampling locations in the same geographic area are concordant.

Historical mark-recapture studies in the Gulf of Mexico have shown movement from south Florida in winter to the northwestern and western Gulf in summer, as well as the reverse. However, our mark-recapture data on large king mackerel tagged in winter off Louisiana suggest that these fish may comprise a different group from fish that have migrated between the western and northwestern Gulf and south Florida. Of 1,513 fish tagged and released off Grand Isle, LA, in the winters of 1983, 1984, and

1985, 31 have been recaptured, all in the western Gulf of Mexico (Louisiana, Texas, and Mexico). Thus, if two groups exist, as our results suggest, mixing of the two groups may be occurring in the western and northwestern Gulf of Mexico in summer.

Biochemical (starch-gel and isoelectric focusing electrophoresis), mark-recapture and morphological studies designed to define king mackerel stocks more precisely are continuing at the National Marine Fisheries Service Laboratory in Panama City, Florida.

## DIFFERENTIATION OF MITOCHONDRIAL DNA IN ATLANTIC HERRING

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The relationships among spawning stocks of Atlantic herring (Clupea harengus harengus) are problematical. Recent studies have advanced the hypothesis that specific environmental attributes essential for growth and survival of larval herring largely determine where herring will spawn. Further, timing of spawning has been hypothesized to be a function of the time necessary for completion of larval growth and metamorphosis constrained by resources within the larval retention area. Implicit in this model is the idea that individual herring belong to defined groups which home to specific spawning sites, i.e., stocks. If this is the case, there should exist high genetic continuity (and identity) among individuals within stocks and relatively lower continuity among stocks. Allozyme investigations have not supported this prediction; despite the availability of a large number of polymorphic markers, genetic heterogeneity among groups is not evident. To examine further genetic relationships among herring stocks, we examined restriction endonuclease cleavage patterns of mitochondrial DNA among three spawning groups in the northwestern Atlantic.

Three discrete samples of spawning Atlantic herring were obtained: fall spawners (Jeffries Ledge, Massachusetts, southern Gulf of Maine; Trinity Ledge, New Brunswick, northern Gulf of Maine) and spring spawners (Gulf of St. Lawrence, New Brunswick). For comparative purposes, spring spawning Pacific herring (Clupea harengus pallasii) were obtained from Puget Sound. mtDNA was extracted from eggs and prepared by rapid phenol extraction and occasionally CsCl ultracentrifugation. Samples were digested with 16 six-base restriction endonucleases and restriction fragments visualized with ethidium bromide after agarose electrophoresis.

Based on cleavage patterns for 14 restriction enzymes, the size of mtDNA was estimated to be 17097 + 128bp in Atlantic herring and 16754 + 173bp in Pacific herring; intraspecific size difference was non-significant. The 294 restriction fragments observed encompassed

approximately 1.7% of the mtDNA genome. Variant cleavage patterns were noted for over half of the enzymes examined and were common both within and among population samples of Atlantic herring. Based on composite cleavage phenotypes for seven enzymes, eleven unique mtDNA restriction clones were observed in 20 completely characterized specimens of Atlantic herring. Two clones were shared by all three geographic samples and one additional clone occurred in the fall northern Gulf of Maine sample and the spring Gulf of St. Lawrence sample; all other clones were locality specific.

The occurrence of unique clones within population samples suggests that there may be some degree of genetic discontinuity among spawning stocks of Atlantic herring. Based on the patterns of cleavage site loss, the origin of these unique clones appears consistent with the isolation of discrete spawning stocks. However, the presence of two clones common to all three localities makes the situation ambiguous. The common occurrence of these clones can be interpreted in at least three ways. First, there may be genetic exchange among populations. Stocks are not completely isolated from each other. Second, individual herring possessing the common clones belong to a single genetic stock which periodically co-occurs with other stocks. While such fish are present at other spawning areas, there is no gene exchange. Third, the common clones are not of recent origin and stocks are reproductively isolated despite phenetic similarity. Limited support for this idea comes from the comparison of Atlantic and Pacific herring. Based on cleavage patterns for 14 enzymes, nucleotide sequence divergence between subspecies was estimated to be  $p = 0.026$ . This level of divergence is substantially less than that predicted on the basis of isozyme differentiation. Thus, the possibility exists that heterogeneous rates of mtDNA divergence may be partially responsible for apparent clonal homogeneity. Increased sample sizes and more rigorous analysis of clonal relationships are obviously necessary to test hypotheses about genetic differentiation and incipient reproductive isolation in Atlantic herring.

#### A NEW MULTIVARIATE APPROACH TO DESCRIBE PACIFIC HERRING STOCKS FROM SIZE AT AGE AND AGE STRUCTURE INFORMATION

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The current assessment and management regime for British Columbia Pacific herring (*Clupea harengus pallasii*) stocks is based largely on information gained from meristic analyses and a coastwide tagging program conducted during the 1930s and 1940s. Although the nature of the fishery has changed from a reduction to a roe fishery beginning in the early 1970s, there has been limited progress in terms of more clearly describing and understanding the unit stocks that should be managed

separately. Recent attempts to utilize morphometric and meristic data, electrophoresis, and mitochondrial DNA analyses to separate stocks have had limited success.

A striking aspect of the earlier attempts to identify stocks in the northeast Atlantic was the fact that ecological factors such as the growth rates, age structure, and times and areas of spawning seemed to provide the best information for stock separation. However, this information had always been analyzed univariately which was not only tedious but also made it difficult to interpret the simultaneous effects of several variables suggesting the need for a multivariate approach.

Consequently, we examined the information available for Pacific herring from spawning ground samples with a variety of multivariate procedures. Pacific herring usually recruit to the fishery at ages 2 and 3 and leave at ages 7 or 8 although most of the catch is ages 3-5. We chose 1979-80 which appeared to represent the most complete coastwide sampling information and from this selected samples which had data on size at age for ages 3-6. We then calculated the mean length and weight at age and the proportion of fish at each age in the samples. Using these twelve variables we calculated the principal components and scores for the samples in each of the five major management areas suspected of containing more than one distinct spawning stock. Plots of the first three principal component axes were examined for possible grouping of samples suggesting unique populations of herring. Discriminant and cluster analyses were then used to attempt to quantify observed sample groupings.

The Queen Charlotte Islands separate clearly into east and west coast stocks which are not separable within themselves. The three spawning areas on the north coast cannot be separated and so suggest a single homogeneous population. The central coast is unclear with the small inlet populations separating out, but the coastal spawners appear to represent two, or possibly one or three spawning populations. The west coast of Vancouver Island is also problematical. The two southern spawning groups are inseparable, but clearly separate from the northern spawning groups which have not been well sampled. The limited data for the three northerly statistical areas suggests either one homogeneous stock, or two composed of the two northernmost spawning areas and a group intermediate between the northern and southern stocklets. Possibly the most confusing area is the Strait of Georgia where fish migrate in through Juan de Fuca and Johnstone Straits to spawn in several distinct localities. There is no clear separation of any groups in this data set which may be a function of sampling the fish on the migration routes before they have reached the spawning areas thereby providing biased learning sets.

The results obtained with this analysis generally follow the pattern of stock groupings identified from the historical tagging information and meristic data which indicates that the analytical approach suggested may be useful for other species as well. All the presently utilized stock

identification techniques for Pacific herring have drawbacks, but it is encouraging that they are at least all leading in the same direction. A major advantage of this technique is that it relies on data collected routinely by most sampling programs for all marine and many freshwater species. It also uses parameters that reflect aspects of the ecological niche of the population, i.e., the growth rate and the reproductive success reflected in the age composition.

#### POPULATION (STOCK) DETERMINATION FOR ATLANTIC CROAKER

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Differences in Atlantic croaker (Micropogonias undulatus) life history and population dynamics characteristics between areas north and south of Cape Hatteras caused us to hypothesize that at least two stocks with overlapping ranges occurred between North Carolina and Virginia. Several methods were employed to determine the validity of this hypothesis and to assess whether differences were genetically or environmentally controlled. During 1982-1983 we collected samples of croaker hemoglobin, liver, muscle, heart, and eye and analyzed them by starch and acrylamide gel electrophoresis. Because spawning adult croaker are very difficult to obtain, we concentrated sampling on postlarvae from two distinct recruitment periods, fall and spring, which we suspected may represent two breeding pools.

Most samples were from North Carolina where the two recruitment groups overlap, but some juvenile fish from Chesapeake Bay and Delaware Bay exhibiting only fall recruitment were analyzed. Ten of 24 screened loci yielded potentially useful genetic variation. They represented four enzyme/protein systems, phosphoglucose isomerase, hemoglobin, transferrin, and parvalbumin. The six hemoglobin phenotypes provided the most useful information with frequencies exhibiting considerable heterogeneity among locations. Although these data were consistent with a two stock hypothesis, the analyses were complicated by ontogenetic pattern changes and small sample sizes from the well-defined breeding pools. Without additional data, especially from spawning adults, our overall conclusions were that genetic heterogeneity in the tested systems was insufficient to support genetic separation of croaker breeding pools.

To complement the biochemical study, a tagging project was carried out in North Carolina waters during 1982-1985. During fall of 1982-1984 nearly 80,000 one year old croaker were tagged, mostly in the Southern Pamlico Sound area. These fish probably had lived in North Carolina

since postlarval recruitment. An additional 18,000 fish of all available ages were tagged in April-May 1985 in northern Pamlico Sound. Although data from this project have so far revealed some interesting movements, they have not suggested different populations in North Carolina.

The strongest evidence for population differences among croaker by geographic area comes from the analysis of stock assessment surveys and age and growth studies. Aging of 2,369 croaker suggested differences in fish from offshore waters and north of Cape Hatteras compared to those south of Cape Hatteras and from inshore, estuarine waters. The former appeared to be larger at age, have greater longevity and lower mortality than the latter. Estuarine trawl survey data from North Carolina (1979-1984, about 180 stations) compared to surveys from other states also revealed differences in population structure centering around middle North Carolina. North of Bluff Shoal, Pamlico Sound, North Carolina, there is strong young-of-the-year fall croaker recruitment that is characteristic of Chesapeake and Delaware Bays. South of Bluff Shoal, young-of-the-year recruitment is strongest during spring. Surveys of inshore commercial fisheries revealed significant differences in croaker landings and size/age of the fish north and south of Bluff Shoal.

Collectively these data indicate that croaker may have northern and southern stocks roughly separated by the area between Cape Lookout and Cape Hatteras. There is no strong evidence for genetic differentiation between these groups, and it seems most likely that they are controlled by the different zoogeographic (environmental) characteristics north and south of Cape Hatteras. Different population dynamics of the two groups may necessitate different management strategies.

SUCCESS OF STOCK IDENTIFICATION TECHNIQUES USED IN THE  
U.S./CANADA SALMON INTERCEPTION RESEARCH PROGRAM IN ALASKA

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In March, 1985, the United States and Canada signed a treaty that provides a framework for management of Pacific salmon in certain coastal areas. The basis of the treaty is equity. Each country receives the benefits of the salmon it produces, regardless of where they are caught. And, portions of the returns of all five species of Pacific salmon, as they approach the coast on their spawning migration, pass through waters of the other country and are intercepted in various commercial net and troll fisheries.

Because the country of origin determines "ownership" of the salmon under the terms of the treaty, regardless of where they were captured, it becomes necessary to be able to identify major stocks or races of salmon.

Anticipating the signing of the treaty, a large tagging program was initiated in 1982 in domestic fisheries in the boundary area between Alaska and British Columbia by both countries. In addition to tagging, three other methods of stock identification were evaluated: 1) scale pattern analysis, 2) a genetic method electrophoresis, and 3) parasite analysis. The scale studies were contracted to the Stock Biology Group in Commercial Fisheries Division of the Alaska Department of Fish and Game. The electrophoresis analyses were joint studies between the National Marine Fisheries Service's Northwest and Alaska Fisheries Center in Seattle, Washington, and the Auke Bay Fisheries Laboratory in Alaska. Identification of parasites was done at the Pacific Biological Station, Nanaimo, British Columbia.

The application of digitizing and computer technology to the counting, measuring and analysis of circuli on fish scales has made possible the processing of large numbers of scales in a short period of time. Distinctive scale patterns that separate stocks can be compared. Each year a baseline sample of scales from the common age group of sockeye salmon (Oncorhynchus nerka) are digitized from each of about 30 lake systems in Alaska, the major Canadian Rivers, plus the transboundary rivers - Stikine, Taku and Alsek Rivers. This baseline sample is compared to mixed-stock samples of unknown origin from the major fisheries to estimate interception rates. Growth differences between sockeye salmon from the large Canadian Rivers and the smaller streams in southern southeast Alaska are quite distinct on the scales. Estimates of interception rates made by the scale method in most cases are very similar to estimates of interception rates based on tagging data.

The sampling procedures for studies on stock separation of sockeye salmon by the genetic method (electrophoresis) are similar to the procedures used for scale studies. A baseline of data is established by sampling fish from each of about 45 sockeye salmon systems in Alaska and British Columbia. This baseline of data is then used to estimate stock composition of samples from mixed-stock fisheries. Ninety loci were screened for genetic variation. Ultimately, only 5 loci in sockeye salmon in southern southeast Alaska proved to be consistently useful for mixed-stock analysis. Last year, we initiated a genetic study of chum salmon (O. keta) in addition to sockeye salmon. Chum tend to be more variable genetically than sockeye salmon so the genetic method should be even more successful in estimating stock composition of unknown mixtures of chum salmon.

A Canadian parasitologist, Dr. Leo Margolis, found several unique "parasite-tags" useful in separating stocks of sockeye salmon. One of the most distinctive parasites is a myxozoan protozoa - Myxobolus neurobius - that lives in the brain. It is acquired during the fresh-water residency and lives in the brain area during the total life span of the fish. In southern southeast Alaska, the incidence of M. neurobius in sockeye salmon is very high - usually greater than 90 percent. In northern British Columbia, the parasite is mostly absent from the major populations of sockeye salmon.

For sockeye salmon, each of the three stock identification methods works in many situations, however, a statistical analysis that combines scale data, genetic data and parasite data appears capable of giving the most accurate interception estimates overall. For the other species of Pacific salmon, it is too early to say which is the best technique for stock separation. However, we do know that the scale method also works on chinook salmon (O. tshawytscha) and the genetic method also works in other areas on chinook, chum, and pink salmon (O. gorbuscha).

### STOCK IDENTIFICATION BY OTOLITH COMPOSITION

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Fish otoliths consist of calcium carbonate crystals of aragonite laid down in a protein matrix of otolin. Their crystalline lattice structure can include other elements during increment formation. Otoliths do not undergo the continual remodeling that mammalian bone does, therefore, once a layer of bone is deposited any trace elements are held in a matrix creating a permanent record of the fishes' food and surrounding water.

Otoliths of 1983 and 1984 young-of-the-year white perch (Morone americana) from the Potomac, Patuxent, Elk, Bohemia, Nanticoke and Choptank Rivers of Chesapeake Bay were examined using a scanning electron microscope equipped with an energy dispersive x-ray analyzer. Preparation for x-ray analysis required otoliths be embedded in a contaminant-free resin, ground sagittally until the nucleus was reached, and polished with 0.25 microns grit diamond paste. To expose daily rings otoliths were etched in 25% acetic acid. A microprocessor was used to identify elements and to integrate the peak areas. All data were normalized to a standard calcium peak before statistical evaluation by stepwise discriminant function analysis. X-ray microanalysis has several advantages over more conventional techniques of element analysis in that it can simultaneously analyze for all elements with an atomic number >11 (Na) and <92 (U), it is non-destructive and can analyze a wide range of elemental concentrations. Elements most often included in the otoliths were Si, Al, S, Na, Cl, Mn, V, Cr, Fe, Cu, Ni, Zn, Hg and the lanthanide series elements. Discriminant analysis of element inclusion within daily rings 3-5 detected significant differences in otoliths among riverine stocks. For 1983 the best discrimination was detected in the Elk and Bohemia Rivers, 61.1% correctly classified, while the poorest discrimination was found in the Potomac River, 38.9% correctly classified. A broader geographical grouping of rivers led to better discrimination. Data from the Choptank and Nanticoke were combined to form the eastern shore, Potomac and Patuxent (western shore), and Elk and

Bohemia (upper bay). These groupings markedly improved discrimination with a high occurring in the upper bay, 86.1% correctly classified, to a low occurring on the eastern shore, 44.4% misidentified. Prior to analyzing 1984 otoliths a comparison of daily ring groups 3-5, 8-10, and 3-5 and 8-10 combined showed the first grouping to yield the best discrimination. Consequently, ring group 3-5 was also used for 1984 analyses. Results from 1984 show better riverine discrimination than 1983 with the best discrimination occurring in the Bohemia River, 77.8% correctly classified, while the poorest occurred in the Nanticoke River, 44.4% correctly classified. The same grouped rivers classification used for 1983 data resulted in a high of 69.4% correctly identified in the upper bay and a low of 61.1% correctly classified on the western shore. Although this method shows limited discriminatory ability in some cases, especially in limited geographical areas, it is a simple, quick, relatively inexpensive procedure that should prove useful in stock identification. Future applications may include an evaluation of stocking programs by elemental tagging, homing accuracy of anadromous stocks, and identification of sea run stocks of many species.

STOCK STRUCTURE OF ATLANTIC SALMON:  
ITS CURRENT STATUS AND FUTURE PERSPECTIVES

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This study focuses on three aspects of stock structure in Atlantic salmon (*Salmo salar*). First, we investigated the pattern of geographic variation in 12 morphometric, 10 meristic and one electrophoretic character (malate dehydrogenase). Secondly, we examined two factors which may bias the conclusions of a stock investigation study: the number of groups included in the analysis and the difficulty of partitioning size from morphometric data. Finally, we discuss areas of future research which are applicable to stock discrimination of Atlantic salmon.

Collections of juveniles from 27 populations were taken within the native range of Atlantic salmon. The overall slope was used to adjust the morphometric characters to the overall mean standard length. The homogeneity of within-group slopes was determined using analysis of covariance (ANCOVA). Discriminant analysis of the meristic and adjusted morphometric characters was used to test for differences among stocks.

Extensive genetic and somatic divergence between European and North American populations was found in all three character sets. Morphometric and electrophoretic characters identified a dichotomy between insular

Newfoundland and continental North American groups. Within the North American Atlantic region, a polarity between anadromous and landlocked populations was evident from meristic and electrophoretic characters. Vertebrae, dorsal, anal and pectoral fin rays, gill rakers, head length, body depth, head width, gape width and malate dehydrogenase (MDH-3,4 [100]) were the most important characters in distinguishing between the regional stocks described above. We rejected the hypotheses that geographic stocks exist among the European populations examined, and that river origin identification of individual specimens is possible.

In the second part of our study, we found that the number of groups included in the analysis altered the conclusions regarding identification of individual river origin. An examination of fewer populations, three from Scotland and four from Newfoundland, would have led to the conclusion that, in addition to regional, each river population may be regarded as a separate stock. As in the complete population set, the Newfoundland and Scotland regional stocks were composed of population samples with overlapping, but significantly different standard lengths. The Newfoundland and Scotland populations with longer standard lengths were more easily distinguished from each other than those composed of smaller specimens.

As a result of the above analysis, it was proposed that an inability to adjust for size was responsible for the high level of identification of individual river origin. An empirical test of five methods (described below) commonly used to adjust for size was devised. Additional specimens were collected from one Canadian to one Scotland stock. These specimens spanned the range in standard length of the 27 populations described above. The Canadian and Scotland geographic stocks were subsequently divided into three types of data sets consisting of non-overlapping, partially overlapping and completely overlapping size groups. Of the examined methods, it is recommended that bivariate regression (based on overall slope) combined with discriminant analysis and principal component analysis based on the total covariance matrix be used in future morphometric analyses. The two above procedures were most consistent in partitioning size, identifying stocks and character selection. The other examined techniques, ANCOVA, principal components based on the within covariance matrix and the shear technique did not partition size from the non-overlapping data set.

The present study indicates that the use of morphometric, meristic and electrophoretic characters will enhance efforts to define and manage regional stocks of Atlantic salmon. However, as well as addressing the statistical problems defined above, additional regions within the native range must be sampled to determine if the development of a simple set of classification functions is possible. This should include a more intensive electrophoretic approach than was possible in this study. Investigating other methods, such as scale meristic, morphometric and micro-nutrient characteristics is also necessary to determine the most practical means of identifying salmon stocks. Annual variation in the

selected characters requires further clarification, so that identification may be based on characters with the most stability.

A PROTOCOL FOR CLONING FISH MITOCHONDRIAL DNA AND EVALUATING  
ITS RESTRICTION POLYMORPHISMS FROM FROZEN TISSUES

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The identification and assessment of fisheries stocks depend on the determination of the genetic relationships between the populations under consideration. In recent years, characterization of mitochondrial DNA (mtDNA) polymorphisms has provided a precise tool for the determination of genetic variation at the base sequence level. This allows an accurate characterization of individual genomes, and thus of population structure and differentiation. However, application of the technique is limited by technical requirements, such as purification of mtDNA from fresh tissue by ultracentrifugation on cesium chloride gradients. Our protocol presents a method for the isolation, cloning, and characterization of mtDNA that does not depend on ultracentrifugation, nor on the availability of fresh tissues. Specimens of the common snook (Centropomus undecimalis Bloch) were collected from Tampa Bay, Naples Bay, and the Indian River estuary, Florida. Reagents were obtained from several vendors; restriction and modification enzymes were procured either from Bethesda Research Laboratories (BRL), Bethesda, Maryland, or from Boehringer Mannheim, Indianapolis, Indiana. Other reagents were obtained from Sigma, St. Louis, Missouri. Fish livers, brains, hearts, kidneys and skeletal muscle samples were frozen at  $-70^{\circ}$  C for a period of fourteen days. One to two grams of tissue from each specimen and from each organ sample were minced on ice by cutting into very small pieces with an electric knife or a razor blade. The minced tissue was then homogenized in a 50 ml Dounce homogenizer with a loose pestle, in about 15 ml of chromatid isolation buffer (CIB) made up of: 0.1 M NaCl, 10 mM EDTA, 10 mM mercaptoethanol, 5.0 ml Triton X-100/L and 30 mM Tris HCl, at pH 8.0. This step ruptured the cells, leaving a suspension from which the nuclei and aggregated chromatin were removed by centrifugation at  $5000 \times g$  for 10 minutes at  $4^{\circ}$  C. The centrifugation was repeated until the supernatant was clear and no pellet was formed, thus obtaining a suspension enriched in mtDNA and mitochondria. The remaining mitochondrial membranes were lysed with 1.0% SDS for 5 minutes at  $37^{\circ}$  C. This step is especially important if fresh tissue is used, but should not be omitted even with frozen tissue preparations, in order to maximize the

extraction of mtDNA. The lysate was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, ratio) to remove proteins that may interfere with subsequent restrictions. A final extraction with 24 volumes of chloroform : 1 volume of isoamyl alcohol was performed to remove traces of phenol. The aqueous (upper) phase was precipitated with 2.5 volumes of 95% ethanol or one volume of isopropanol for 2 hours at  $-20^{\circ}$  C. The DNA was pelleted by centrifugation at  $12,000 \times g$  for 30 minutes at  $4^{\circ}$  C. The pellet was rinsed with 70% ethanol, dried in vacuo and resuspended in about 30 microliters of TE buffer: 10 mM Tris-HCl, 1mM EDTA, pH 8.0. Samples were stored at  $-20^{\circ}$ C or over chloroform at  $4^{\circ}$  C.

The DNA was digested with restriction endonucleases, and the DNA fragments were separated by electrophoresis on 1% agarose gels (Maniatis, J., E.F. Fritsch, and J. Sanbrook. 1982. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, 545 pp.). The electrophoresis buffer used was TAE: 0.4 M Tris-HCl, 0.2 M glacial acetic acid, 10 mM EDTA disodium salt, pH 8.0. The DNA was then transferred to nitrocellulose filters (Southern, E.M. 1979. Gel electrophoresis of restriction fragments. In R. Wu (ed.), Methods in enzymology, Vol 68, Recombinant DNA. Academic Press, New York, pp. 152-182.)

The mitochondrial fragments generated by endonuclease restrictions were identified by hybridization to radiolabelled heterologous mtDNA probes. Our probes consisted of cloned plasmid DNA which contained the vector pBR322 and the complete bovine mtDNA genome (kindly supplied by Drs. Hauswirth and Laipis, University of Florida, Gainesville, Florida). The probe DNA was labelled with  $^{32}$ PdCTP by nick translation, and hybridization conditions were a modification of Maniatis et al., (1982). Filters were prehybridized for one hour at  $65^{\circ}$  C and then hybridized overnight at the same temperature with  $1 \times 10^6$  cpm/ml of radiolabelled probe DNA. Autoradiographs were obtained by exposing the filters to Kodak X-Omat AR X-ray film.

For cloning purposes, DNA enriched for mtDNA was isolated from snook brain tissue according to the above protocol. The DNA was restricted with Hind III and the fragments thus generated were separated by electrophoresis on a 1% agarose gel in TAE buffer. The mitochondrial bands were cut from the gel, and purified by freeze extraction via repeated freezing and thawing followed by centrifugation through a siliconized fiberglass wool filter. The fragments were combined with DNA from the bacterial plasmid pT7-2 (obtained from Gene Scribe<sup>TM</sup> U.S. Biochemical Corp. Cleveland, Ohio) which was also restricted with Hind III and treated with calf intestinal alkaline phosphatase. T4 DNA ligase was added to generate chimeric molecules containing the plasmid and one snook mitochondrial fragment. These molecules were used to transform Escherichia coli cells rendered competent by calcium chloride treatment (Maniatis et al., 1982). Transformed cells were selected by ampicillin resistance, which indicates plasmid uptake. We confirmed that these plasmids contained mtDNA by preparing plasmid DNA from the transformed

cells by alkaline lysis. We then restricted the plasmid DNA with Hind III and hybridized it to radiolabelled bovine mtDNA.

The DNA extracted by our protocol is enriched in mtDNA and is pure enough for direct quantification and restriction. Contamination by nuclear DNA is inevitable, since the nuclear membranes break during freezing, and some membrane breakage occurs during homogenization of fresh tissue. However, the DNA extract should be enriched for mtDNA by a factor of up to 100. Our protocol allowed the identification of three mtDNA fragments generated by Hind III restrictions of total DNA extracts: 3.67, 5.87, and 7.28 kbp. This suggests that the snook mtDNA genome is about 16.82 kbp.

The total DNA yields were consistent between tissue samples. We obtained a total DNA yield of about 0.9 micrograms DNA/g of tissue. However, the mtDNA fraction varied as a function of the tissue used. The estimated mtDNA components were highest from brain DNA extracts, lower from heart and skeletal muscle, and lowest from liver and kidney. The brain tissue yielded mtDNA of sufficient purity and quantity to allow visualization of restriction bands on 1% agarose gels stained with 2.5 micrograms of ethidium bromide/ml. Our cloning experiment resulted in the insertion, into the Hind III site of the polylinker sequence of plasmid pT7-2, of three DNA fragments. These fragments had molecular weights corresponding to those of the snook mtDNA fragments generated by restriction with Hind III. The identity of these clones was confirmed by transferring the cloned fragments from the agarose gel to nitrocellulose filters, and hybridizing the filters to radiolabelled bovine mtDNA probes. Thus, we substantiated the identity of two independent clones of the 3.67 kbp fragment and three clones of the 7.28 kbp fragment. Verification of the cloning of the 5.87 kbp mtDNA fragment is currently under way.

Final confirmation of the identity of our clones, and of their applicability as molecular probes required the demonstration that they hybridize to the same bands identified by bovine mtDNA probes. Conducting such an experiment also gives an indication of the relative sensitivity of homologous and heterologous probes. Therefore, we digested snook mtDNA with Hind III, Pst I, Bam HI, Pvu II, and Eco RI. The fragments were separated by electrophoresis and identically transferred to two nitrocellulose filters by a southern sandwich blot transfer (Maniatis et al., 1982). The DNA on one filter was hybridized to a radiolabelled probe consisting of the complete bovine mitochondrial genome. The DNA on the other filter was hybridized only to the radiolabelled 3.67 kilobase subunit of the snook mitochondrial genome.

Several points are worthy of note. First, our cloned probe does hybridize to the 3.67 kbp Hind III fragment of snook mtDNA. Second, the signal intensity of that hybridization is considerably greater than that generated by the bovine probe. Finally, the use of the homologous probe reveals a Pvu II fragment of about 0.7 kbp that does not hybridize to the bovine probe. These results clearly demonstrate that the use of

homologous probes significantly increases the sensitivity of our techniques. Work in progress is evaluating the restriction fragments identifiable by using each of the cloned snook mtDNA fragments as a molecular probe.

SENSITIVITY ANALYSIS OF THE FMP-LS MAXIMUM-LIKELIHOOD  
STOCK-IDENTIFICATION MODEL USING MULTI-LOCUS GENETIC CHARACTERS

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We performed numerical experiments to evaluate the bias and precision of stock composition estimates from the fmp-ls maximum-likelihood stock-identification model (e.g., Fournier et al. 1984. Can. J. Fish. Aquat. Sci. 41:400-408) using hypothetical multi-locus characters. Bias and precision were examined in relation to the number of stocks being resolved, the number of loci available and the difference in allele frequency among stocks (i.e., stock separation) at each locus, using Monte Carlo simulations with different levels of sampling error in the mixture and learning samples. Bias decreased with increasing stock separation and number of loci available. For a given stock separation and mixture sample size, bias increased with the number of stocks resolved due to reduced contributions from individual stocks, and hence, greater sampling error; bias was not affected by the number of stocks resolved in simulations where mixture contributions remained constant. Learning sample size had little effect on bias for realistic sample sizes (>20), especially where genotype frequencies in the random samples were corrected to conform to Hardy-Weinberg expectations. These results provide guidelines for reducing the complexity of genetic stock identification problems. We suggest that mixing proportions be estimated for all learning samples individually rather than for aggregates of learning samples; this circumvents the problem of weighting stocks a priori and permits correction of learning samples thought to be in Hardy-Weinberg equilibrium. Individual stock estimates can then be summed by grouping stocks with the poorest separation potential. The tradeoff between decreased bias and loss of stock resolution can be examined graphically to determine the most useful degree of grouping for the problem at hand. We illustrate this procedure with a real example from mixed-stock fisheries on sockeye salmon (*Oncorhynchus nerka*) along the British Columbia - Alaska coast.



A RESEARCH ADMINISTRATOR'S PERSPECTIVE  
OF THE STOCK IDENTIFICATION PROBLEM

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Over the past two days we have been exposed to presentations on a wide range of research dealing with stock identification of fishery resources. Now, we move on to the panel discussion.

Before giving the principal speakers an opportunity to comment further on the topic, or to open the session to the audience for questions or comments, I would like to comment briefly on problems I have regarding some of the techniques used in stock identification, namely, that of immunogenetic and electrophoretic methods. I should note that I speak from an outsider's viewpoint since I am not a geneticist, but a biologist-administrator who has responsibility to direct research initiatives or provide comments on projects planned by other agencies.

Workshops such as this are valuable means for scientists to present results of their research and to discuss technical aspects with colleagues. Workshops, however, rarely address the broader question of whether the original objectives of the research are being met or devote much time to entertain negative views of research initiatives. For myself, I began having some reservations on the utility of immunogenetic and electrophoretic techniques in stock identification for fishery management purposes several years ago.

Fishery administrators and managers are interested in determining if a resource subjected to a fishery is a single "unit" stock or is made up of more than one self-sustaining unit (subpopulation). Marr and Sprague (1963) described four general methods used to investigate population structure. These are (1) phenotypic qualities, (2) movement, (3) vital statistics, and (4) genetic characteristics. While all papers presented at this workshop fall into one of these categories, it is interesting to note that there has been an abundance of presentations on genetic research at this workshop. At this time, I would like to provide my observations on immunogenetic and electrophoretic techniques as they relate to stock identification of skipjack tuna (Katsuwonus pelamis) and milkfish (Chanos chanos).

The skipjack tuna is an important commercial tuna species, especially in the Pacific where recent catches have exceeded 500,000 metric tons. The stock structure of skipjack tuna in the Pacific is important since many nations fish this species. The Southwest Fisheries Center, Honolulu Laboratory of the National Marine Fisheries Service carried out extensive immunogenetic and electrophoretic research on skipjack tuna from 1960 to 1973. Other agencies involved in stock identification of the skipjack tuna include the Inter-American Tropical Tuna Commission (IATTC) and the South Pacific Commission (SPC).

The number of population "units" (subpopulations) postulated for the Pacific skipjack tuna resource based on immunogenetic and electrophoretic

techniques has varied from one to seven; there have been suggestions of additional population "units".

Fujino (1970) conducted a comprehensive study of the esterase enzyme by electrophoresis and described a two-population "unit" structure for the western Pacific. Figure 1 shows the demarkation zone separating the two "units" proposed by Fujino (1970). The solid line and the broken line represent the eastern range limit for the northern winter and northern summer periods, respectively. Superimposed on the skipjack tuna population structure are some results of skipjack tuna tag and recovery data based on the SPC skipjack tuna tagging program (Kearney 1983). Figure 1 shows that there is considerable movement of skipjack tuna across the boundary in both directions; thus, the tagging results appear to be in conflict with Fujino's two "unit" structure. Another example of the shortcoming of the electrophoretic technique is shown by Fujino's (1969) study comparing skipjack tuna from the Atlantic, eastern Pacific and western Pacific (Fig. 2). The results suggest that the Atlantic samples could not be separated from the western Pacific samples on the basis of a combination of gene frequencies of the Y blood group and the serum esterase system. Since it is unlikely that the Atlantic and western Pacific skipjack tuna resources represent a single management "unit", the lack of genetic separation must point to other factors. In assessing the problems of using genetic characters to examine population structures of the skipjack tuna, researchers have noted that the skipjack tuna has a low level of variable polymorphism suitable for population studies; thus, it would be difficult to demonstrate differences in population "units" by genetic characteristics even if separate population "units" of skipjack tuna did exist.

Another marine species which has been studied extensively is the milkfish in the central and western Pacific (Winans 1980). Although the milkfish is a coastal species and is commonly found in estuarine or lagoon habitats, conditions which are suitable for establishment of discrete population units, Winans (1980) was only able to separate milkfish specimens collected from various locations between Hawaii and the Philippines into three or four groupings (Fig. 3). The lack of genetic differentiation was reported to be due to gene flow which resulted from movement of milkfish from one location to the other by larval dispersal. A priori, on the basis of life history information of the milkfish, I would expect a fishery administrator in the region to "manage" the milkfish resources by much smaller units than Winans' electrophoretic study would suggest.

In summary, I find it difficult to reconcile some of the results of the immunogenetic and electrophoretic work with what is known of the biology and movement of some marine species. I suspect that administrators and scientists, especially geneticists, may not be responding to the same set of questions. One basic question is whether the self-sustained population "unit" of the administrator's dictionary is the same as self-sustained population "unit" of the geneticist's dictionary. For the administrator, he recognizes that some mixing may occur between population "units" in his universe; what he needs is a measure of the degree of mixing to ensure that he can manage each "unit" separately. For the geneticist, even a slight "leakage" which allows for gene flow would result in a larger envelope of self-sustained population "units" than desired by the administrator.

Finally, it appears that administrators and scientists need to have a continuous dialogue regarding stock identification problems.

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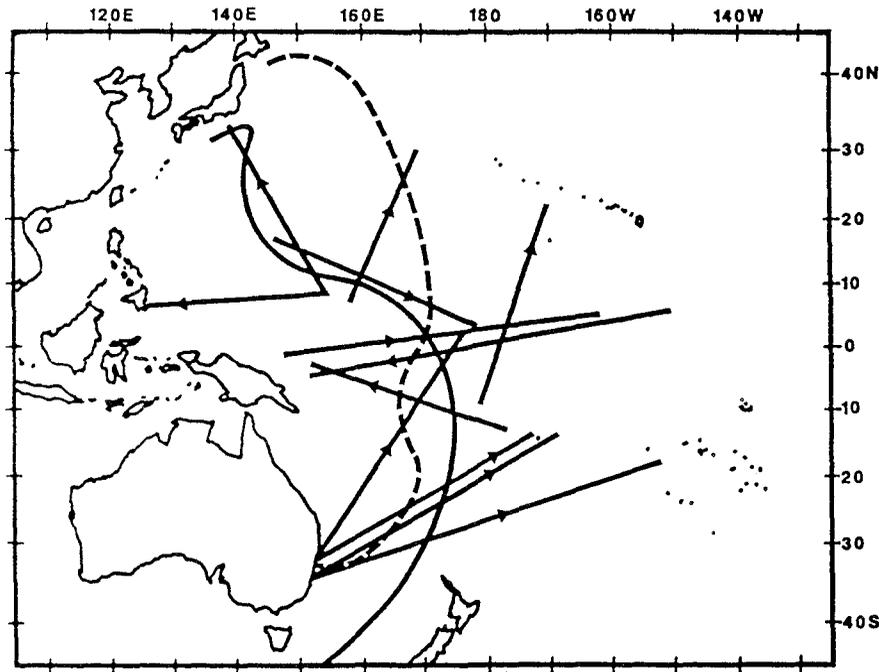


Figure 1. Proposed range of skipjack tuna subpopulations in the western Pacific Ocean. Eastern limit of range in the northern winter (southern summer) and northern summer (southern winter) are indicated by a thin solid line and a broken line, respectively (Fujino 1970). The thin lines with arrows depict straight line representations of movements of skipjack tuna tagged by the SPC Skipjack Programme (from Kearney 1983).

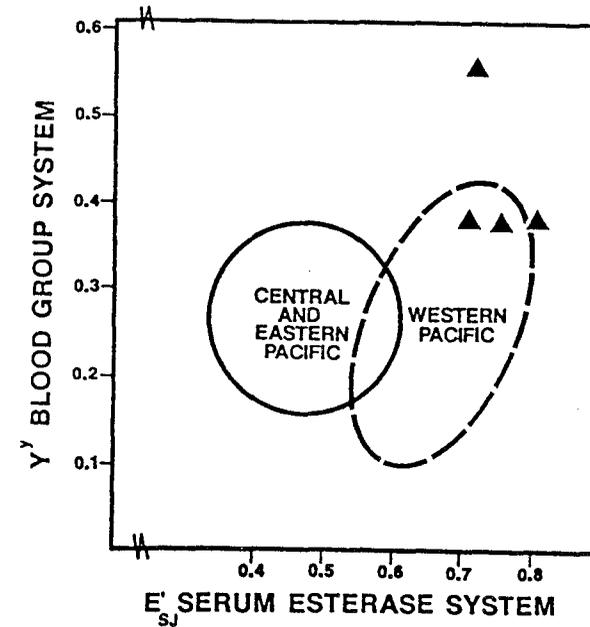
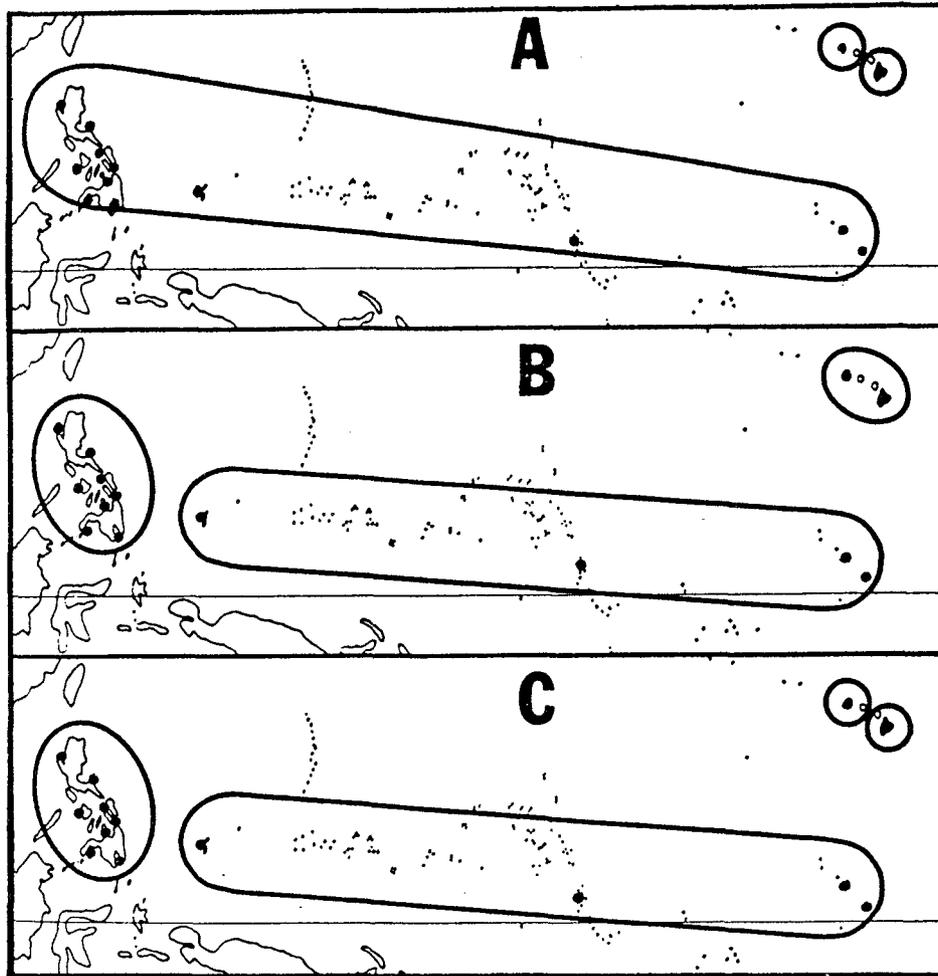


Figure 2. Differentiation of skipjack tuna subpopulations by the combination of gene frequencies of the Y blood group and serum esterase systems. Rejection ellipse in solid line is for the central-eastern Pacific and that in dotted line for the western Pacific (at 5% significance level). Plotted are also individual values (indicated by solid triangles) of four lots of the Atlantic samples (from Fujino 1969).



Locus	Within			Between			
	PHIL	EPI	HI	PHIL-EPI	PHIL-HI	EPI-HI	
A	PGM	N.S.	N.S.	*	N.S.	***	**
	PRO-2	N.S.	N.S.	***	N.S.	***	***
B	GPI-A	N.S.	*	N.S.	***	***	***
	PGDH	**	N.S.	N.S.	***	***	***
C	EST-2	N.S.	N.S.	***	***	***	***
	AK	N.S.	N.S.	**	***	***	***

N.S.  $P > 0.05$ ; \*  $0.05 > P > 0.01$ ; \*\*  $0.01 > P > 0.001$ ; \*\*\*  $0.001 > P$ .

Figure 3. Results of tests of heterogeneity of gene frequency (within) and tests of independence of gene frequency and location (between) by G-statistic. The resultant geographic patterns are illustrated. Solid circles represent milkfish sample sites (adapted from Winans 1980).



## PANEL AND AUDIENCE DISCUSSION

After the panel moderator (Richard Shomura) presented his opening remarks, the panel, composed of the plenary session speakers, was invited to open the discussion with audience participation. The following are the edited remarks made from the audio and video recordings of the closing session of the workshop.

SHOMURA: I have had my say and now am going to open it to the panel to either raise questions or make some points and then open the discussion to the audience.

AVISE: I'd like to comment on your remarks. I'm a geneticist and not normally concerned with stock identification in quite the same way discussed at these meetings. I would like to raise a provocative point perhaps, but it seems to me that what many of you, as practicing fishery biologists, are interested in describing as stocks will not necessarily bear any relationship at all to what geneticists are trying to distinguish. Let me just give a hypothetical example. Let's suppose that you are charged with managing a series of 16 farm ponds, and you stock them all with large numbers from the same parental stock. All the farm ponds will be genetically identical whether it be differentiated by mitochondrial DNA or allozymes or anything else. That would be irrelevant to your requirement for managing those units. I would think you would manage each pond separately and not overharvest a particular pond and drive it into extinction. The management unit in that sense would be complete and it would be irrelevant to have any specific genetic information. The relevant fact is that you have sixteen farm ponds to manage separately. Put another way, suppose that we stock those same sixteen farm ponds with two genetically different stocks and half the farm ponds get genetic stock A, and half get genetic stock B. The geneticist would go in and might be able to distinguish stocks A and B from one another but that again would be irrelevant to your management concerns which you would still have to focus on trying to maintain the population within each of those sixteen ponds separately. It seems to me that this sort of concern has come up time and time again at these meetings. A real live example that comes to mind deals with the salmon stocks in the Pacific northwest and the sharing of these stocks between Canada and the United States. As I understand it for purposes of management the concern was simply to distinguish where the salmon had originated. It doesn't matter whether they are genetically different. A parasite label is perfectly fine even though there may be no genetic distinction whatsoever between the Canadian and American salmon. It is irrelevant to the management goal, which was simply to describe where the salmon had come from, and that can be done with a non-genetic marker. It doesn't matter whether they are genetically different for that particular management perspective as I see it.

It seems to me that in many cases what we need is a clarification of what are the objectives of the fishery biologist in a given study. Is it necessary to know what genetic differentiation may be or is that going to be irrelevant to the management concerns in a particular instance? It may sound like a strange statement coming from a geneticist, but I'm not really sure that you really want to know what the actual stocks are. There may be some cases where actual stocks need to be defined, but in many cases it seems not to be what particularly interests fishery managers.

COOK: I deal with salmon stocks quite regularly in the northwest. I think that the overall perspective that you (Avisé) utilize is not necessarily useful to managers. The situation is a little finer than that. You have two different types of management issues: allocation issues and then true management issues. The allocation objectives are strictly along political boundaries which bears no correlation at all to any underlying perspective of stock as we would have it as biologists. But if you investigate individual systems as a biologist on a finer scale, particularly relating to salmon, the biologists are very interested in maintaining the genetic integrity of the stock and various components of the stock. For example, on the Fraser River and its many different tributaries the goal is to maintain the stock level within each of the different subpopulations or subgroups. So you really have two different issues at the management level. I think that the goal of the manager is to maintain all components of the stock and harvest them at their particular optimal rate of exploitation, not over-harvest those stocks that normally sustain a very low level of exploitation. We had a Pacific salmon case in which the return per spawner ratio for hatchery fish is quite high, sometimes 10-15 to 1, whereas for a wild stock you have a return per spawner ratio of 2 or 3 to 1. If you harvest, based on the higher ratio, you are essentially pushing the wild stock down to a very low level of productivity. On a more subtle scale within the Fraser River, for example, you have stocks that can sustain high yields because they are large, highly productive, stocks. Thus, they can sustain a very high exploitation rate. There also are a number of minor, smaller, stocks that need to be identified and exploited at a much lower rate so that you can keep the overall yield to the fishery at a high level. If you ignore the stock uniqueness you are going to wipe out the smaller, less productive components of the resource and your yields will go down. You have essentially decreased the genetic diversity of the resource. I think there is a very fundamental underlying relationship between managers and biologists. The goals of the managers should be compatible with the goals of the biologists. We both want to maintain this genetic diversity.

AVISÉ: I am glad you see the problem. If I were listening to the same kind of discussion in the agricultural realm, people

would be talking about seed banks and preserving the diversity of genetic resources. They would be interested in harvesting and maintaining different seedbank populations which they have in stock holds (or whatever) from different places to try to maintain genetic diversity in the species. It seems like I've heard very little of that sort of talk in these meetings and it is a very important issue. The possible desirability of maintaining or identifying where genetically distinct units may be in populations with the goal of preserving diversity within species is an important issue. This is where the geneticist can contribute to the discussion by helping to identify what may be somewhat different genetic units in nature and suggesting ways to identify genetic resources that are out there. I can give a specific example. A number of years ago I got involved in Geomys pinetis, the pocket gopher, work because of the concern about one population that had been recognized as a distinct species which was protected under the Endangered Species Act. The concerns were about its management during the course of a genetic survey of that endangered species and its common conjugate in the southeast. We found that the "endangered species" was not genetically distinct by any criteria that we could find from surrounding populations of the common species. Yet within the "territory" there was a major genetic break recognized by a number of genetic criteria distinguishing eastern populations from western ones. From the standpoint of preserving genetic diversity in that complex we can clearly define what we think are the major evolutionary units, and they did not coincide with what had been recognized as classical taxonomy as the genetic unit. I think genetics can contribute to the identification of different genetic stocks, but I still question whether that is going to be a primary concern to most management decisions.

WINANS: The key word that John (Avisé) uses is "may". We may be able to detect genetic differences among different taxonomic groups or within a taxonomic group, or we may be able to detect patterns of genetic differentiation, and then perhaps we may not be able to detect differences. I couldn't tell Christmas Island milkfish from other milkfish from across the Pacific Ocean. But a manager might well say this species will be managed differently from those from Fanning Island. What my study gave was a glimpse of the genetic differentiation and I said across the equator they were genetically similar. We couldn't detect differences.

SHOMURA: I think we're looking at different problems. The administrator is expecting that we can look at these management units and differentiate them. Management units, the definition of this as John Avisé mentioned, are stocks as the geneticist envisions them. But in terms of a manager, especially with pelagic marine resources, this problem is going to become more and more critical. I realize the point you made on the milkfish

study was not from a manager's perspective but looking at genetic differentiation.

BERT: I think Gary Winans' comments relate to dispersal and gene flow, for example, an isolated island and its population of fish. When we're looking at gene flow at the molecular level, we observe this flow using electrophoresis or mitochondrial DNA, as we were taught in school - we all know that a very small amount of gene flow will maintain homeostasis among different populations and genetic identity, but that may be a very different kind of gene flow in the management sense. In the management sense, if you fish out an island how soon are you going to get enough gene flow, i.e., enough individuals flowing back in to repopulate your now fished out population? So there is another problem with using genetic techniques in the management sense that also needs to be considered and that is gene flow from the molecular point of view is very different from the fisheries management point of view. It can take a long time to replenish a stock that may be genetically identical to an adjacent stock depending on the dispersal patterns and life history strategies of the organisms that are involved.

UTTER: I think you raised a very good point. The point has been alluded to throughout the workshop here and that has been explicitly expressed now, to some degree by Gary Winans and others on the panel. It concerns the fact that what appears to be a homogeneous unit genetically may in fact not be, depending on the tools you are using. Particularly in the case of electrophoresis where it has been pointed out that we are looking at a very small amount of the total genome. Therefore when you do find a difference that is consistently stable over time and throughout the range of the individual, meeting all the criteria of a valid genetic substructure between two groups, you have positive data. This has to be seriously considered in the management of the resource. But the absence of such differences does not justify managing resources, at least on even good sensible electrophoretic data, as a panmictic unit at all. Therefore, as Richard Shomura has expressed the concern over marine species, you may not get the answers you are looking for as managers through electrophoresis. This does not mean that you should not use this technique, because the method is fairly straightforward and not terribly expensive and should be utilized at the initiation of any stock identification program. It is to the advantage of the management agency to collect good sets of electrophoretic data initially and see what the data express. Then at that point, if these data are not sufficiently informative for management needs, move on to other methods, such as mitochondrial DNA procedures, which are somewhat more labor intensive and expensive, but nevertheless are potentially more

informative as well. Theoretically you do have an increase in the precision of stock separation through use of these methods. There are a myriad of other tools that are available to the manager and these have to be used and such tools should be used in concert with each other and not as an all or nothing type of an approach.

One point that I wish to make is that there is a difference between marine and freshwater species. I have been involved in salmonids for most of my career. I've had the good fortune of working with a group of animals that do have very distinct genetic units because of their life history, and as a consequence we have identified many different stock units. These have been used effectively by our group and by others. What I am trying to say is that some animals have life histories well suited for use of genetic markers to identify stock units and can be used in effective resource management. In marine species you do have much greater potential for gene flow and dispersal. In such cases genetic methods have more limited use over the same geographic range, although over broader geographic ranges there is certainly documented evidence that you have distinct subunits as Gary Winans' studies have shown in milkfish. There is a recent paper out by Bulestein from the University of Stockholm which examines the case of the gene diversity or the equivalent of the Wright's F statistics, where you measure the amount of substructuring within a species. The paper documents what is predictable, that freshwater species are highly substructured and that anadromous species are somewhat less, because of the possibility of gene flow amongst the different demes. The lowest level of substructuring are the marine species.

SAILA: I'd like to address briefly the apparent question raised concerning this workshop and where we are now with reference to stock identification. I personally am coming out of this workshop very, very much impressed with the progress that has been made recently in many areas. The impression I have gotten was that significant advances were being made in numerous fields. For example, optical image analysis, mitochondrial DNA work, advances in isoelectric focusing, high pressure liquid chromatography applications, advances in analytical chemistry, x-ray spectrometry, the electron microprobe elemental analysis, atomic absorption, and monoclonal antibodies; all of these are new tools which I think have applications. It seems to me that I revert back to a statement that I made earlier and that is, we have to define the problem very explicitly. We have techniques available now where we can identify fish just as we can identify each other as individuals if we are willing to allot sufficient funding. On the other hand, we must apply specific tools to specific problems if we're interested in identifying the effect on the fish population of a perturbation such as a pollution event. Perhaps biochemical techniques, which identify organics or analytical

techniques which identify elements, would be very adequate for distinguishing the impact from the non-impacted populations. If the problem is how many stocks are in the entire Pacific Ocean, then this has very different base dimensions than what is the effect of nuclear power plant A in a particular estuary on a fish population adjacent to it. I also believe that concomitant with the beautiful advances that have been made in various types of genetic biochemical and analytical chemical techniques, there are now advances rapidly occurring in ways to analyze these data statistically. We should keep in mind that we can now essentially statistically resolve questions much more precisely than in the past. We are coming into a dilemma to some extent where, for example, Rod Cook indicated that there may be up to 256,000 pieces of information which can be used for some types of classification. Clearly there has to be a very, very careful screening of this information to determine which ones are appropriate for the purposes of, and what number of variables are manageable for, a given statistical technique. For example, I alluded to the fact that limiting discriminate functions isn't dead, but certainly they need a larger sample size than another technique that is based on components analysis. We have to become aware of how we match statistical analyses to the available data we have, either biochemical or genetic data. There is a genetic problem and there is a geo-political problem of assigning an individual with a certain probability to Canada or the U.S. I think these are two different classic problems that should be recognized as such.

SHOMURA: I'm looking at some of these management issues strictly from a very narrow perspective and am extremely interested in the morphometric approach. You really can't just go out and collect masses of morphometric data and plug it in and try to make something out of it. But if you had some sort of preconceived ideas of what you're looking at, then with these statistical methods that have arisen in recent years you could get a much more powerful method of analyzing these data. I recall Bill Royce's original work on yellowfin tuna morphometrics. He collected thousands of measurements and the best that he could conclude was that, and I am putting it simplistically, the yellowfin of the eastern Pacific and the yellowfin from a thousand miles to the west probably didn't come from the same population.

BOGDANOWICZ: Given that we as scientists can define or solve a problem in a decade versus short term funding to solve a problem in 2 years, how do we compromise the need for really long term research versus the funds that we're getting? We can't even define the problem in three years. How do you reconcile the managers who want a quick fix and the scientists who need a decade to get a good handle on what's going on? We have heard the term "temporal stability" come up more than once and trying

to forecast something based on one or two years of research regardless of the technique used could be totally inappropriate.

SHOMURA: You really need to sample over some time frame to look at seasons, size, year class, over the range of distribution before you can make any definitive statement.

ROLON: I work with the staff of the Caribbean Fishery Management Council. One of the goals of my coming to this meeting was to determine whether we have sufficient tools to make recommendations to our managers for the allocation of resources. One problem that I see is that there is a tendency to use terms that are quite different as if they were interchangeable. For example, the terms management unit and gene pool are used as one word and that is a big mistake. Another component of our problem is that we have to use the best available data, whatever that means. Right now in the western Caribbean we are looking at an electrophoretic study and analysis conducted on swordfish. There are a lot of people betting that our swordfish come from the south Atlantic and that it's not part of the northeast Atlantic stocks. Management strategies will differ depending on the outcome of this research. I think that this workshop has been very excellent from the quality and the number of experience papers presented and I see this as another step toward better communication between scientists and managers. Another point should be expressed - that we are looking at a snapshot of what is happening in the whole evolutionary history of a species or a stock. We are trying to get tools that will allow us to forecast what would happen and try to stop the decline in the fishery.

KUMPF: We have talked about funding and availability of resources. What does it cost per unit sample? What does it cost to set up a particular methodology? I like what Fred Utter said that you don't have a perfect method that will solve all your problems. But, I would like to get a relative feel for the costs, exclusive of obtaining samples.

UTTER: I would like to speak to that based on setting up a basic electrophoretic project. You will need four power units that cost about 500 dollars a piece. You will also want to have an ultra cold freezer (-80o C) for storage of samples. I believe this is absolutely necessary for long-term storage and for resampling individuals to avoid having to go out and collect them again. The freezer may cost \$5,000 for a good unit. Beyond that your costs are not extremely high. For equipment you will need a total of not more than \$5,000. Your major expense beyond manpower for the project is going to be chemicals. That may run as high as \$5,000.

SHAKLEE: Basically, all I'd like to do is point out that there is no simple answer to your question of how much it costs. It's

partly a function of the magnitude of the operation. In the state of Washington, we are in the process of setting up a laboratory to do large volume analyses of salmon stocks. I think the department is investing in the neighborhood of hundreds of thousands of dollars, not \$10,000 or \$20,000. But we're not talking about a small scale preliminary investigation of the technique, we're talking about the application of the technique on a large scale with a number of individuals and adequate equipment. So within this one technique, if you're talking how much it costs to do as a preliminary first operation with one person you might be talking \$20,000 for the first year exclusive of salaries, sample costs, etc. Yet, if you're talking about using it in a practical large scale stock identification program either involving a large number of stocks and a large number of species and individuals, then you can quickly be talking on a scale of hundreds of thousands of dollars. Clearly, the criterion that has to be applied is whether or not the questions that need to be answered are of such a commercial or economic scale that they justify the level of investment necessary hopefully to generate an answer.

SHOMURA: In your estimates, Jim (Shaklee), you pretty much discounted the cost of collecting samples. When you're looking at it from a state situation, fairly localized, the cost of sampling is fairly small relative to when you're looking at the resources we've looked at in the Pacific.

KUMPF: The reason I discounted sample collection cost is that it would be roughly the same for each method, but I can see that the sample needs for a mitochondrial DNA project would be different from a morphometric study.

SHAKLEE: Well, the function of the sample size needed to answer the question and also how the samples have to be collected and protected or preserved between the time of collection and the time of analysis are also important factors. The reason I avoided any discussion of sample cost is that it is so highly specific to the question being addressed, both geographical and in terms of the logistics of collecting the animal. In some cases, the cost of collecting samples can be orders of magnitude greater than the cost of analysis. In other cases it is in fact a small fraction of the cost of analysis.

AVISE: I find your question on costs very hard to answer because there are so many considerations. One way to summarize the cost of mitochondrial DNA work is to note that I used to have a protein electrophoretic lab and it was supported by grants that were roughly half the size of those that I currently have for supporting the mitochondrial DNA lab. In terms of the normal operations, once the lab is set up it's not that different. I'm able to process far fewer samples of mitochondrial DNA with that

grant than I could have processed with electrophoresis. In terms of the capital expense it's going to depend on exactly how the procedure is done. During the meeting here you heard several alternative ways in which one might approach the mitochondrial DNA technique. In our particular lab the most expensive piece of equipment is the ultracentrifuge which may be twenty or thirty thousand dollars or more for the machine itself, exclusive of rotors. So that's a major capital outlay, but there are ways to get around that. There are other techniques of analysis that don't require an ultracentrifuge, but we prefer its use for our current purposes. My last grant was for three years and it was about \$150,000 (\$50,000 a year). The majority of that goes to personnel. This included one full time lab technician and several graduate students that were supported outside the grant.

COOK: I've been dealing with scale analysis for quite awhile. Part of the reason I've been well funded over time, perhaps at a lower level, is because its an extremely inexpensive route to go. If it answers the questions for the managers and the people interested in the problem, and its the cheapest tool for them to use, they will fund the work. The comparison of scale analysis and electrophoresis, as to which method is going to work, isn't a valid question. In order to solve problems you really need to focus in several different directions to address an important problem. You have to realize that one particular technique is not going to answer all the questions. You may have to use an alternative technique. Perhaps you should start with the cheapest technique which may be the computerized analysis of morphometrics or scales and if that does not work then go to the next least expensive technique and on up the chain until you've lost support from the people interested in addressing the allocation or the management issue. Scale analysis has been extremely inexpensive to use. Agencies with very small budgets are approaching us to look at this method. We're looking at capitalization costs of around ten to twenty-five thousand dollars. The manpower requirements are very very low because we can process a few thousand samples a day on some occasions, if your samples are well organized, and for scale collection it is often simply a matter of writing a letter to a brother agency or to someone that may have these samples on file and exchange acetate impressions. It can be very inexpensive because it doesn't require one full salary of an individual to address these problems. We're looking at small tribal organizations being able to do things that required a quarter of a million dollars just five years ago. I'm probably the only person from the private sector to pick up on this technique, and I feel that there is significant potential for financial return, not necessarily for stock identification alone, but for other types of applications.

MACIOROWSKI: The technique issue is not really the problem. The issue, as Dr. Saila said, is the objective. A myriad of tools,

techniques, test systems and experiments exist that you can use to attack the problem. Each of them have value to a specific question and objective, but in no specific problem are they all equally valid. The issue really is defining questions and objectives and looking at an evaluation process. The basic researcher is always interested in basic research and always feels that he needs to conduct more basic research to build a data base. The manager is often faced with the situation where he has to make a management decision now based on whatever available data exists although these data may be very highly uncertain and they may not be very extensive, but those drawbacks do not allow postponement of making a decision.

LUNDSTROM: One of my purposes of coming here was to get a view of some of the contemporary techniques that are being used today. I was surprised, but very pleased, to find a number of quite unrelated techniques that are peacefully coexisting and finding very useful application. Immunology is a field which was at its height probably 25-30 years ago. We do have numbers of technological innovations in immunology which produced some outstanding applications particularly in human genetics. Hopefully, we'll be able to make some of this extend to fishery genetics. Using monoclonal antibodies turns out to be an expensive technique. Of course cost depends on whether you're just using monoclonal antibodies or are developing the antibodies. The development part is very labor intensive and costly. However, once you have the antibody which essentially forms a type of biochemical reagent it can in fact be produced very cheaply, and can be used reasonably inexpensively. If you're talking about analyzing thousands and thousands of samples, then like everything else it also is an expensive technique. Our particular interest initially was for species identification and we've been doing this for about two and a half years. The first year our budget, excluding salaries, was about \$30,000-\$40,000. The situation has changed somewhat in the last year with the acquisition of proper automated instrumentation costing approximately \$150,000. We have reached the point now where we are able to look at a number of different applications, including stock identification. With the capital equipment in place, the research phase budget is approximately \$100,000 a year involving myself and three other full-time people.

AVISE: Let me point out, that I think it's perhaps a very limited perspective to try to count the benefits of any of these approaches solely in terms of the immediate benefits to management. For example, electrophoresis that was developed in a pure research context in the mid-1960s wasn't done in response to concerns about stock identification or anything of this sort. It was developed to answer a fundamental question of evolution and how much variation characterized the natural population. Many of the applications that have subsequently spun off, such as

stock identification, certainly wouldn't have been counted in the 1960s as one of the immediate benefits of the technique. In respect to mitochondrial DNA, I think we're in a very similar stage right now. We're at a stage where we're trying to develop not only things that are of immediate practical significance to resource managers, but we're trying to evaluate a whole conceptual approach to the study of evolution. To me, mitochondrial DNA is particularly exciting because it's the first time we're able to study an asexually transmitted molecule in reproducing species. There are a number of reorientations of thinking that are brought about by simply having the realization and studying the ramifications of the consequences of loaded inheritance. It's far too early to begin to assess the kinds of benefits that may eventually come out of this approach. I think it's far too limited to set down and do a cost-benefit analysis.

WINANS: To set up a morphometrics research project the basic required instrument is a digitizer which costs between \$1,000-\$2,500. A computer system with appropriate software is a critical acquisition. Added to these fixed costs is sample collection and labor. If someone gave me \$5,000, I'd be rich.

COOK: The hardware for the type of optical systems that I showed with digitizers, monitors and microscope costs about \$15,000. If you buy the computer software the cost is approximately an equivalent amount. If you develop the software inhouse it would take about 1-1/2 to 2 years.

CASEY: About 20 years ago I tried to find out what electrophoresis was all about. I talked to people in our lab and I thought that we could differentiate spawning stocks. Our researchers responded that they could tell the difference between samples, but not conclude that they were the same stocks. I think the managers have trouble with that response. It seems to me the techniques are at hand and they are not that expensive from what we have heard. Isn't there some technique that could be used right now to at least get part of the question answered? When are we going to be able to prove our case, and get support for this work? My bottom line comment on this is that I would be willing to supply biological material for the evaluation of some wide ranging species such as blue shark or swordfish. If nothing shows up it's fine, but I don't want to be under the gun five years from now to get large numbers of samples and not be able to supply them. So if any of you want to see me after the meeting I would be happy to arrange a proper sampling protocol. I can give you some ideas on what the problems are going to be. Blue shark, for example, is probably the most abundant large pelagic shark in the Atlantic, and occurs on both sides of the Atlantic, but none of the international fishing community is exploiting it yet. It certainly is going to be harvested, and it is very important in our U.S. recreational fishery, so I would like to see this

species looked at genetically and the stocks delineated, before it gets to be a critical issue.

CRATEAU: I want to get back to what John Avise said earlier about those farm ponds and it would be important to me to have some way of distinguishing between those pond stocks. I am involved in trying to restore striped bass in the Gulf of Mexico. One question being asked by the people in the Gulf is why not use Atlantic stocks, and I am trying to determine whether there is a genetic difference and if so perhaps a certain genetic characteristic may lend itself to be used in restoring the Gulf of Mexico stock. There is now one small population of Gulf stock striped bass left in the Apalachicola River system, and we are trying to save that stock to restore the Gulf of Mexico striped bass population. I would like to show that there is some genetic difference. The ultimate answer to the question of whether this particular striped bass does have some adaptive characteristics would be demonstrated through stocking programs to see if in fact it does live longer, grow faster and have a higher survival rate.

STEVENSON: I would like to offer a look into the future that we all need to think about. I think it is clear from attending this workshop that there will continue to be advances in technology and in the kinds of statistical tests that can be applied to data in stock identification. We are going to be learning more in the future resulting in even more information being available to resource managers. Their job is going to become even more difficult than it is now, in sorting through this information and figuring out what to do with it. It will put a burden on the managers and probably also on your assessment people who, I think, are sort of going to be caught in the middle.

HELLE: I like the analogy of the snapshot that Miguel Rolon from the Caribbean Council suggested. What we really need to look at is a much broader period of time, i.e., much bigger picture. We are looking at these problems in our own lifetime and in the terms of that context, but we need to look at it on an evolutionary time cycle. More in terms of what are the fish life cycles? How many life cycles of a chinook salmon am I going to see in my lifetime and be able to study? We need to look at what we are leaving future generations. We have an urgent problem in the lack of communication between researchers, managers and the general public. Let me give you a case in point. When offshore oil development was being considered in the Gulf of Alaska, one of the city managers from a small village along the coast said we shouldn't care if there is major pollution along the coast because it won't affect the salmon anyway, because we will build hatcheries and have salmon whether we have polluted waters or not. There was a total lack of understanding of the basic problem. If habitats are eliminated you won't have the salmon. In the public's mind a hatchery can replace wild stocks.

Hatcheries do not replace wild stocks. Wild stocks supply our storehouses of genetic diversity for the future. Hatchery breeding and production is a selection process. This results in a homogeneous stock transplanted over wide areas, and that is exactly what we should not be doing if we are going to leave future generations something worthwhile. We need to be able to communicate with managers better and tell them that they have an evolutionary crisis on their hands right now. A case in point is the homing behavior of salmonids. Homing is highly inheritable. If you take an animal out of its indigenous environment, salmon in particular, and you transplant that fish to another stream, a few will come back, but not at the high returns that result from indigenous stock. If the hatchery raised stock was acclimatized to the hatchery locale, you could get an instant success. The first hatchery that was built in Alaska did not get their king salmon eggs from Alaska but got them from Washington. Chinook salmon eggs were transplanted from a hatchery on the Columbia River. There is a very virulent virus, called IHN virus, in sockeye salmon and every time you try to bring sockeye salmon into a hatchery, you get an outbreak of this disease. This virus is present everywhere in the natural environment that we have looked where sockeye are, but Alaskan king salmon are apparently immune to this virus. However, king salmon from Washington, Oregon, and California are not immune to this virus. Some kings returned to the hatchery, but they also strayed into all sorts of streams all over the area. If these imports interbreed with a wild fish are you going to tear down natural resistance to IHN virus? I think we do have a management crisis on our hands. I think you can manage a hatchery and keep your diversity high, and I think you can do it by proper location of the hatcheries and proper management of the broodstock.

SCOTT: As I see it the attendees are basically divided into two groups, those that have their stocks pre-defined and are looking for ways to discriminate between them because they've already decided on the units to manage. Then on the other hand there are those people that presented papers, who are looking to their data to tell them whether or not they have separate stocks, and if these units should be of concern to management. I fall into the latter group. We are getting very good at discriminating differences and as Saul Saila and others have pointed out, we're starting to get to the level of individuals. The question I have, after we discern differences, are those differences important and if they are important, on what time scale? Brown talked about the fact that the response time scale for a local resource might be quite different than the response time scale on an evolutionary standpoint and obviously it's different. I think that in terms of fishery management we're concerned about that local response time. I have looked to the geneticist first to tell me what differences mean in terms of evolutionary processes, and if we remove that genome from this population as a whole,

does it mean anything in the long run? I guess that depends on your assumption as to how things have evolved to their present state. If we take a stochastic point of view then it probably doesn't matter if you wipe out a genome, because it is equally likely that something will come back and replace it. However, if some deterministic force establishes the present state, then removing the genome may be very important, because some selection pressure is pushing us towards that divergence. I also wonder what are the overall objectives. Are these objectives population fitness, and maximizing genetic diversity? If they are, are they somewhat contrary to more traditional fishery management objectives, such as maximizing yield or production?

UTTER: Very little is really known about predicting what's going to happen when you remove a stock or portion of it. About the only thing that most biologists have to go on is what has happened when stocks have been removed. I would think the species dynamics of an area certainly changes. Obviously, when stock removal occurs you sometimes have results that are indeed drastic, but as far as I can tell they're unpredictable at this point.

AVISE: I hardly know where to begin. You've raised so many issues that are novel to the discussion. In a very fundamental sense it's even debatable whether it is desirable to maintain or contradict diversity in our world. That's a point of contention and we certainly do have a lot of biological diversity, and I find it wonderful to explore and to study and to marvel at. But someone else might be perfectly happy with a monoculture situation with very low diversity. There are alternative ways in which a given pattern of partitioning of genetic information could have arisen in the evolutionary process. One alternative is that there were particular selection regimes or other zoogeographic barriers that might have in a deterministic fashion molded the particular pattern that we observe today. The other possibility being the stochastic explanation that simply involves the sorting of lineages independently in a particular determinism. The question is whether there are predictable ways in which evolutionary processes work to mold the current data distribution to genetic information.

SCOTT: The immediate time frame of interest is that local response time for the group of individuals that we tend to determine as a stock. In many cases it really doesn't matter if they're genetically distinct or not, we have already predetermined the units we are dealing with. I'm looking to a data set or multiple sets to try and tell me if there is some reason to try and segregate our distinct groupings or units. Do we treat them as a single unit or do we treat them as separate units?

SHAKLEE: I'd just like to say I think there are two issues which are very different in their time frames. The question of stock identification as it has been presented, I think, at this workshop deals with short term problems and short term questions about how in fact you address management questions related say to commercial exploitation which has implications today and next year and for the next ten years. This question of maintaining genetic diversity should be seen more in the context of a thousand generations from now, not today or next week or next year, and for that reason the criteria that you use and the interpretations and the value judgements that have to be placed on a particular data set are very, very different. I think that it's important to make a distinction between the two kinds of goals and the context in which the evaluation of the resource and the decision making process is carried out. I think for the management of fisheries you're generally concerned with much shorter term processes than you are if you're talking about maintaining genetic diversity in an evolutionary time scale.

HELLE: I think the point is that management decisions must be made whether we can provide the answers or not. And so that's why often we're under the gun looking at stock identification, so we can describe a stock and say we're trying to do it in a two or three year period. Whether we differentiate stocks or not, the management decisions are going to be made from year to year for a particular fishing season. Often the scientist is too conservative with his opinion, and I think the manager needs a broader scientific background to manage effectively, and I think it should come from the scientist. Based on the evidence that we know today, why not play it conservative? Why not assume that all this evidence points to the presence of diverse stocks and why not manage them that way until it's proven different? Often it's the other way around, because it's more complicated to manage on the basis of diverse stocks, managers assume that stock units are all the same until proven different. I think we need to turn that whole thing around and manage from the best scientific information available and go from there.

BERT: I'd just like to question whether managing for genetic diversity and evolutionary set versus managing for an immediate issue in a pragmatic sense isn't somewhat of a moot point. Those of us who deal in immediate fisheries management issues recognize that social, political and economic problems so far outweigh any defense of evolutionary quality in a management decision. I don't think management will ever, or at least in the immediate future, manage for genetic diversity, unless that question becomes the most critical issue, which presumably is only in endangered species.

COOK: I think that is happening in areas of agriculture now when they are very, very concerned about the seed stock, the genetic

stock for grains, for example. The decisions being made in those areas are by people very atuned to the idea that the gene pool has been dramatically reduced, because virtually all grass lands around the world are plowed under and are under agricultural situations. They are very much interested in maintaining resources for genetic diversity.

KUMPF: I just wanted to make one concluding point on this issue and that is that we don't really manage stocks, what we manage is people through various management measures. It is up to the scientist and especially people like our plenary speakers to provide the information so that managers can develop strategies to manage the fishery and manage the people that harvest the fishery.

I would like to spend a few minutes concerning the logistics and support of this meeting. It really takes a team effort to put on a meeting of this kind, to plan and organize and carry it out. We had some eighty attendees, and I'd like to acknowledge some of the members of this team that put the workshop together. Some of the people that were involved in putting together all the materials that you're taking home - Carol Parker, Rita Bloechel, Nancy Butowski, Rosalie Vaught, and Lyman Barger who ran all the audio visuals during this meeting, and also Gary Nelson, who is doing the video taping. The organizing committee - Churchill Grimes, Allyn Johnson and Gene Nakamura. And the conveners plus Richard Shomura, our panel discussion moderator, who we decided actually came the farthest to this workshop. I really want to thank the plenary speakers. They set the stage on the first day and then all the contributors should be recognized: they shared their experiences the second day. And a special note of thanks to our Canadian colleagues who came all the way down here to share their experiences and expertise. We certainly are grateful for that. But basic to the success of such a gathering is really the mix of all the people that attended and participated. You are the ones that deserve the credit for such a successful meeting. Thank you.

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